

UCSF

UC San Francisco Electronic Theses and Dissertations

Title

Neuronal nitric oxide synthase and PDZ domains

Permalink

<https://escholarship.org/uc/item/9tj7n4zh>

Author

Brenman, Jay,

Publication Date

1997

Peer reviewed|Thesis/dissertation

NEURONAL NITRIC OXIDE SYNTHASE AND PDZ DOMAINS

by

Jay Brenman

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

BIOMEDICAL SCIENCES

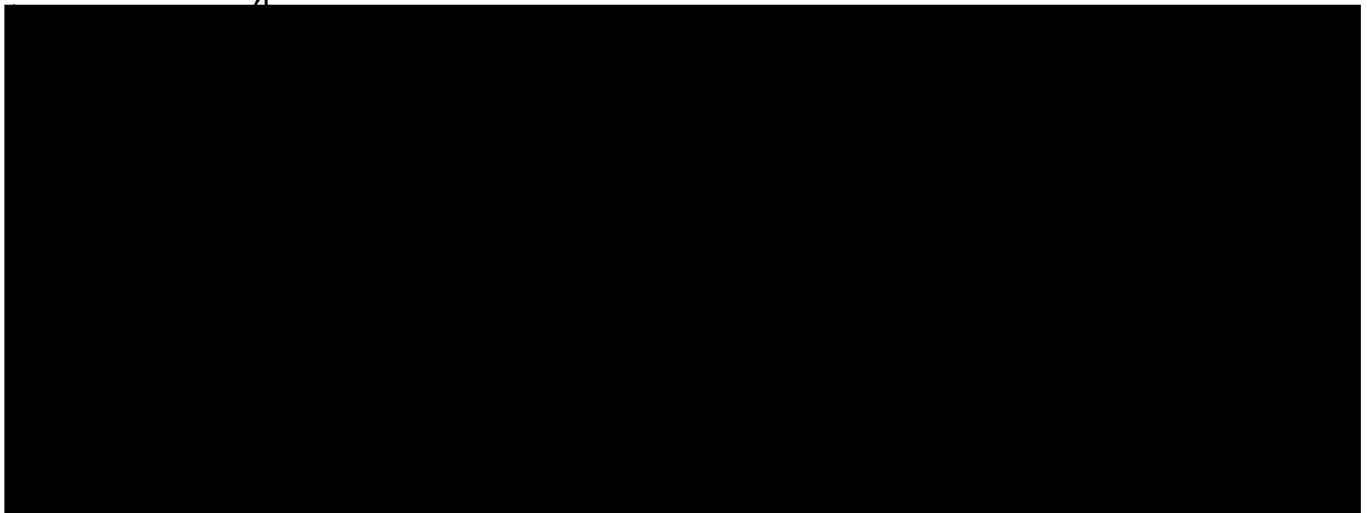
In the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Date

University Librarian

Degree Conferred:

ACKNOWLEDGMENTS

This thesis and my work as a graduate student are first and foremost a reflection of the continuous support and encouragement provided by my wife, Julia Murray Brenman. Without Julie's incredible understanding, patience, and willingness to share me with the "lab," none of this would be possible. On many beautiful weekend days, I groveled toward the laboratory, knowing that somehow Julie would be able to understand that I had some experiments to tend to. To her, this must have meant harming some poor defenseless animal, running one of a seemingly endless supply of gels or pipetting small quantities of essentially water. How did she understand this?

I acknowledge all the collaborators, who have at some point helped me. Of course, particular thanks go to past and present members of the Bredt laboratory. During my time in the Bredt lab, I have enjoyed myself about as much as I think you are supposed to in graduate school. This is no doubt a reflection of my fellow graduate students within the lab, who are all quite impressive people and provided a warm and spirited research environment. In fact, all of us have authored publications together. Thank you Karen Christopherson, Sarah Craven, Aaron McGee, and Hugh Xia. I also had the pleasure of sharing the lab with two great researchers who tried to keep the lab functioning on a daily basis, Dan Chao and Rick Topinka. Thanks everyone for putting up with me and being supportive.

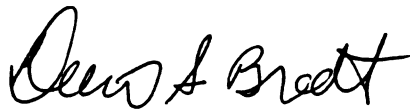
I also acknowledge all the faculty who have served on my orals and thesis committees for causing me to think carefully about my scientific approach and providing guidance in an endless number of areas. I thank my thesis committee - Dr. David Copenhagen, Dr. Robert Edwards, Dr. David Morgan, and Dr. Louis Reichardt - for their invaluable time and guidance.

Particular thanks and gratefulness are reserved for David S. Bredt, my thesis advisor. I think David has taught me all the basics one would ever need to know to succeed in science within the laboratory. I think his method of teaching was particularly effective for me, as well as enjoyable. Being on the same page as your mentor definitely makes your thesis time more pleasant. In testament to my thankfulness, David, I can simply say I would not have done anything different in graduate school.

I have been extremely "lucky" in graduate school in that much of my work as a graduate student has been published in scientific journals. Some of these publications are reproduced in my thesis. Chapter one is a review that was published in *Current Opinion in Neurobiology* (1997). Chapters two through five have also been published in various journals. Complete citation references and copyright approval follow the advisor's statement.

Involvement of Co-authors - Thesis Advisor's Statement:

Jay Brenman's thesis work resulted in the publication of five manuscripts on which he is listed as first author, one manuscript where he is listed as co-first author, and numerous other papers where he is listed as co-author. In all five papers where Jay is first author, Jay did the vast majority of the work. The experiments in these papers were designed and carried out by Jay independently. A paper in Neuron, published in 1996, was co-authored by Jay Brenman and Noam Cohen at Johns Hopkins University, School of Medicine. Jay and Noam contributed equally to this paper published in Neuron. The work described in this thesis meets the standards required for completion of the Ph.D. program.



David S. Bredt, M.D., Ph.D.

Current Opinion in Neurobiology
Current Biology Ltd.
New York, NY

July 30, 1997

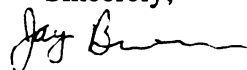
To whom it may concern:

I would like to request permission to include in my thesis dissertation a copy of the paper cited below:

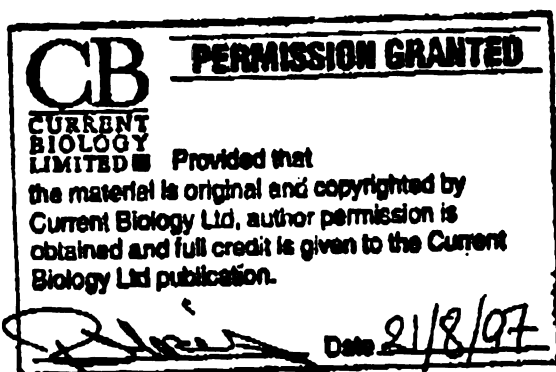
Brenman, J. E., and Bredt, D. S. (1997). Nitric oxide signaling at the synapse. *Current Opinion in Neurobiology* 7, 374-378.

The dissertation will be microfilmed by University Microfilms Incorporated and they request permission to supply single copies upon demand. Please respond by FAX to (415) 476-4929. Thank you for your consideration.

Sincerely,



Jay Brenman
Biomedical Sciences
UC San Francisco



UCSF LIBRARY

Cell
Cell Press
1050 Massachusetts Avenue
Cambridge, Massachusetts 02138

July 30, 1997

To whom it may concern:

I would like to request permission to include in my thesis dissertation a copy of the papers cited below:

Brenman, J. E., Chan, D. S., Xia, H., Aldape, K., and Brecht, D.S. (1995). Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 82, 743-752.

Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Brecht, D. S. (1996). Interaction of nitric oxide synthase with the synaptic density protein PSD-95 and alpha-1 syntrophin mediated by PDZ motifs. *Cell* 84, 757-767.

*Cohen, N. A., *Brenman, J. E., Snyder, S. H., and Brecht, D. S. (1996). Binding of the inward rectifier K⁺ channel to PSD-95 is regulated by protein kinase A phosphorylation. *Neuron* 17, 759-767.

* these authors contributed equally

The dissertation will be microfilmed by University Microfilms Incorporated and they request permission to supply single copies upon demand. Please respond by FAX to (415) 476-4929. Thank you for your consideration

Sincerely,

Jay Brenman
Jay Brenman
Biomedical Sciences
UC San Francisco

Permission granted subject to citation
of the original manuscript, and notation
that copyright is held by Cell Press.
(Our permission is contingent on
permission of the author.)

Jessica A. Donatelli
8/1/97
for Jay Brenman only
in his thesis
dissertation

UCSF LIBRARY

The Journal of Neuroscience
Society for Neuroscience
Washington, DC

July 30, 1997

To whom it may concern:

I would like to request permission to include in my thesis dissertation a copy of the paper cited below:

Brenman, J. E., Christopherson, K. S., Craven, S. E., McGee, A. W., and Bredt, D. S. (1996). Cloning and characterization of postsynaptic density 93 (PSD-93), a nitric oxide synthase interacting protein. *The Journal of Neuroscience* 16, 7407-7415.

The dissertation will be microfilmed by University Microfilms Incorporated and they request permission to supply single copies upon demand. Please respond by FAX to (415) 476-4929. Thank you for your consideration.

Sincerely,


Jay Brenman
Biomedical Sciences
UC San Francisco



Permission is granted by the
Society for Neuroscience provided
the original *The Journal of
Neuroscience* reference is always
cited.

NEURONAL NITRIC OXIDE SYNTHASE AND PDZ DOMAINS

by

Jay Brenman



Robert H. Edwards
Chair, Thesis Committee

ABSTRACT

PDZ motifs are multifunctional protein-protein interaction domains of about 100 amino acids that help organize signaling cascades and cluster ion channels at areas of cell-cell contact. In skeletal muscle, nNOS (which contains one PDZ domain) is localized to the sarcolemma of fast-twitch muscle fibers where production of nitric oxide opposes contractile force. We show that nNOS partitions with skeletal muscle membranes owing to association of nNOS with the dystrophin complex, named for the protein product mutated in Duchenne muscular dystrophy. In both *mdx* mice (a mouse model of dystrophinopathy) and humans with Duchenne muscular dystrophy, there is selective loss of nNOS protein and catalytic activity from muscle membranes. We also demonstrate selective loss of sarcolemmal nNOS in patients with Becker muscular dystrophy, a milder dystrophy but also caused by mutations in dystrophin.

To molecularly define the protein(s) responsible for subcellularly targeting nNOS to synaptic membranes in brain and muscle, we performed

yeast two hybrid screening. In brain, we identify two interacting PDZ containing proteins, PSD-95 and a novel related protein, PSD-93. These PSD proteins target nNOS to synaptic membranes and are coexpressed in neuronal populations. These observations could explain the well known coupling of nNOS activity to NMDA type calcium channels. PSD-95 directly binds to both nNOS and the NMDA receptor, thus directly linking a calcium channel to a calcium dependent enzyme.

In skeletal muscle, nNOS interacts with the PDZ containing protein syntrophin, a dystrophin binding protein. nNOS isoforms lacking a PDZ domain, do not associate with PSD-95 in brain nor with skeletal muscle sarcolemma.

In addition to PDZ-PDZ interfaces, PDZ domains of PSD-95 can also bind to ion channels that end in the C-terminal amino acid consensus sequence of "T/S X V" (threonine/serine X valine). Two inwardly rectifying potassium channels, which have loose consensus sequences, can associate with PSD-95. We find the critical consensus serine residue is also an excellent substrate for phosphorylation by protein kinase A (PKA). Stimulation of PKA in cells causes rapid dissociation of the channels from PSD-95. This work identifies a mechanism for dynamically regulating ion channel interactions with synaptic membranes.

TABLE OF CONTENTS

Acknowledgments	iii
Advisor Statement	v
Abstract	ix
List of Figures and Tables	xiv
 CHAPTER ONE	 1
Introduction - An Overview of Neuronal Nitric Oxide Synthase and Synaptic PDZ Proteins	
General introduction	2
Nitric oxide and skeletal muscle	3
Nitric oxide and hippocampal synaptic plasticity	5
Nitric oxide neurotoxicity	6
Synaptic regulation of nitric oxide	7
Modular PDZ domains and neuronal signal transduction	9
Conclusions	11
Figures and Tables	12
 CHAPTER TWO	 17
Nitric Oxide Synthase Complexed with Dystrophin and Absent from Skeletal Muscle Sarcolemma in Duchenne Muscular Dystrophy	
Abstract	18
Introduction	19
Results	23
Discussion	31

Materials and Methods	36
Figures and Tables	41
CHAPTER THREE	57
Interaction of Nitric Oxide Synthase with the Synaptic Density Protein PSD-95 and α-1 Syntrophin Mediated by PDZ Domains	
Abstract	58
Introduction	59
Results	62
Discussion	70
Materials and Methods	74
Figures and Tables	79
CHAPTER FOUR	98
Cloning and Characterization of Postsynaptic Density 93 (PSD 93), a Nitric Oxide Synthase Interacting Protein	
Abstract	99
Introduction	100
Results	102
Discussion	108
Materials and Methods	113
Figures and Tables	116

CHAPTER FIVE	131
Binding of the Inward Rectifier K⁺ Channel Kir 2.3 to PSD-95 is Regulated by Protein Kinase A Phosphorylation	
Abstract	132
Introduction	133
Results	137
Discussion	143
Materials and Methods	146
Figures	150
 CHAPTER SIX	 164
Conclusions and Future Directions	
Summary	165
Discussion	170
PDZ domains in genetic organisms	172
Figures and Tables	175

LIST OF FIGURES AND TABLES

CHAPTER ONE

Figure 1-1: Protein Domain Structure of NOS Proteins	13
Figure 1-2: Synaptic Interactions of nNOS, NMDAR and PSD-95	15
Table 1-1: PDZ Domains Bind Differentially to Peptide Ligands	16

CHAPTER TWO

Figure 2-1: Extractability of nNOS and eNOS in Skeletal Muscle	42
Figure 2-2: The N-Terminus of nNOS is not Required for Activity	44
Figure 2-3: Association of nNOS and the Dystrophin Complex	47
Figure 2-4: nNOS is Absent from <i>mdx</i> Membrane Fractions	49
Figure 2-5: nNOS is Absent from <i>mdx</i> Sarcolemmal Membranes	51
Figure 2-6: nNOS is Absent from Dystrophic Sarcolemma	53
Figure 2-7: nNOS Protein is Decreased in Dystrophic Muscle	55
Table 2-1: NO Synthase Activity is Diminished in <i>mdx</i> Muscle	56

CHAPTER THREE

Figure 3-1: <i>In situ</i> Hybridization for PSD-93, PSD-95 and nNOS	80
Figure 3-2: PSD-95 and nNOS Colocalization in Neurons	82
Figure 3-3: nNOS Binds to PSD-95 via PDZ Domains	84
Figure 3-4: Expression of Splice Variants in nNOS Knockouts	86
Figure 3-5: nNOS Knockouts Have Catalytically Active nNOS	89
Figure 3-6: nNOS Isoforms Lacking PDZ Domains are Mislocalized	91
Figure 3-7: Syntrophin Binds nNOS via PDZ Domains	93

Figure 3-8: Direct Binding of nNOS to Syntrophin	95
Table 3-1: Interactions between nNOS and PSD-93/95	96
CHAPTER FOUR	
Figure 4-1: Alternative Splicing of PSD-93	117
Figure 4-2: Northern Analysis of PSD-93	119
Figure 4-3: In situ Hybridization of PSD-93	121
Figure 4-4: Immunoblots of PSD-93 and PSD-95 on Brain	123
Figure 4-5: PSD-93 is Postsynaptic and PSD-95 Presynaptic in Cerebellum	125
Figure 4-6: PDZ Repeats of PSD-93 interact with nNOS and NMDAR	127
Figure 4-7: The PDZ is Required but not sufficient for nNOS Binding	129
Table 4-1: Interactions of nNOS and PSD-93 in Two-Hybrid	130
CHAPTER FIVE	
Figure 5-1: PKA Phosphorylates Serine 440 of Kir2.3	151
Figure 5-2: Inward Rectifiers with tSXV Homology	153
Figure 5-3: Kir2.3 Binds to PSD-95	155
Figure 5-4: Kir2.3 and PSD-95 are Coexpressed in Hippocampus	157
Figure 5-5: Kir2.3 Associates with PSD-95 in Brain	159
Figure 5-6: PKA Phosphorylation Inhibits the Kir2.3/PSD-95 Interaction	161
Figure 5-7: Kir2.1 and Kir2.3 but not Kir3.1 interact with PSD-95	163

CHAPTER SIX

Figure 6-1: Schematic Diagram of nNOS in Skeletal Muscle	176
Figure 6-2: Schematic Diagram of nNOS and the NMDA Receptor	178
Figure 6-3: MAGUK Domain Protein Structure	180
Figure 6-4: PSD-93 Immunolocalization in Purkinje Neurons	182
Figure 6-5: Schematic Diagram of <i>Drosophila</i> Photoreceptors	184
Figure 6-6: Mislocalization of TRP in <i>inaD</i> mutants	186
Figure 6-7: Mislocalization of Let-23 receptor in <i>lin-7</i> mutants	188
Table 6-1: A Sampling of PDZ Domain Containing Proteins	189

CHAPTER ONE

Introduction

An Overview of Neuronal Nitric Oxide Synthase and Synaptic PDZ Proteins

Introduction

Nitric oxide (NO) functions as a major endogenous mediator. Though NO was first identified as the endothelial derived relaxing factor, it is now clear that the brain is the primary source of NO in the body. In the peripheral nervous system, where physiological functions for NO are best characterized, NO often acts like a classical neurotransmitter. In the intestine, neuronal NO synthase (nNOS) is concentrated in axonal varicosities in a subset of myenteric neurons (Bredt et al., 1990). Calcium influx through N-type channels activates nNOS via calmodulin (Mashimo et al., 1996). NO then diffuses into the adjacent smooth muscle, where it stimulates guanylyl cyclase and elicits relaxation. Mutant mice deficient in nNOS have severe intestinal dysfunction and a hypertrophic stomach (Huang et al., 1993; Mashimo et al., 1996).

Functions for NO in the CNS remain more elusive. Pharmacological studies with NOS inhibitors initially provided evidence that NO mediates aspects of synaptic plasticity including hippocampal long term potentiation (LTP) (Schuman and Madison, 1994). NO may influence synaptic efficacy by regulating neurotransmitter release (Meffert et al., 1996). While physiological levels of NO serve signaling functions, excess NO causes excitotoxic brain injury. The primary stimulus for NO synthesis in brain is calcium influx through N-methyl-D-aspartate (NMDA) type glutamate receptors.

To understand NO actions in brain requires identification of the functional connection of NOS with NMDA receptors. This selectivity is mediated, in part, by compartmentalization of nNOS with NMDA receptors at certain synaptic sites (Aoki et al., 1993). Synaptic localization is regulated by the N-terminus of nNOS which encodes a PDZ protein motif (Brenman et al., 1996) (Figure 1-1). Recent studies from numerous laboratories indicate that

PDZ domains are modular protein motifs that are implicated in signal transduction in neurons and other cells. Indeed the PDZ domain of nNOS appears to tether the synthase to protein complexes at the postsynaptic density that contain NMDA receptors.

Nitric oxide and skeletal muscle

Although endogenous NO was originally appreciated as a mediator of smooth muscle relaxation, more recent studies indicate a role for NO in skeletal muscle. nNOS mRNA is expressed at high levels in human skeletal muscle (Nakane et al., 1993), where nNOS mRNA is alternatively spliced yielding a muscle specific isoform, nNOS μ (Silvagno et al., 1996). nNOS μ contains an additional 102 base pair exon, which is inserted between exons 16 and 17 and encodes a 34 amino acid insert between the calmodulin- and FMN-binding domains (Figure 1-1) (Silvagno et al., 1996). In muscle, nNOS protein is enriched in fast twitch muscle fibers where NO opposes contractile force (Kobzik et al., 1994). Additionally, NO produced by cultured myocytes facilitates myoblast fusion (Lee et al., 1994).

A striking aspect of skeletal muscle nNOS is that the synthase is concentrated beneath the plasma membrane or sarcolemma. nNOS occurs at skeletal muscle sarcolemma owing to its association with the dystrophin complex (Brenman et al., 1995). Patients with Duchenne muscular dystrophy and *mdx* mice that lack dystrophin evince a loss of nNOS from the sarcolemma. Certain mutations in the rod like domain of dystrophin that cause Becker's dystrophy result in loss of sarcolemmal nNOS but not other components of the dystrophin complex (Chao et al., 1996).

The loss of dystrophin in Duchenne dystrophy disrupts the normal link between the extracellular matrix and the myofiber cytoskeleton (Campbell, 1995). This results in sarcolemmal damage and myofiber necrosis. Sarcolemmal instability in Duchenne dystrophy leads to repeated rounds of myofiber degeneration and subsequent regeneration. Because dysregulation of nNOS mediates neuronal injury in stroke (Huang et al., 1994), redistribution of nNOS from sarcolemma to cytosol in Duchenne dystrophy could conceivably contribute to myofiber necrosis. On the other hand, NO also plays a role in cellular differentiation and the loss of sarcolemmal nNOS signaling may contribute to failed muscle regeneration in Duchenne dystrophy.

A genetic approach using nNOS^{-/-} mouse has been recently employed to determine whether nNOS mediates certain properties of muscle that are specifically abnormal in Duchenne dystrophy (F. Silvagno and D.S.B., unpublished). These studies show that mice lacking nNOS^{-/-} in muscle do not suffer from sarcolemmal instability characteristic of Duchenne dystrophy. On the other hand, myoblasts from nNOS mutant mice hyperproliferate and show impaired differentiation in culture (F. Silvagno and D.S.B., unpublished). A similar role for NO in suppressing proliferation of developing *Drosophila* has recently been reported indicating that NO may play a general role in growth arrest (Kuzin et al., 1996). An alteration in myocyte differentiation is also evident in muscular dystrophy, and this may be related to loss of sarcolemmal nNOS.

Detailed biochemical studies have been used to determine the specific protein in the dystrophin complex that binds to nNOS. This work shows that the PDZ protein motif at the N-terminus of nNOS binds to a similar motif near the N-terminus of syntrophin (Chao et al., 1996), a dystrophin associated

protein. Syntrophin directly binds to dystrophin and links nNOS to the dystrophin complex. It seems likely that the PDZ domain of syntrophin also associates with other muscle proteins. Sarcolemmal loss of syntrophin associated proteins in Duchenne dystrophy may participate in the loss of sarcolemmal integrity that accompanies this disease.

Highest densities of nNOS in myofibers occur at the endplate in the depths of the postsynaptic folds, similar to the distribution of dystrophin and syntrophin. Muscle-derived NO mediates activity-dependent synaptic suppression in myocyte-neuronal co-cultures (Wang et al., 1995). Through this mechanism NO may help prevent polyneuronal innervation of myofibers. Synaptic suppression by NO appears to be mediated by cGMP, and cGMP dependent protein kinase is concentrated at neuromuscular junctions together with nNOS (Chao et al., 1997).

Nitric oxide and hippocampal synaptic plasticity

NO is a lipophilic molecular mediator that readily diffuses across cellular membranes. NO can therefore contribute to both anterograde and retrograde signaling at the synapse. These properties make NO uniquely suited to mediate induction of hippocampal long term potentiation, a process that requires information transfer from the postsynaptic to the presynaptic terminal. Indeed, studies with NOS inhibitors suggest a role for NO in some but not all forms of hippocampal LTP. Injection of NOS inhibitors into the postsynaptic CA1 pyramidal neuron blocks LTP consistent with the proposal that NO functions as a retrograde messenger (Schuman and Madison, 1994).

The role for NO in LTP has been quite controversial. Some investigators find no role for NO in LTP and others find that NO plays only a

minor role. These disparate findings may be resolved by a recent study with NOS mutant mice. Both endothelial NOS (eNOS) and nNOS activities are found in the hippocampus (O'Dell et al., 1994). Mice that lack either eNOS or nNOS have essentially normal LTP, while mutant mice deficient in both eNOS and nNOS have substantially decreased LTP in the stratum radiens but have normal LTP in the stratum oriens (Son et al., 1996). Therefore, it appears that NO plays a necessary role in synaptic plasticity at some but not all hippocampal synapses.

Nitric oxide neurotoxicity

A toxic role for endogenous NO was first characterized in the immune system, where NO mediates certain cytotoxic actions of macrophages (Hibbs et al., 1987). In addition to killing tumor cells and pathogens, NO can be neurotoxic. The primary stimulus for NO synthesis in central neurons is activation of NMDA type glutamate receptors (Garthwaite, 1991). Overactivity of NMDA receptors is implicated in numerous neurodegenerative processes including stroke, Alzheimer's disease, Huntington's chorea, and amyotrophic lateral sclerosis (Choi, 1994). A causal role for NO in NMDA mediated degeneration was first implied by neuroprotective effects of NOS antagonists in culture (Dawson et al., 1991). Recent studies with NOS deficient mice indicate that endogenous NO plays a central role in neurodegenerative processes. Mice lacking nNOS are resistant to a variety of neurotoxic insults including cerebral ischemia, MPTP-induced Parkinsonism, and striatal malonate lesions (Huang et al., 1994; Przedborski et al., 1996; Schulz et al., 1996). On the other hand, cerebral ischemic lesions are enhanced in mutant mice lacking eNOS (Huang et al., 1996). Taken together

these studies suggest that excess NO biosynthesis by neurons contributes to cell death while endothelial-derived NO is neuroprotective, presumably by dilating blood vessels.

Because of the urgent need for effective therapeutics for treatment of neurodegenerative diseases, numerous groups and pharmaceutical companies have explored the use of NOS inhibitors in stroke and other excitotoxic processes. As might be expected, NOS inhibitors that antagonize both eNOS and nNOS are not effective neuroprotective agents because they compromise cerebral blood flow (Huang et al., 1994). However, drugs that selectively inhibit nNOS effectively prevent neuronal injury in animal models of stroke (Zhang et al., 1996) and Parkinson's disease (Hantraye et al., 1996).

Synaptic regulation of nitric oxide

nNOS is a calcium / calmodulin dependent enzyme and is therefore regulated by the steep gradients of calcium that occur in the vicinity of open calcium channels. In brain, NOS is functionally coupled to Ca^{2+} influx through N-methyl-D-aspartate (NMDA) type glutamate receptors (Garthwaite and Boulton, 1995), while other Ca^{2+} pools are poorly linked to NOS. Many of the diverse synaptic functions of NO in brain are intrinsically linked to the NMDA receptor. Thus, NO mediates synaptic plasticity associated with the NMDA receptor but does not play a major role in other pathways. nNOS mutant mice are resistant to NMDA-mediate neurotoxicity but are sensitive to kainate-mediated injury (Dawson et al., 1996).

The mechanism for selective coupling of nNOS to calcium influx through NMDA receptors appears to involve targeting of nNOS to the

postsynaptic density. nNOS was first characterized from brain supernatants, which facilitated enzyme isolation. Subsequent studies demonstrate that the majority of nNOS in brain occurs in particulate fractions. Detailed ultrastructural analysis of primate visual cortex indicates that 85% of nNOS immunoreactive profiles are identified as axonal or dendritic profiles. nNOS immunoreactivity in spines is concentrated over thick postsynaptic specializations of plasma membranes (Aoki et al., 1993). Because NMDA receptors also reside at postsynaptic densities, NMDA receptors and nNOS occur in proximity to one another in some dendritic spines. The N-terminal PDZ domain of nNOS links the synthase to synaptic membranes in brain (Brenman et al., 1996). To identify molecules that anchor nNOS to the postsynaptic density, nNOS interacting proteins were obtained by two-hybrid analysis. The nNOS interacting proteins in brain, postsynaptic density-95 (PSD-95) and PSD-93 share homology to skeletal muscle syntrophin and contain PDZ protein motifs (Brenman et al., 1996). Again, nNOS interacts with PSD-95 / 93 through a PDZ / PDZ binding interface.

PSD-95 and related proteins are abundant components of the postsynaptic density in forebrain (Cho et al., 1992). They have a modular structure comprised of three PDZ domains, an SH3 domain and a C-terminal region homologous to yeast guanylate kinase. In addition to binding nNOS the PDZ domains of PSD-95 interact with the C-terminal tails of certain ion channels including the NMDA receptor (Kornau et al., 1995). PSD-95 can mediate assembly of a tight ternary complex containing nNOS and NMDA receptor subunits (K. Christopherson, W. A. Lim and D. S. B., submitted). This macromolecular complex likely underlies the efficient coupling of nNOS to calcium influx through NMDA receptors (Figure 1-2).

Modular PDZ domains and neuronal signal transduction

PDZ motifs, like those in nNOS and PSD-95, are common multifunctional protein-protein interaction domains that help organize signaling cascades. In addition to direct interaction of PDZ motifs, these domains can also bind to specific C-terminal peptide sequences. The PDZ domain of PSD-95 binds to peptides terminating Glu - (Ser / Thr) - X - (Val / Ile) - COOH (where X can be any amino acid). Numerous ion channels in brain including NMDA receptors (Kornau et al., 1995), Shaker type K⁺ channels (Kim et al., 1995), and certain inwardly rectifying K⁺ channels (Cohen et al., 1996), end with this consensus and are found to associate with PSD-95. An elegant structural study of a co-crystal of PSD-95 with its cognate C-terminal peptide explains the binding specificity (Doyle et al., 1996). A carboxylate binding loop in the PDZ domain interacts forms specific interactions with the C-terminal Val / Ile - COOH. A histidine residue in the PDZ domain forms a critical hydrogen bond with the hydroxyl side chain of the Ser / Thr at the -2 peptide position. While most PDZ domains have a conserved carboxylate loop, the histidine residue which binds the peptide Ser / Thr is not generally conserved suggesting that other PDZ domains likely bind distinct C-terminal peptides

Two recent studies demonstrate systematic methods for identifying the specific C-terminal ligands for orphan PDZ domains (Songyang et al., 1997; Stricker et al., 1997). One study used a chemical peptide library and determined the binding specificity for several distinct PDZ domains (Songyang et al., 1997). This work identifies two families of PDZ domains. One family, including PDZ domains of PSD-95 selected peptides with the consensus Glu - (Ser / Thr) - X - (Val / Ile) - COOH. In contrast, another

family of PDZ domains, including those of LIN-2, p55 and Tiam-1 selected peptides with three hydrophobic C-terminal amino acids. The second study used a C-terminal peptide display strategy to select ligands for PDZ domains (Stricker et al., 1997). This work showed that the PDZ domain of nNOS binds best to peptides terminating Gly-Asp-X-Val-COOH. Preference for Asp at the -2 peptide position is mediated by Tyr-77 of nNOS and mutating this residue to His changes the binding specificity from Asp-X-Val-COOH to Thr-X-Val-COOH. Guided by the Asp-X-Val-COOH consensus, candidate nNOS interacting proteins were identified including glutamate and melatonin receptors. These studies emphasize a general role for PDZ domains in binding specific sequences at the C-termini of protein targets (Table 1-1).

Molecular scaffolds such as PSD-95 contain multiple PDZ domains that help assemble macromolecular signaling complexes at the postsynaptic density. A critical mechanism of synaptic plasticity involves dynamic regulation of ion channel interactions with the cytoskeleton. PKA and CamK mediate hippocampal synaptic plasticity (Kandel and Abel, 1995; Nicoll and Malenka, 1995) perhaps by causing a redistribution of ion channels at the postsynaptic density of "silent synapses" (Isaac et al., 1995; Liao et al., 1995). A recent study indicates that certain K⁺ channel interactions with PSD-95 are regulated by PKA phosphorylation (Cohen et al., 1996). All class 2.0 inwardly rectifying potassium channels (IRKs) terminate with a PSD-95 binding sequence of Arg-Arg-Glu-Ser-X-Ile. Importantly, the critical C-terminal Ser also occurs in a perfect consensus for phosphorylation by cyclic nucleotide regulated kinases. Indeed, elevations of cAMP lead to phosphorylation of the -2 serine in the tail of Kir 2.3. This phosphorylation causes a rapid dissociation of Kir 2.3 from PSD-95 (Cohen et al., 1996). Since IRK channels set the resting membrane potential, redistribution of these channels would

alter the excitability of the synapse and could play a role in synaptic plasticity. Because cGMP dependent protein kinase has a similar substrate specificity as PKA, it is possible that NO mediated synaptic plasticity may also utilize this pathway.

Conclusions

NO signaling modulates diverse aspects of synaptic function. Because NO is not cleared from the synapse by uptake pumps or degradatory pathways, regulation of NO biosynthesis is critical. A complex mechanism for regulation involves targeting of nNOS to specific synaptic sites. One class of interacting proteins, including PSD-95 in brain and syntrophin in skeletal muscle, binds to the N-terminus of nNOS in a PDZ / PDZ domain interaction. Other proteins may also regulate nNOS at the synapse. In fact, a protein inhibitor of nNOS (PIN-1) was recently found to bind within the catalytic domain of nNOS and block activity (Jaffrey and Snyder, 1996). A detailed characterization of the nNOS complex is critical for understanding how synaptic nNOS activity participates in synaptic plasticity and neurodegeneration. PSD-95 contains multiple protein-binding motifs, and it is likely that other associated proteins also modulate nNOS signaling.

Figure 1-1. Schematic alignment of the NOS proteins. The neuronal NOS (nNOS) uniquely has a N-terminal PDZ motif and can be alternatively spliced to yield nNOS μ . Abbreviations: CaM, calmodulin; FMN, flavin adenine mononucleotide; FAD, flavin adenine dinucleotide; eNOS, endothelial NOS; iNOS, inducible NOS.

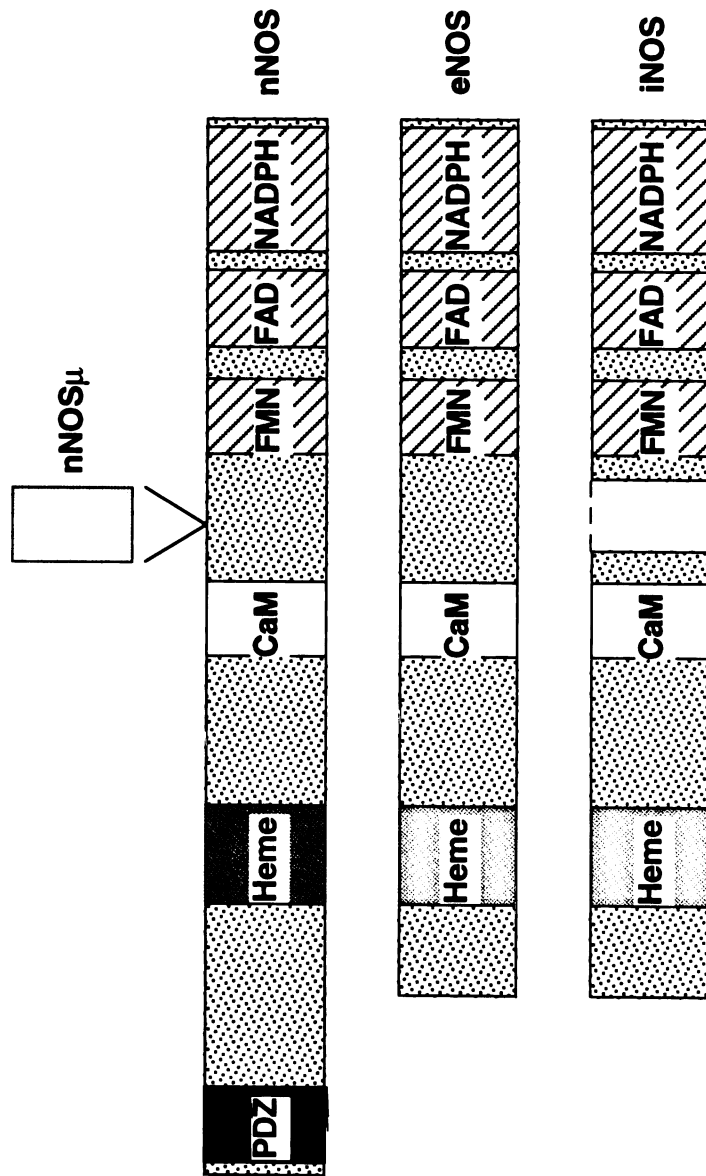


Figure 1-1

Figure 1-2. Schematic model depicting postsynaptic interactions of PSD-95 with NMDA receptor (NMDAR) and nNOS. Shaded boxes in PSD-95 and nNOS correspond to PDZ motifs. Abbreviation: CaM, calmodulin; Glu, glutamate; L-Arg, L-arginine; NO, nitric oxide.

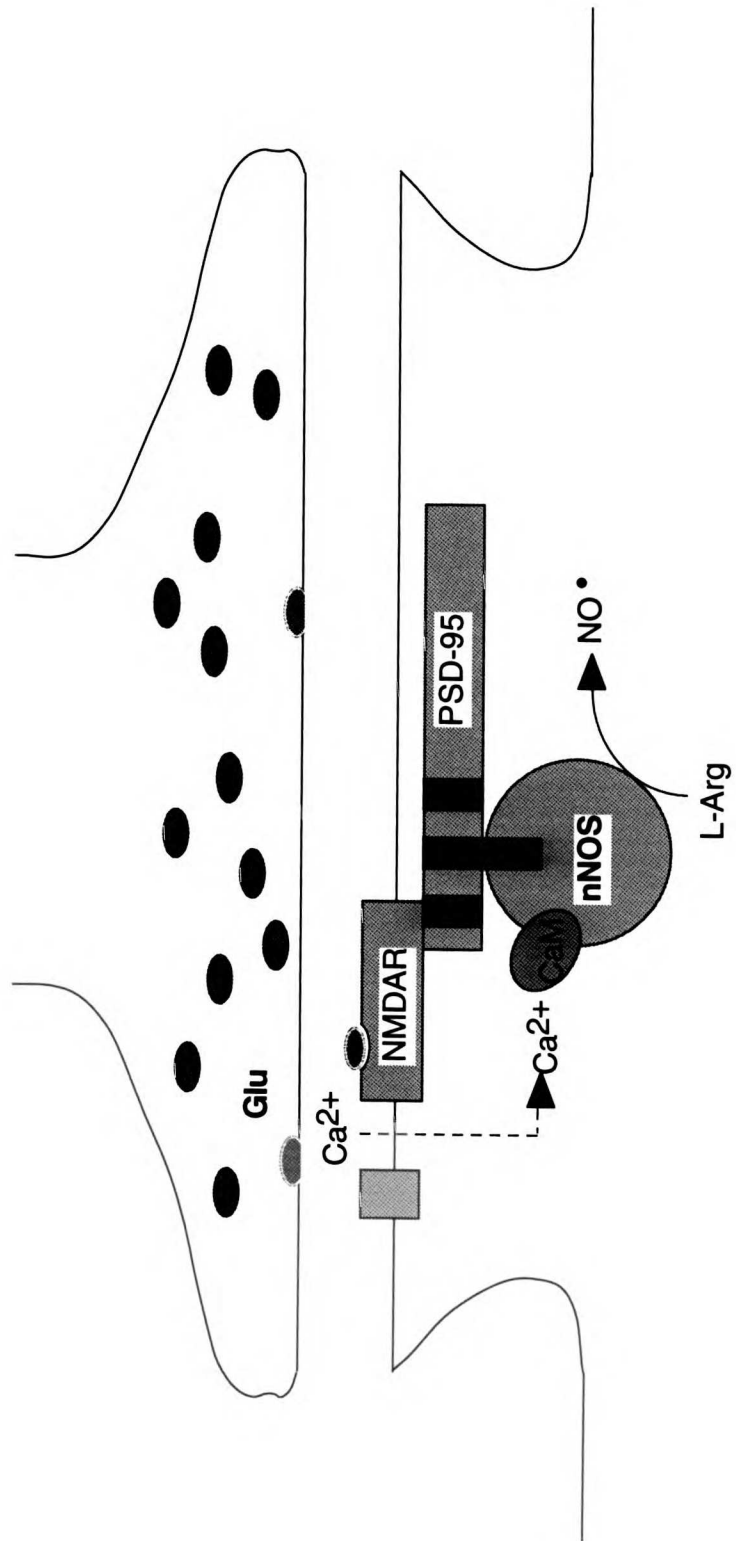


Figure 1-2

Table 1-1.

PDZ domains differentially bind specific carboxy-terminal peptides

<u>Classification</u>	<u>C-terminal peptide</u>				<u>Examples</u>	
		Positions				
	-3	-2	-1	0	PDZ	
Group I	E	(T/S)	X	(V/I)	PSD-95	NMDAR, Kv1.0, Kir 2.0
Group II	X	(Y/F)	X	(F/V)	p55	Glycophorin C (?)
Group III	G	D	X	V	nNOS	Melatonin rec. (?), GluR 6C (?)

CHAPTER TWO

Nitric Oxide Synthase Complexed with Dystrophin and Absent from Skeletal Muscle Sarcolemma in Duchenne Muscular Dystrophy

Abstract

Nitric oxide (NO) is synthesized in skeletal muscle by neuronal NO synthase (nNOS), which is localized to sarcolemma of fast twitch fibers. Synthesis of NO in active muscle opposes contractile force. We show that nNOS partitions with skeletal muscle membranes due to association of nNOS with dystrophin, the protein mutated in Duchenne muscular dystrophy (DMD). The dystrophin complex interacts with an N-terminal domain of nNOS, which contains a GLGF motif. *mdx* mice, and humans with DMD, evince a selective loss of nNOS protein and catalytic activity from muscle membranes, demonstrating a novel role for dystrophin in localizing a signaling enzyme to the myocyte sarcolemma. Aberrant regulation of nNOS may contribute to preferential degeneration of fast twitch muscle fibers in DMD.

Introduction

Nitric oxide (NO) is a major endogenous mediator involved in diverse developmental and physiological processes (for review see, (Bredt and Snyder, 1994; Marletta, 1993; Moncada and Higgs, 1993; Nathan and Xie, 1994). Because NO is a short lived free radical, regulation of signaling occurs largely at the level of NO biosynthesis. Three mammalian NO synthase (NOS) genes have been identified and each forms NO from the guanidino nitrogen of L-arginine in a unique cytochrome P450 type reaction which consumes NADPH. Endothelial and neuronal NOS enzymes (eNOS and nNOS, or types I and III respectively) are discretely expressed in specific tissues and rapidly transduce signaling events in a calcium dependent manner. eNOS activity accounts for endothelium dependent blood vessel relaxation, while nNOS occurs discretely in a variety of cell types including neurons, epithelial cells, mesangial cells and skeletal muscle cells. Physiological actions for nNOS have been best characterized in the peripheral nervous system where NO functions as a non-adrenergic-non-cholinergic transmitter in numerous pathways including the gastrointestinal and urogenital tracts (Bult et al., 1990). In contrast to these calcium dependent enzymes, a largely inducible calcium independent NOS (iNOS or Type II) is expressed at highest levels in immunologically activated cells. NO_x formed in this pathway functions in a non-specific immune response that mediates certain cytotoxic and bacteriocidal actions of activated macrophages (Hibbs et al., 1987).

In addition to controlling diverse cellular processes, NO also participates in certain pathophysiological conditions. Deregulation of nNOS in brain is associated with glutamate receptor overactivity and contributes to

neuronal damage in animal models of stroke (Dawson et al., 1992). Mutant mice lacking nNOS are relatively resistant to neuronal damage following focal cerebral ischemia. iNOS activity contributes to cellular damage in a variety of immunological disorders such as rheumatoid arthritis and septic shock (Huang et al., 1994).

Maintenance of physiological NO signaling in the face of this potential toxicity requires tight regulation of NOS at numerous points. In addition to transcriptional control, NOS proteins are all regulated by calmodulin (Bredt and Snyder, 1990; Cho et al., 1992), which links NO formation to increases in cellular calcium. A more complex level of regulation is reflected by targeting of NOS proteins to intracellular membranes. This subcellular targeting restricts NO signaling to specific targets within a limited microenvironment while minimizing aberrant toxic pathways. Membrane association of endothelial type NOS (eNOS) is mediated by two fatty acid modifications (Robinson et al., 1995). Co-translational N-terminal myristoylation of eNOS is necessary for membrane association. In addition, eNOS is post-translationally palmitoylated by a dynamic process regulated by a specific agonist, bradykinin. nNOS, which lacks consensus sequences for fatty acid modification, also occurs largely in particulate fractions (Hecker et al., 1994). The majority of nNOS immunoreactivity in neurons is associated with rough endoplasmic reticulum and specialized electron dense synaptic membrane structures while in skeletal muscle nNOS is associated with the sarcolemma (Kobzik et al., 1994).

Understanding mechanisms for targeting of nNOS within cells will help identify pathways associated with NO signaling and how disruption of this transduction machinery contributes to disease processes. The regular and defined structure of skeletal muscle makes it an ideal tissue in which to

address these questions. Furthermore, recent studies identify a major role for NO in skeletal muscle. nNOS is expressed at higher levels in human skeletal muscle than in human brain (Nakane et al., 1993). In mature skeletal muscle nNOS is enriched in fast twitch muscle fibers where NO opposes contractile force (Kobzik et al., 1994). Additionally, NO produced by cultured myocytes facilitates myoblast fusion (Lee et al., 1994) and mediates activity-dependent synaptic suppression in myocyte-neuronal co-cultures (Wang et al., 1995).

These physiological actions of NO in muscle are facilitated by restriction of nNOS protein to the sarcolemmal membrane (Kobzik et al., 1994). The sarcolemma of skeletal muscle is a complex structure reinforced by an actin containing cytoskeleton. In addition to ubiquitous structural elements such as spectrin, skeletal muscle sarcolemma contains a unique network formed around dystrophin and related proteins (Ervasti and Campbell, 1993). Dystrophin was originally identified by positional cloning as the gene product mutated in Duchenne muscular dystrophy (DMD) (Monaco et al., 1986). Subsequent studies have identified a family of intracellular and transmembrane glycoproteins in a dystrophin associated complex which links the extracellular matrix with the actin based cytoskeleton. Recent studies indicate a major role for this complex in neuromuscular development and disease. α -Dystroglycan, an extracellular glycoprotein linked to dystrophin serves as a physiologic receptor for agrin, which mediates clustering of acetylcholine receptors (Campanelli et al., 1994; Gee et al., 1994). However, these conclusions are controversial. On the other hand, disruption of dystrophin or other proteins in this complex results in muscular dystrophy in both humans and animals (Campbell, 1995). Despite these data, it not known how the dystrophin complex mediates signal transduction, nor is it clear why

disruption leads to muscle disease. Indeed, none of the previously identified dystrophin associated proteins have known catalytic activities.

In this report we demonstrate that sarcolemmal nNOS is complexed with dystrophin in skeletal muscle. The dystrophin complex interacts with the N-terminus of nNOS which contains a motif of previously unknown function present in a family of cytoskeletal associated proteins including syntrophins, known binding partners of dystrophin. In human DMD and *mdx* mice, which lack dystrophin, nNOS is absent from the sarcolemma and accumulates in the cytosol. This derangement of nNOS is specific for dystrophin abnormalities as nNOS disposition is unaffected in other muscular diseases. These observations provide the first molecular evidence for a specific intracellular signaling molecule linked to the dystrophin associated complex and suggest roles for NO in processes of neuromuscular development and disease associated with this complex.

Results

nNOS but not eNOS is Present in Cytoskeletal Extracts from Skeletal Muscle

Previous work indicates that the majority of nNOS in human and rat skeletal muscle occurs in membrane fractions (Nakane et al., 1993). We similarly find that approximately 80% of the total nNOS protein in mouse skeletal muscle associates with the particulate fractions (data not shown). To understand mechanisms for membrane association of nNOS, we characterized the extractability of nNOS from mouse quadriceps and found that nNOS is anchored to both microsomal membranes and to cytoskeleton (Figure 2-1b). Thus the majority of nNOS protein remained membrane associated following extensive washing of skeletal muscle heavy microsomes (Mitchell et al., 1983) with 0.5 M NaCl. Solubilization of washed membranes with 0.5% Triton X-100 released about 1/2 of this particulate nNOS with the remainder in an insoluble cytoskeletal pellet (Figure 2-1b). By contrast, eNOS, which is membrane associated due to N-terminal myristoylation (Busconi and Michel, 1993), was quantitatively solubilized from these same preparations by Triton X-100 (figure 2-1c).

This differential fractionation suggested that unique determinants in nNOS anchor this isoform to the skeletal muscle cytoskeleton. The amino acid sequence of nNOS contains a 230 amino acid N-terminal domain which is not present in eNOS (figure 2-2a). Beyond this extended N-terminus of nNOS the two proteins share >60% sequence identity. Similar enzymatic activities of eNOS and nNOS suggest that the unique N-terminus of nNOS may not be required for catalytic activity. To analyze the role of the extended

N-terminal region of nNOS we constructed a deletion mutant, nNOS Δ 1-226, lacking the first 226 amino acids. Expression vectors containing full length nNOS and nNOS Δ 1-226 were transiently transfected into COS cells. Three days following transfection NOS catalytic activity was measured in tissue homogenates. Kinetic characteristics of NOS activity for the nNOS Δ 1-226 mutant was essentially indistinguishable from the full length isoform. Both constructs displayed similar V_{max} , K_m for arginine as well as regulation by calcium / calmodulin (figure 2-2 c, d).

Rather than regulating catalytic activity we reasoned that the N-terminal domain of nNOS may instead target nNOS to skeletal muscle sarcolemma. Within this domain, nNOS contains a 66 amino acid motif which bears homology to a heterogeneous family of signaling enzymes that share the property of being localized to specialized cell-cell junctions (figure 2-2b). Proteins containing this motif, which is named "GLGF" for a conserved tetrapeptide (Cho et al., 1992), include: *dlg-1*, the product of the lethal discs-large tumor suppressor gene that localizes to the undercoat of the septate junction in *Drosophila* (Bryant and Woods, 1992); disheveled (*dsh*), a gene required for planar cell polarity in *Drosophila*; PSD-95, a brain-specific protein that localizes to the post-synaptic density in mammals (Cho et al., 1992); ZO-1, 2, peripheral membrane proteins that localizes to tight junctions (zona occludens) of epithelial and endothelial cells (Jesaitis and Goodenough, 1994; Willott et al., 1993); and certain protein tyrosine phosphatases, such as PTP1E, which are thought to localize at the junction between the plasma membrane and the cytoskeleton. Interestingly, we and others (Ponting and Phillips, 1995) noted homology to syntrophins (*syn*), a family of recently cloned dystrophin-binding proteins, which co-localize with NOS beneath the sarcolemmal membrane of skeletal muscle (Adams et al., 1993; Ahn et al., 1994). In fact,

syntrophins are more closely related to NOS in this domain than any other known gene (figure 2-2b), suggesting a possible role for dystrophin in regulating the sarcolemmal localization of nNOS.

Association of nNOS and Dystrophin in Skeletal Muscle Extracts

We evaluated possible association of nNOS with dystrophin by means of succinylated wheat germ agglutinin (sWGA)-Sepharose affinity chromatography. nNOS which lacks glycosylation sites is not expected to adhere to a wheat germ column while dystrophin, which is bound to a glycoprotein complex, is routinely purified by this technique (Ervasti et al., 1991). To ensure specificity of this association we conducted parallel experiments with *mdx* mice which specifically lack dystrophin due to a non-sense mutation (Sicinski et al., 1989), but express nNOS at near normal levels. Quadriceps from wild type and *mdx* mice were homogenized and solubilized in buffer containing 0.5 M NaCl and 1% digitonin. Solubilized homogenates were applied to succinylated (WGA)-Sepharose columns which were extensively washed with buffer containing 0.5 M NaCl and 0.5% triton X-100. Tightly bound proteins were affinity eluted with 0.3 M N-acetyl-D-glucosamine (NAG). Western blot analysis reveals presence of nNOS in NAG eluates from wild type but not *mdx* tissue (2- 3a).

We also conducted analogous experiments and evaluated association of dystrophin with nNOS using a 2' 5' ADP agarose column, which tightly binds nNOS at its C-terminal NADPH binding motif (Bredt and Snyder, 1990). Dystrophin has no nucleotide binding site (Koenig et al., 1988) and would not be expected to adhere to a 2' 5' ADP column. To ensure specificity of a potential dystrophin / nNOS interaction, we conducted parallel purifications

from skeletal muscle of nNOS "knockout" mice which are devoid of nNOS protein (Huang et al., 1993) yet express dystrophin at normal levels. Salt washed heavy microsomes from wild type and nNOS knockout mouse quadriceps were solubilized in 1% digitonin and allowed to adhere to 2' 5' ADP agarose columns. The columns were extensively washed with buffers containing 0.5 M NaCl and 0.5% Triton X-100. Tightly bound proteins were eluted with buffer containing 20 mM NADPH. Western blot analysis revealed presence of dystrophin in NADPH eluate fractions from wild type but not nNOS knockout tissue (figure 2-3b).

To directly evaluate binding of dystrophin associated complexes to the N-terminal domain of nNOS we coupled a protein containing glutathione-S-transferase (GST) fused to the first 299 amino acids of nNOS to glutathione beads. The GST-nNOS (1-299) beads and control GST beads were incubated with solubilized homogenates of mouse skeletal muscle. After extensive washing of the beads, bound proteins were eluted with sample buffer. Western blotting indicated that GST-nNOS (1-299) beads but not control beads retained dystrophin protein (figure 2-3c). Other proteins evaluated, such as myosin, were retained in very small but equivalent amounts by both GST and GST-nNOS (1-299) beads (data not shown) indicating specificity of the association with dystrophin.

In order to quantitate enrichment of nNOS by sWGA chromatography we conducted larger scale purifications from rat skeletal muscle tissue. Western blot analysis for nNOS in equally loaded fractions (5 μ g / lane) from sWGA chromatography revealed a striking enrichment of nNOS in the NAG eluate (figure 2-3d). nNOS was typically purified approximately 100-fold by sWGA chromatography; thus, equal nNOS immunoreactivity was detected in 20 μ g "load" and 200 ng NAG eluate fractions (data not shown). To evaluate

the retention of nNOS by this column, fractions from sWGA chromatography were reloaded onto SDS gels with 6-fold more total protein in "load" and "flow through" lanes (21 μ g) than in wash and eluate lanes (3.5 μ g) (figure 2-3e). Clearly, the majority of particulate nNOS adhered to the sWGA column as minimal nNOS is present in the "flow through". We probed this same blot with antisera to dystrophin and found that essentially all the solubilized dystrophin adhered to the sWGA column and dystrophin was enriched to a somewhat greater degree than nNOS by this procedure (~250 fold purification). The somewhat higher recovery of dystrophin appears to be due to slow dissociation of nNOS from the sWGA column (dystrophin complex) as evidenced by some "bleeding" of nNOS with the 500 mM NaCl and triton X-100 wash steps (figure 2-3e). We also probed this same blot with an antibody to eNOS which is similar to nNOS but lacks a GLGF domain. eNOS did not specifically adhere to the sWGA column and only residual amounts of eNOS were found in NAG eluate fractions.

To determine whether nNOS associates with dystrophin complexes eluting from sWGA we conducted immunoprecipitation experiments. NAG eluate samples were incubated for one hour with a monoclonal antibody to a-dystrophin and immunocomplexes pelleted with anti-mouse IgG linked to protein A sepharose. Western blot analysis revealed potent immunoprecipitation of nNOS with 2.0 μ g / ml (12 nM) a-dystrophin. Control immunoprecipitations lacking the primary dystrophin antibody or containing an alternate monoclonal (a-myc, 12 nM) (BABCO, Berkeley, CA) did not precipitate detectable nNOS demonstrating specificity of the interaction. (figure 2-3f).

nNOS is Displaced from Sarcolemma and Accumulates in Cytosol of *mdx* Skeletal Muscle

Dystrophin contains binding sites for F-actin (Koenig et al., 1988) and b-dystroglycan (Ibraghimov-Beskrovnya et al., 1992), such that dystrophin partitions with both the cytoskeleton and the sarcolemma of skeletal muscle (Ervasti and Campbell, 1993). A similar distribution for skeletal muscle nNOS raises the possibility that the subcellular localization of nNOS is mediated by dystrophin binding. The absence of dystrophin leads to a dramatic reduction of other dystrophin-associated proteins, including a- and b-dystroglycan and syntrophins, in the sarcolemma of *mdx* mice (Ohlendieck and Campbell, 1991), and patients with DMD (Ervasti et al., 1990). We first evaluated the distribution of nNOS in *mdx* mice. Overall nNOS levels and enzyme activity were modestly decreased (~90% of control levels) in skeletal muscle from *mdx* mice (data not shown). Subcellular analysis revealed that nNOS distributed with dystrophin in membrane associated and cytoskeletal fractions from wild type skeletal muscle (figure 2-4 a, b). However, in preparations from *mdx* mouse, nNOS was quantitatively solubilized from microsomal membranes washed with 0.5 M NaCl; no nNOS protein was detected in detergent extract or cytoskeletal fractions (figure 2-4a).

NOS catalytic activity was also absent from particulate fractions of *mdx* skeletal muscle. As previously reported in rat (Kobzik et al., 1994), NOS activity was enriched in the particulate fraction of mouse skeletal muscle homogenates (Table 2-1). NOS activity in the soluble fraction of *mdx* skeletal muscle occurred at levels 75% greater than wild type, but NOS activity was not detectable in the particulate fraction from *mdx* muscle. In brain, NOS specific activity was nearly equivalent in soluble and particulate fractions.

This distribution was unchanged in *mdx* brain suggesting that proteins other than dystrophin anchor nNOS to neuronal membranes.

Immunohistochemical studies further identified an absence of sarcolemmal nNOS in *mdx* skeletal muscle. Using a polyclonal antiserum we found that nNOS immunofluorescence was restricted to the sarcolemma of a subset of skeletal muscle fibers (figure 2-5), which were previously noted to be fast twitch (Kobzik et al., 1994). However, nNOS immunoreactivity was absent from the sarcolemma of *mdx* muscle (figure 2-5). To determine whether this derangement of nNOS was specific for dystrophin abnormalities, we evaluated the distribution of nNOS in *dy* mice which have severe muscular dystrophy due to absence of an extracellular matrix protein, merosin, but have a normal distribution of dystrophin at the sarcolemma (Sunada et al., 1994). We found that nNOS is present normally at the sarcolemma of *dy* dystrophic mice (figure 2-5).

We conducted several control experiments to ensure for specificity of the immunofluorescence. No specific labeling was present in muscle tissues incubated with "pre-immune" serum or without primary antiserum (data not shown). Most importantly, labeling was not present in transgenic mice containing a targeted mutation (Huang et al., 1993) of the nNOS gene (figure 2-5).

nNOS is Absent from Skeletal Muscle Sarcolemma in Duchenne Muscular Dystrophy

In addition to animal models we evaluated the localization of nNOS in human muscle tissues. A total of twenty human tissues were evaluated including thirteen normal specimens, and seven from patients with DMD.

Skeletal muscle cross sections were processed for immunofluorescence for nNOS, dystrophin, and spectrin. All normal specimens showed colocalization of nNOS, dystrophin and spectrin beneath the sarcolemma of muscle fibers (figure 2-6 a, b) indicating that nNOS shares a similar distribution in both human and rodent skeletal muscle. In all seven biopsies from patients with DMD, the disruption of dystrophin resulted in absence of nNOS staining of sarcolemma (figure 2-6 a, b). Normal sarcolemmal labeling for spectrin confirmed that this structural cytoskeleton was not disrupted in the DMD tissues (figure 2-6a). Two independently raised nNOS antibodies (see methods) yielded similar immunofluorescent staining patterns, and no specific labeling was found in muscle sections incubated without primary antibody (figure 2-6c).

Absence of dystrophin in DMD results in disruption of the dystrophin associated glycoprotein complex and a dramatic reduction in overall levels of certain dystrophin associated proteins in muscle. To evaluate total nNOS levels in skeletal muscle tissues from human DMD, we conducted western blot analysis. Strikingly, we found that nNOS levels are dramatically reduced in human dystrophic muscle (figure 2-7a). Densitometric scanning of nNOS immunoreactive bands in equally loaded western blots revealed approximately 75% decrease of nNOS in DMD tissues. Western blots were also probed with an antibody to dystrophin which was essentially absent from all cases of DMD examined (figure 2-7b). Immunoblotting for spectrin confirmed that similar amounts of protein were loaded in all cases and that the structural cytoskeleton was intact (figure 2-7c).

Discussion

This study defines a role for the dystrophin complex in tethering nNOS to skeletal muscle sarcolemma. nNOS was originally characterized from brain cytosol which facilitated biochemical isolation of the enzyme (Bredt and Snyder, 1990). Subsequent studies indicate that the majority of nNOS activity and immunoreactivity is present in the particulate fraction of tissue extracts (Hecker et al., 1994; Kobzik et al., 1994). Thus over 60% of NOS activity is associated with the particulate fraction of cortical extracts (Hecker et al., 1994) and over 85% of NOS immunoreactivity in monkey visual cortex is identified as axonal or dendritic profiles (Aoki et al., 1993). Membrane association of eNOS is mediated by fatty acid modifications; however, particulate nNOS in skeletal muscle and brain (J.E.B and D.S.B, unpublished) is not solubilized by detergent extraction implying association of nNOS with the cytoskeleton. Our finding that nNOS remains membrane associated in brain of *mdx* mice implies that distinct nNOS anchoring complexes likely exist in other tissues. Possible candidates in brain include complexes formed about dystrophin related proteins, utrophin (Matsumura et al., 1992), and the 87 kD postsynaptic protein identified from *Torpedo* electric organ (Wagner et al., 1993) as well as homologues of protein 4.1, such as NF-2, which have been shown to bind GLGF domains *in vitro* (Marfatia et al., 1994).

The GLGF domain is a recently identified protein motif that is present in a heterogeneous family of enzymes. This observation has motivated suggestions that GLGF domains may regulate enzyme activities or alternatively that this motif may function as a protein-protein interaction domain (Cho et al., 1992). We find that deletion of the GLGF domain of

nNOS does not alter NOS catalytic activity in transfected cells. On the other hand, we find that sepharose beads linked to a 299 amino acid fusion protein containing the GLGF domain in nNOS selectively retain dystrophin from skeletal muscle extracts, indicating that this domain is capable of interacting with the dystrophin associated complex. The presence of GLGF domains in a diverse group of signaling enzymes suggests that some of these proteins may also interact with dystrophin complexes and that the family of dystrophin associated proteins may far exceed the structural glycoprotein complex which occurs stoichiometrically with dystrophin in skeletal muscle.

The association of nNOS with the dystrophin glycoprotein complex sheds light on protein interactions involved in maintaining the complex. Numerous data establish that the cysteine-rich and C-terminal domains of dystrophin are necessary and sufficient to establish the glycoprotein complex (Ahn and Kunkel, 1995; Campbell, 1995; Suzuki et al., 1994). Gel overlay assays suggest that determinants within the cysteine rich domain of dystrophin are necessary for binding b-dystroglycan which links dystrophin to the sarcolemma (Suzuki et al., 1994). The region(s) of dystrophin which interacts with syntrophin remains uncertain. The GLGF domain of syntrophin occurs near the N-terminus of the protein, analogous to the location of the GLGF domain in nNOS. *In vitro* binding assays show that the C-terminal half of syntrophins interacts with a protein fragment encoded by two C-terminal exons of dystrophin, exons 73 and 74 (Ahn and Kunkel, 1995; Suzuki et al., 1995; Yang et al., 1995). However, an *mdx* mouse containing a transgene for a truncated dystrophin protein lacking exons 71-74 fully restores syntrophins to dystrophin associated complexes in skeletal muscle (Rafael et al., 1994). Together with the results of this study these data suggest that the C-terminal half of syntrophins interacts with protein encoded by exons 73-74 of

UCSF LIBRARY

dystrophin, and that the GLGF domains of syntrophins and nNOS interact with a distinct region of dystrophin or perhaps with a distinct protein of the dystrophin complex. Interaction with this second site likely requires either post-translational modifications of dystrophin or the presence of other proteins which are not replicated by *in vitro* translation conditions.

These results suggest potential roles for nNOS in neuromuscular signaling and disease associated with dystrophin. The dystrophin associated glycoprotein complex has been proposed to mediate neurite-induced clustering of acetylcholine receptors via binding of α -dystroglycan to agrin (Campanelli et al., 1994; Gee et al., 1994), a neuron-derived component of the extracellular matrix (Nitkin et al., 1987). Binding of nNOS, and potentially other enzymes containing a GLGF motif (figure 2-2), to dystrophin, completes the link between the extracellular matrix and intracellular signal transducing enzymes. NO, which modulates synaptic connectivity (Wu et al., 1994) and synaptic efficacy (Schuman and Madison, 1994) in brain, may similarly influence formation and stabilization of neuromuscular synapses. Indeed, very recent studies show that NO serves as a retrograde messenger at neuromuscular synapses and mediates pre-synaptic suppression, a process that prevents polyneuronal innervation of muscle (Wang et al., 1995). Targeting of nNOS to dystrophin, which occurs at extrajunctional sarcolemma may help eliminate formation of extrajunctional synapses (Sealock et al., 1991). The dystrophin related protein, utrophin, is selectively present at the crests of neuromuscular synaptic folds (Bewick et al., 1993). The absence of nNOS at neuromuscular junctions of *mdx* mice (D.S.C., J.E.B. and D.S.B, in preparation) suggests that nNOS does not significantly interact with utrophin complexes and supports the notion that pre-synaptic suppression by NO may reflect its role in prevention of extrajunctional synapses.

Aberrant translocation of nNOS from sarcolemma to cytosol in DMD and *mdx* muscle has implications for the pathogenesis of muscular dystrophy. We are currently evaluating nNOS distribution in a variety of muscle diseases and dystrophies. In preliminary studies we find that nNOS occurs normally at the sarcolemma in human neurogenic muscle atrophy, central core disease, and in severe childhood autosomal recessive muscular dystrophy (D.S.C., K.A, J.E.B. and D.S.B, in preparation) suggesting specificity of the defect of nNOS in DMD. Whereas nNOS accumulates in the muscle cytosol of *mdx* mice we find that overall nNOS levels in DMD tissue are significantly decreased compared to normal human muscle. Reasons for this peculiar difference are not clear. *mdx* mice lack skeletal muscle dystrophin, yet display a considerably more mild phenotype than human DMD patients (Bulfield et al., 1984). It is therefore possible that disease progression in DMD causes transcriptional down-regulation of nNOS expression. This seems unlikely because we find that other animal and human muscle disorders are not associated with changes in nNOS levels. Alternatively, nNOS may be less stable in the cytosol of human skeletal muscle and would therefore be more rapidly degraded in DMD.

Derangement of NO metabolism and reaction of NO with superoxide is responsible for tissue damage in certain diseases including autoimmune (Nathan and Xie, 1994) and neurodegenerative processes (Dawson et al., 1992; Huang et al., 1994). Free radical oxygen intermediates, which occur at high levels in skeletal muscle (Reid et al., 1992; Reid et al., 1992), are known to contribute to cytotoxic damage in various muscle diseases including DMD (Davison et al., 1988). Altered regulation of nNOS in dystrophic muscle may augment the toxic interaction of NO and superoxide and contribute to myofiber necrosis. Fast-twitch muscle fibers are preferentially affected in

DMD. However, dystrophin and previously identified components of the dystrophin associated complex are equally distributed between fast- and slow-twitch fibers (Ohlendieck et al., 1991). The selective enrichment of nNOS in fast twitch muscle fibers (Kobzik et al., 1994) could help explain the preferential degeneration of this fiber type seen in DMD (Webster et al., 1988). Selective nNOS antagonists, such as 7-nitroindazole, which protect neurons from excitotoxic damage associated with cerebral ischemia (Huang et al., 1994) and enhance force production in skeletal muscle may also be therapeutic in DMD.

Materials and Methods

Tissue Extraction and Western Blot Analysis

Mouse quadriceps skeletal muscle was homogenized in 10 volumes (w/v) of Buffer A (25 mM TrisHCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF) and heavy microsomes were prepared by a standard protocol with minor modifications (Mitchell et al., 1983). Nuclei were pelleted by centrifugation at 1,000 x g. The supernatant was then centrifuged at 20,000 x g yielding supernatant "S1". The resulting heavy microsomal pellet was resuspended in buffer A containing 500 mM NaCl, incubated for 30 min at 4°C with agitation and centrifuged at 15,000 x g yielding supernatant "S2". This resulting pellet was resuspended in buffer A containing 500 mM NaCl + 0.5% triton X-100, incubated for 30 min at 4°C with agitation and centrifuged at 15,000 x g yielding supernatant "S3" and a final pellet "P".

Tissue extracts were resolved by SDS-PAGE (7.5% acrylamide) and proteins were transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were incubated overnight with primary antisera (bNOS, Transduction labs, 1:250; eNOS, Transduction labs, 1:250; dystrophin, Novacastra, 1:100; spectrin, Novacastra, 1:100) diluted in TrisHCl buffered saline (TBS) containing 3% bovine serum albumin (BSA). Immunoreactive bands were visualized by ECL according to the manufacturer's specifications (Amersham).

Affinity Chromatography

For sWGA affinity chromatography mouse quadriceps from wild type (WT) and *mdx* mice were homogenized and solubilized in 10 volumes of buffer B (50 mM TrisHCl pH 7.4, 200 mM NaCl, 1mM PMSF) containing 1% digitonin. 4 mg of solubilized membranes from wild type and *mdx* mice were circulated for 1 hr with 250 μ L of sWGA agarose (Vector Labs) at 4 $^{\circ}$ C. Columns were washed sequentially with 5 mL of buffer B containing 0.1% digitonin, buffer B containing 0.1% digitonin and 500 mM NaCl, buffer B containing 500 mM NaCl and 0.5% triton X-100. Columns were affinity eluted with 1 mL of buffer B containing 0.3 M N-acetyl-D-glucosamine (NAG) with 0.1 % digitonin. Chromatography of rat skeletal muscle followed a similar procedure except 20 mg of solubilized membranes were loaded onto 1 ml of sWGA which was washed with 10 ml of buffers containing 500 mM NaCl and triton X-100 and eluted with 3 ml of 0.3 M NAG.

For 2'5' ADP affinity chromatography, mouse quadriceps from wild type (WT) and nNOS knockout (NOS $^{-/-}$) mice were homogenized in 10 volumes (w/v) of Buffer B and heavy microsomes were prepared and solubilized in buffer B containing 1% digitonin. 4 mg of solubilized membranes from wild type (WT) and nNOS knockout (NOS $^{-/-}$) mice were applied to 150 μ L columns of 2'5' ADP agarose (Sigma). Columns were sequentially washed with 5 ml of buffer B containing 0.1% digitonin, 1 ml of buffer B containing 500 mM NaCl and 0.1% digitonin, 1 ml of buffer B containing 500 mM NaCl and 0.5% triton X-100. Columns were affinity eluted with 1 mL of buffer B containing 20 mM NADPH and 0.1% digitonin.

GST-Fusion Proteins

A GST-nNOS (1-299) construct was generated by cloning sequences encoding the first 299 amino acids of rat brain NOS into the EcoR1 site of the pGEX-2T vector. GST fusion proteins were expressed in *Escherichia coli* and purified on glutathione sepharose beads according to the manufacture's specifications ((Smith and Johnson, 1988); Pharmacia)). Two mg of solubilized skeletal muscle membranes were incubated with control (GST) or GST-nNOS (1-299) beads for 1 hour. Beads were washed with buffer containing 0.5% triton X-100 + 300 mM NaCl, and proteins eluted with 150 µl of loading buffer.

Dystrophin Immunoprecipitation

Monoclonal antibodies (2 µg) to dystrophin or myc-epitope (BABCO) were added to 1 ml aliquots of NAG eluate (15 µg) and samples were incubated on ice for 1 hour. Rabbit anti-mouse IgG (10 µg) (Cappel, Durham NC) was then added, and after 30 minutes 50 µl of protein A sepharose was used to precipitate antibodies. Protein A pellets were washed 3 times with buffer containing 200 mM NaCl and 0.1% triton X-100. Immunoprecipitated proteins were denatured with loading buffer and resolved by SDS-PAGE.

Antibodies, Immunohistochemistry and Immunoblotting

Unfixed skeletal muscle samples were flash frozen in liquid nitrogen cooled isopentane, sectioned on a cryostat (10 mm) and melted directly onto glass slides. Sections were then post-fixed in 2% paraformaldehyde phosphate buffered saline. Tissues were "blocked" in PBS containing 1% normal goat serum. Monoclonal antibodies to dystrophin (Sigma, 1: 200), nNOS (Transduction Labs, 1:100), spectrin (Novacastra Laboratories, 1:50) or a

polyclonal nNOS antibody (Kobzik et al., 1994), 1:250) were applied to sections overnight at 4° C. For indirect immunofluorescence secondary goat anti-rabbit FITC, or donkey anti-mouse Cy -3 conjugated antibodies were used according to the manufacturer's specifications (Jackson Laboratories, 1:200).

Mammalian Cell Transfections

Neuronal NOS cDNAs were cloned into the mammalian expression vector pcDNA 3 (Invitrogen). Monkey COS cells were grown in culture medium consisting of DMEM (Gibco BRL) supplemented with 10% fetal bovine serum. Cells were plated in 10 cm dishes at a density of $2 \times 10^4 \text{ cm}^{-2}$ and transfected the following day using calcium phosphate as previously described (Bredt et al., 1991). Two days following transfection cells were washed with PBS, harvested in 2 ml of buffer containing 25 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and disrupted using a polytron.

NOS Catalytic Assays

Quadriceps skeletal muscle from wild type and *mdx* mouse were homogenized in 10 volumes of buffer containing 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1mM EGTA, and 0.1 M NaCl. The homogenate was centrifuged at 20,000 x g yielding the soluble fraction. The pellet was extracted in the same buffer containing 0.5 M NaCl, and centrifuged at 20,000 x g yielding the particulate fraction. Aliquots from these fractions were assayed in 125 μl reactions containing 100,000 cpm ^3H -arginine (60 Ci mmol^{-1}), 1 mM NADPH, 400 μM $\text{Ca}^{2+}_{\text{free}}$, 1 μM calmodulin, 3 μM each of tetrahydrobiopterin, FAD, and FMN. After incubation for 25 minutes at 22 °C, assays were terminated

with 4 mL H₂O. Samples were applied to 0.5 mL Dowex AG50WX-8 (Na⁺-form) columns. ³H-citrulline was quantified by liquid scintillation spectroscopy of the 4 mL flow-through. Crude homogenates of transfected COS cells were assayed using an identical procedure.

Characterization of Human Tissues

Human tissues were obtained from the pathology department at University of California San Francisco. Clinical diagnosis of DMD was made on the basis of onset and progression of disease, creatine kinase in serum and histologic study of the biopsied muscles. Tissues were snap frozen in liquid nitrogen cooled isopentane. For western blotting, cryostat sections were collected into plastic tubes and sonicated in buffer containing 50 mM TrisHCl pH 7.4, 1mM EDTA, 1mM EGTA, and 1mM PMSF. Crude protein samples (200 µg per lane) were resolved by SDS PAGE and analyzed by western blotting as described above. Immunohistochemistry was performed on 10 µm human tissue samples as described above. To ensure specificity immunofluorescent nNOS staining was performed using two independently raised antisera. The first antiserum (a-nNOS 1) reacts only with determinants in the amino terminal domain of nNOS (Bredt and Snyder, 1994), while the second (a-nNOS 2) reacts only with the C-terminal region (Transduction Labs). Unless otherwise noted all histologic sections were labeled with a-nNOS 1.

UCSF LIBRARY

Figure 2-1. Differential extractability of nNOS and eNOS in skeletal muscle homogenates.

a) Western blotting indicates that significant nNOS remains in an insoluble pellet (P) following sequential extraction of mouse quadriceps homogenates with 100 mM NaCl (S₁), 500 mM NaCl (S₂), and 0.5% triton (S₃). nNOS is present in each of these fractions. b) eNOS occurs only in membrane associated fractions. Probing the same blot with an eNOS monoclonal antibody indicates that eNOS is completely extracted by 500 mM NaCl (S₂) and 0.5% triton (S₃). eNOS is absent from cytosol (S₁) and the insoluble pellet (P). After subcellular fractionation and 7.5 % SDS-PAGE (100 µg protein / lane), nNOS and eNOS were sequentially detected by protein immunoblot. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

UCSF LIBRARY

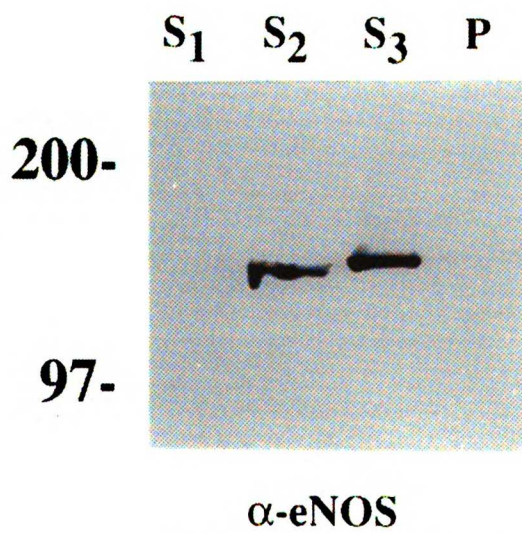
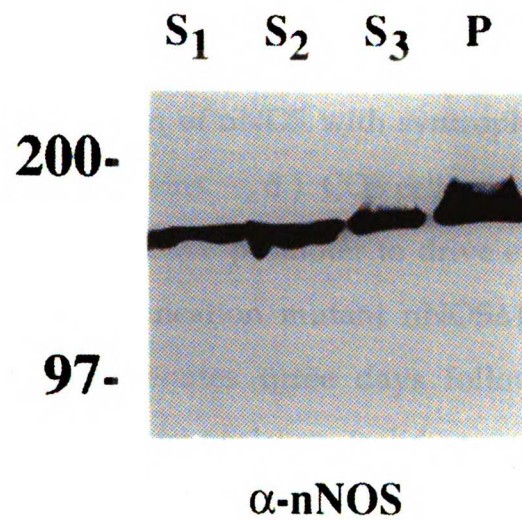


Figure 2-1

Figure 2-2. The extended N-terminus of nNOS contains a GLGF domain that is not required for enzyme activity. a) Schematic alignment of cofactor binding domains of eNOS and nNOS indicating N-terminal myristoylation of eNOS and extended N-terminus of nNOS. The jagged line indicates the region deleted for the nNOS Δ 1-226 mutant. b) Alignment of the GLGF / dystrophin binding domain of nNOS with syntrophins and a family of other cytoskeletal associated proteins. c, d) COS cells were transfected with 10 μ g of expression vector using a CMV promoter to drive expression of either (c) full length nNOS or (d) a truncation mutant nNOS Δ 1-226. NOS activity was measured in cell homogenates three days following transfection in the presence of either 200 μ M free calcium (solid squares) or 2 mM EDTA (solid circles). Kinetic constants ($V_{\max}$ and K_m) were calculated by Scatchard plot analysis. Data are means of triplicate determinations which varied by less than 10 %. This experiment was replicated twice with similar results.

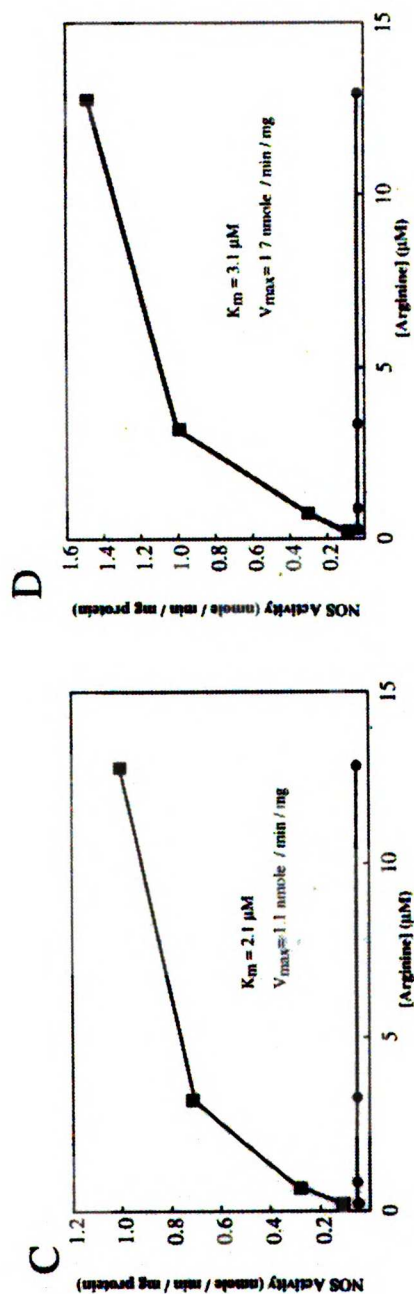
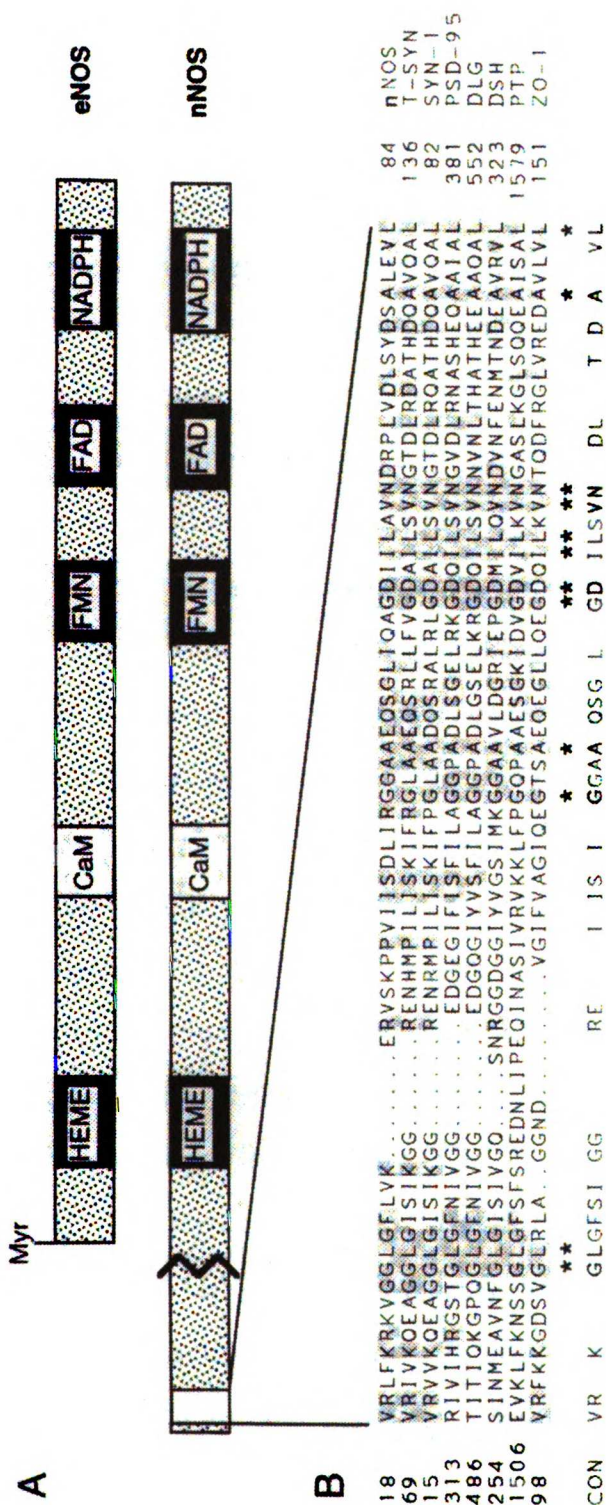


Figure 2-3. Association of nNOS and dystrophin in skeletal muscle.

(a, b) Dystrophin associated glycoprotein complex and nNOS were purified by succinylated wheat germ agglutinin (sWGA) and 2' 5' ADP agarose chromatography respectively from wild type, *mdx*, and NOS knockout (NOS^{-/-}) skeletal muscle. a) Western blotting indicates that total nNOS levels in crude extracts are similar in wild type and *mdx* muscle. nNOS coelutes with dystrophin on sWGA affinity column in muscle homogenates from wild type (WT) mouse. nNOS does not adhere to a sWGA column in extracts from *mdx* mouse.

b) Western blotting for dystrophin reveals that dystrophin levels are equivalent in crude samples from wild type and nNOS knockout skeletal muscle. Dystrophin coelutes with NOS on a 2' 5' ADP affinity column in muscle homogenates from wild type (WT) but not nNOS knockout mouse.

c) Glutathione sepharose beads bound to GST or GST nNOS (1-299) were incubated with solubilized skeletal muscle membranes. After extensive washing, the beads were eluted with 0.2% SDS, and proteins were separated by SDS-PAGE and retention of dystrophin protein was analyzed by Western blotting. For (a-c) protein homogenates (200 µg / lane) and aliquots of affinity eluates (150 µL / lane) were resolved on 7.5% (a-NOS) or 6% (a-dystrophin) SDS gels, transferred to PVDF membranes and immunoreactive bands were visualized by ECL.

d) Western blotting for nNOS from equally loaded (5 µg / lane) fractions from sWGA chromatography shows large enrichment of nNOS in NAG eluate fractions. L, load ; F, flow through; W1, 500 mM NaCl wash; W2, 500 mM NaCl and 0.5 % triton X-100 wash; E, 0.3 M NAG eluate.

e) Reloading samples from sWGA column with 6-fold higher protein in load and flow through (21 μ g / lane) than wash and eluate fractions (3.5 μ g / lane) shows purification of nNOS and dystrophin but not eNOS by sWGA.

f) Immunoprecipitation from NAG eluate fractions indicates that a monoclonal antibody to dystrophin (12 nM) potently precipitates nNOS (lane 1). Control experiments containing a monoclonal anti-myc antibody (12 nM) or lacking any primary antibody (lanes 2, 3 respectively) fail to precipitate nNOS. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

UCSF LIBRARY



Figure 2-3

Figure 2-4. nNOS is displaced from particulate fractions of *mdx* skeletal muscle.

(a) Subcellular fractions (S₁, S₂, S₃ and P) of mouse quadriceps from wild type (WT) and *mdx* mice (age matched at 7-8 weeks) were prepared as described in figure 2-1. Western blotting shows that nNOS in skeletal muscle of *mdx* mice is largely extracted from membranes with 100 mM NaCl (S₁) and is completely removed with 500 mM NaCl (S₂). In wild type mice (WT), the majority of nNOS remains membrane associated following the 500 mM NaCl wash. Approximately half of this remaining nNOS is removed by a 0.5% triton extraction (S₃) and half is present in an insoluble pellet (P). (b) Dystrophin is enriched in detergent extract (S₃) and cytoskeletal pellet fractions (P) in wild type mice and is absent from *mdx* muscle. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

UCSF LIBRARY

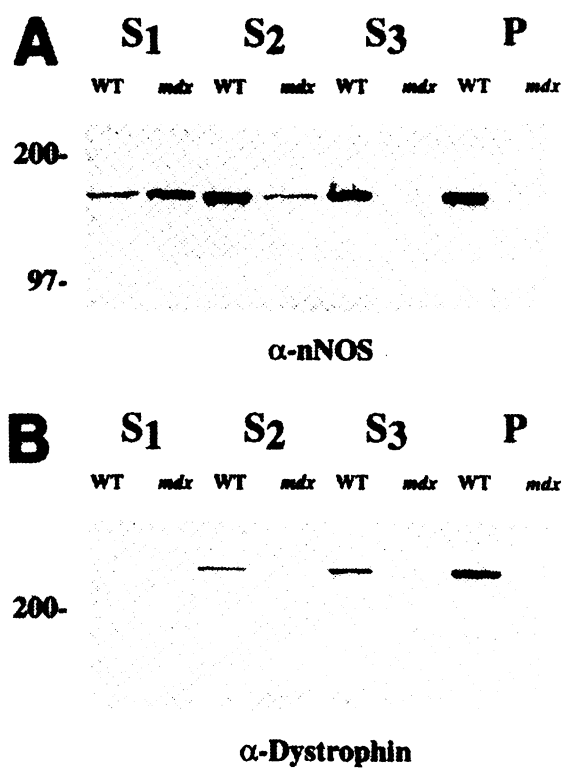


Figure 2-4

Figure 2-5. nNOS is selectively absent from sarcolemma of *mdx* skeletal muscle. Immunofluorescent staining for nNOS in quadriceps of wild type, *mdx*, *dy* and nNOS knockout mouse was performed using a affinity purified polyclonal antibody. a) nNOS immunostaining is present at the surface membranes of skeletal muscle fibers from wild type (WT) mouse, but is absent from b) *mdx* mouse skeletal muscle sarcolemma. c) Homozygous *dy* dystrophic mice display normal sarcolemmal nNOS labeling of intact fibers. d) Skeletal muscle from NOS knockout mouse is entirely devoid of immunostaining. Cryostat muscle sections from wild type, *mdx*, *dy* and nNOS knockout mouse were stained under identical condition using an affinity purified nNOS antiserum and a FITC linked secondary antibody. Magnification, X 188.

UCSF LIBRARY

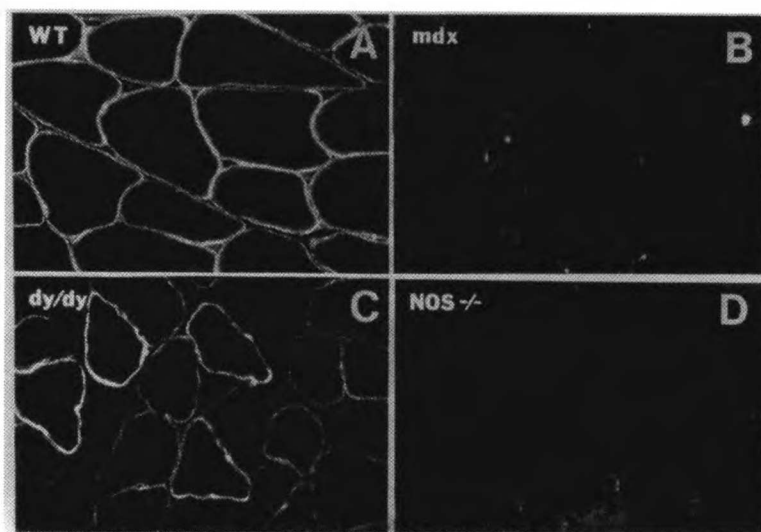


Figure 2-5

Figure 2-6 nNOS is absent from sarcolemma of DMD muscle fibers

(A) Skeletal muscle cryosections of normal (N1) or DMD (D1) skeletal muscle were immunostained with antibodies to dystrophin, nNOS and spectrin. (B) Representative muscle sections from two additional normal patients (N2, N3) and two DMD cases (D2, D3) were labeled with antibodies to dystrophin and nNOS. (C) Control experiments using two independently generated nNOS antisera showed similar staining of human tissues. No immunofluorescence was detected in the absence of primary (1^0) antibody. Magnification, X 122.

UCSF LIBRARY

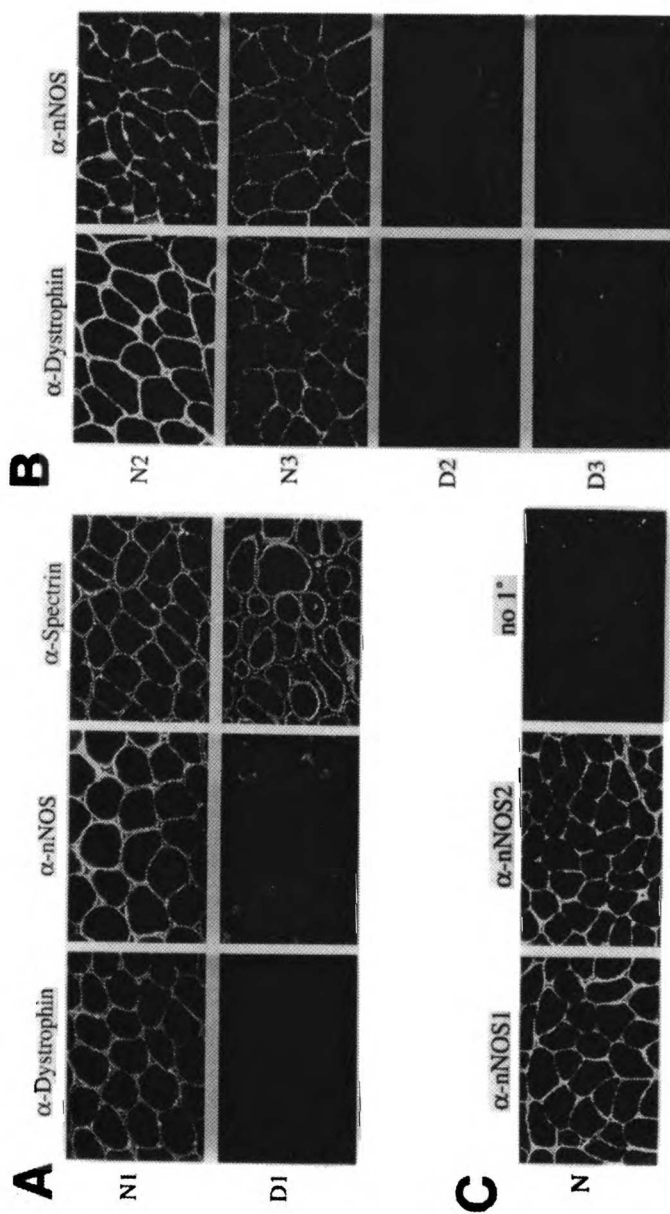


Figure 2-6

Figure 2-7. nNOS levels are diminished in DMD skeletal muscle

Skeletal muscle tissue homogenates from three cases of DMD and three normal muscle biopsies were resolved by SDS / PAGE. a) Immunoblot analysis confirms that dystrophin is essentially absent from DMD muscle. b) nNOS levels are significantly decreased in all three cases of DMD. c) Spectrin levels are nearly equal in normal and DMD biopsies. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

UCSF LIBRARY

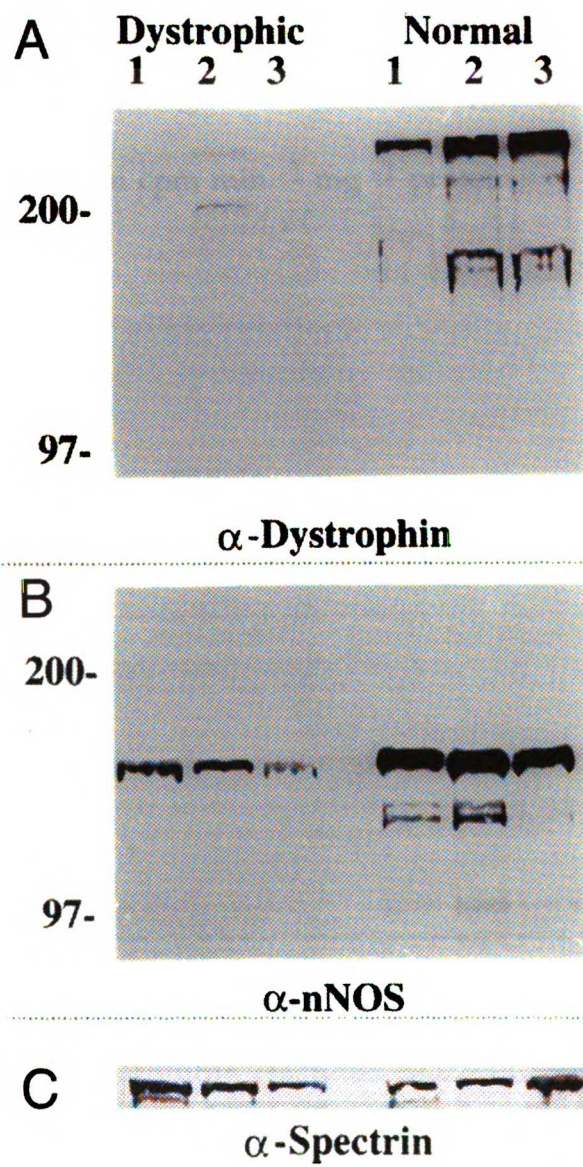


Figure 2-7

TABLE 2-1 NO synthase activity in extracts from wild type and *mdx* mouse tissues

NO synthase activity in cpm min⁻¹ mg⁻¹ protein

	Wild type	<i>mdx</i>
Skeletal Muscle		
Soluble Fraction	33.3	58.3
Particulate Fraction	252	<0.5
Brain		
Soluble Fraction	1,020	1,060
Particulate Fraction	1,080	1,210

Particulate and soluble fractions of quadriceps skeletal muscle from wild type and *mdx* mouse were prepared and assayed for NOS activity as described in "Experimental Procedures". Data are means of triplicate determinations which varied by less than 10%. This experiment was repeated three times with similar results.

CHAPTER 3

Interaction of nitric oxide synthase with the synaptic density protein PSD-95 and α -1 syntrophin mediated by PDZ domains

UCSF LIBRARY

Abstract

Neuronal NO synthase (nNOS) is concentrated at synaptic junctions in brain and at motor endplates in skeletal muscle. Here, we show the N-terminus domain of nNOS, which contains a PDZ protein motif, interacts with the second PDZ motif in postsynaptic density-95 protein (PSD-95) and a related novel protein, PSD-93. nNOS and PSD-95 are coexpressed in numerous neuronal populations, and a PSD-95 / nNOS complex occurs in cerebellum. PDZ domain interactions also mediate binding of nNOS to skeletal muscle syntrophin, a dystrophin associated protein. nNOS isoforms lacking a PDZ domain, identified in nNOS Δ/Δ mutant mice, do not associate with PSD-95 in brain or with skeletal muscle sarcolemma. Interaction of PDZ containing domains therefore mediates synaptic-association of nNOS, and may play a more general role in formation of macromolecular signaling complexes.

UCSF LIBRARY

Introduction

Endogenous NO derives from L-arginine as the product of a complex enzymatic reaction catalyzed by a family of three NO synthase (NOS) proteins (Bredt and Snyder, 1994; Moncada and Higgs, 1993; Nathan and Xie, 1994). The neuronal isoform (nNOS or type I) is prominent in neurons and also occurs in epithelial cells of lung, secretory cells of certain endocrine glands and skeletal muscle fibers (Kobzik et al., 1994; Schmidt et al., 1992). The endothelial NOS isoform (eNOS, type III) is abundant in all vascular endothelial cells and accounts for the endothelial derived relaxing factor described by Zawadzki and Furchgott (Furchgott and Zawadzki, 1980). The inducible isoform (iNOS, type II) is rapidly upregulated in essentially all cell types following appropriate immunological stimuli and serves a non-specific immune function (Hibbs et al., 1987).

Physiological studies have demonstrated numerous functions for neuron-derived NO. In the peripheral nervous system NO functions as a non-adrenergic-non-cholinergic neurotransmitter mediating actions of autonomic motor neurons on vascular and nonvascular smooth muscle. In brain, NO has been implicated in several forms of synaptic plasticity (Schuman and Madison, 1994). To help clarify functions for nNOS throughout the body, nNOS Δ/Δ mutant mice were generated with a targeted disruption of exon 2, which contains the predicted starter methionine codon (Huang et al., 1993). Initial analysis of nNOS mutant mice suggested a complete absence of nNOS protein and mRNA in brain and peripheral tissues. However, residual NOS catalytic activity was detected in brain at levels up to 8% of that found in wild type animals (Huang et al., 1993). nNOS Δ/Δ mice suffer from hypertrophic pyloric stenosis (Huang et al., 1993), consistent with electrophysiological studies showing that NO mediates non-

adrenergic-non-cholinergic inhibitory junctional potentials at enteric sphincters. nNOS Δ/Δ mice are significantly resistant to brain injury following cerebral ischemia (Huang et al., 1994), consistent with a role for NO in this form of excitotoxicity (Dawson et al., 1992). nNOS mutant mice display an increase in aggressive behavior and excess and inappropriate sexual behavior (Nelson et al., 1995).

Synaptic actions of NO are facilitated by subcellular targeting of nNOS to specialized membrane structures. Biochemical studies indicate that nNOS partitions largely with membrane associated and cytoskeletal subcellular fractions (Brenman et al., 1995; Hecker et al., 1994). Electron microscopy identifies over 80% of nNOS immunoreactivity in monkey visual cortex as axonal or dendritic profiles (Aoki et al., 1993). Membrane-associated nNOS has also been identified at the sarcolemma of fast twitch skeletal muscle fibers (Kobzik et al., 1994). Sarcolemmal targeting is mediated by association of nNOS with the dystrophin glycoprotein complex (Brenman et al., 1995). The absence of dystrophin in Duchenne muscular dystrophy results in translocation of nNOS from sarcolemma to muscle cytosol.

The N-terminal domain of nNOS is unique to the neuronal isoform and contains a PDZ motif of ~100 amino acids that is found in a diverse group of cytoskeletal proteins and enzymes (Cho et al., 1992). (Brenman et al., 1995) Because this domain mediates association of nNOS with the dystrophin complex (Brenman et al., 1995), we have now used the yeast two-hybrid system to identify interacting proteins. Screening a brain library demonstrates that the PDZ containing domain of nNOS binds to PDZ repeats in postsynaptic density 95 (PSD-95) (Cho et al., 1992) and a novel related protein, PSD-93. PSD-95 is co-expressed with nNOS in several neuronal populations in the developing and mature nervous system, and a specific

PSD-95 / nNOS interaction can be detected in transfected cell lines and solubilized cerebellar membranes. In skeletal muscle, we determine that association of nNOS with the sarcolemma is mediated by direct binding of nNOS to α 1-syntrophin, a protein of the dystrophin complex containing a PDZ motif (Adams et al., 1993; Adams et al., 1995). Residual catalytically active nNOS isoforms we now identify in nNOS Δ/Δ mice, that specifically lack a PDZ motif, do not interact with PSD-95, nor do they associate with the dystrophin complex of skeletal muscle sarcolemma. These data demonstrate a physiological role for PDZ domain interactions in organizing proteins at synaptic membranes, and identify one potential function for PDZ domains in PSD-95 and α 1-syntrophin.

UCSF LIBRARY

Results

The PDZ containing domain of nNOS can bind to similar motifs in PSD-95 and to a related protein, PSD-93

We fused the PDZ domain of nNOS (amino acids 1-195) to the DNA binding domain of GAL4 and screened a human brain library for interacting proteins using the yeast two-hybrid system (Fields and Song, 1989). Two families of interacting clones were identified from a screen of 10^6 plasmids (table 3-1A). One family represented isolates encoding the PDZ motifs of PSD-95, and the other family encoded PDZ repeats of a related novel gene product.

PSD-95 was originally identified as an abundant detergent insoluble component of brain postsynaptic density (Cho et al., 1992). Subcellular and electron micrographic studies have determined that PSD-95 occurs at both pre- and postsynaptic membrane specializations, similar to the distribution of nNOS (Aoki et al., 1993; Kistner et al., 1993). PSD-95 contains three PDZ repeats, an SH3 domain and a region homologous to guanylate kinase (Cho et al., 1992). Our cloning and sequencing of the related gene predicts a protein of 93 kD, PSD-93, that has the same domain structure as PSD-95 and shares 60% amino acid identity (the nucleotide sequence of PSD-93 has been deposited in GenBank).

nNOS and PSD-95 are co-localized in adult and developing neurons

If the association of nNOS with PSD-95 identified in yeast is physiologically relevant, the two proteins must be co-expressed in neurons. *In situ* hybridization in rat brain (figure 3-1a, b) demonstrated co-expression

of nNOS and PSD-95 transcripts in several neuronal populations, particularly in cerebellar granule and basket cells, which have previously been shown to express high densities of nNOS and PSD-95 proteins (Bredt et al., 1990; Kistner et al., 1993). On the other hand, PSD-93 occurred at highest densities in cerebellar Purkinje neurons, complimentary to the distribution to nNOS and PSD-95.

We also compared expression of nNOS with PSD-95 / 93 during embryonic development. Using *in situ* hybridization, we found that nNOS containing cells in embryonic day 15 (E15) rat were differentially co-expressed with either PSD-95 or PSD-93 (figures 3-1c, d). As previously reported, transient nNOS neurons were detected in developing cerebral cortical plate, olfactory epithelium, and sensory ganglia (Bredt and Snyder, 1994). In all of these neuronal groups we found PSD-95 mRNA. nNOS was also developmentally expressed in certain non-neuronal cells including chromaffin cells of the adrenal gland and secretory cells of the submandibular gland. PSD-93 mRNA and nNOS mRNA were co-expressed in these glands, while PSD-95, which is reported to be neuron specific (Cho et al., 1992; Kistner et al., 1993), was absent.

Co-localization of nNOS and PSD-95 proteins was evaluated by immunohistochemical staining of adjacent sections from E19 rat. Within the olfactory system, both PSD-95 and nNOS were enriched in dendritic specializations in olfactory cilia and in axonal processes projecting to the olfactory bulb, which itself does not contain either nNOS or PSD-95 (figure 3-2 a-d). nNOS also occurs in fetal myenteric neurons and its absence is associated with hypertrophic pyloric stenosis. Immunohistochemical analysis revealed a colocalization of nNOS with PSD-95 in myenteric neurons (figure 3-2 e-h). nNOS and PSD-95 were similarly colocalized in embryonic

cerebral cortex. Staining for both proteins was enriched in the intermediate zone and developing cortical plate, while lesser staining was found in the subplate region. The ventricular zone was devoid of staining (figure 3-2 i, j).

nNOS interacts with the second PDZ motif of PSD-95

We found that an nNOS-PSD-95 complex was immunoprecipitated from COS cells cotransfected with expression vectors for nNOS and the PDZ repeats of PSD-95, indicating that this interaction occurs in a cellular environment (figure 3-3a). To evaluate formation of a nNOS / PSD-95 complex in brain, we conducted immunoprecipitation studies. Though only a small fraction of PSD-95 can be solubilized from brain densities with non-denaturing detergents (Cho et al., 1992; Kistner et al., 1993), we specifically immunoprecipitated an nNOS / PSD-95 complex from cerebellum (figure 3-3b, c), where both proteins are co-expressed at high levels.

Functions for PSD-95 are largely unknown. Recent studies show that the second PDZ motif of PSD-95 interacts with certain ion channels in brain, including NMDA receptors and voltage gated K⁺ channels, that contain a carboxy terminal tSXV consensus sequence (Kim et al., 1995; Kornau et al., 1995). To determine which domain(s) of PSD-95 bind to nNOS, we engineered yeast constructs encoding appropriate fragments of PSD-95 fused to the GAL4 activation domain. Constructs encoding the second PDZ motif of PSD-95 interacted with nNOS while those lacking this region were inactive (Table 3-1A).

To biochemically evaluate these interactions, we generated a fusion protein linking glutathione-S-transferase (GST) to the first 386 amino acids of PSD-95 (G-PSD). Solubilized brain extracts were incubated with glutathione

beads linked to GST or G-PSD. Following extensive washing with buffer containing 350 mM NaCl and 0.5% triton X-100, bound proteins were eluted with loading buffer. Western blotting indicated selective retention of nNOS to G-PSD beads (figure 3-3d). eNOS, which is 60% identical to nNOS but lacks a PDZ motif, was not retained by the G-PSD beads indicating specificity of the interaction with nNOS (figure 3-5d).

Because the second PDZ motif of PSD-95 binds to both nNOS and to tSXV containing ion channels, we determined whether these binding sites are independent or overlapping. Previous studies demonstrated that a peptide, corresponding to the carboxy terminal nine amino acids of NMDA receptor type 2B, blocks interaction of NMDA receptor with PSD-95 (Kornau et al., 1995). Similarly, we found that this NMDA receptor peptide potently blocks association of nNOS with PSD-95 (figure 3-3e). Half maximal inhibition of binding was achieved at <10 μ M NMDA receptor peptide, while control peptides were inactive at 200 μ M. As a control we found that K_v 1.4, a voltage-dependent K⁺ channel containing a tSXV sequence, was displaced from PSD-95 by similar concentrations of NMDA receptor peptide. We also found that the second PDZ domain of PSD-93, expressed as a GST-fusion protein, binds to nNOS in a manner competitive with the NMDA receptor peptide (data not shown).

Catalytically active nNOS isoforms lacking a PDZ motif occur in nNOS Δ/Δ mice

To further characterize the importance of the PDZ domain, we took advantage of our observation that mice carrying a targeted disruption of exon 2 of nNOS, express residual nNOS isoforms specifically lacking the PDZ

domain. $\text{nNOS}^{\Delta/\Delta}$ mice were generated by deleting the first translated exon (Huang et al., 1993), which is exon 2 of nNOS in both mouse and human (Hall et al., 1994) see below) and which encodes the PDZ motif. Northern blotting and 5'RACE-PCR cloning analyses demonstrate that residual nNOS mRNAs are expressed in brain of $\text{nNOS}^{\Delta/\Delta}$ mice resulting from alternative splicing which skips the targeted 1.1 kB second exon (figure 3-4). Interestingly, PCR and sequence analysis indicated that this alternative splice also occurs in wild-type mice and represents ~5% of total NOS mRNA (figure 3-4c, lane 2).

Western blotting of purified brain extracts identified residual nNOS proteins of 136 and 125 kD from $\text{nNOS}^{\Delta/\Delta}$ (figure 3-5a, lanes 3, 4). These 136 kD (nNOSb) and 125 kD (nNOSg) bands from $\text{nNOS}^{\Delta/\Delta}$ did not cross react with antibodies to eNOS or iNOS (data not shown). Failure of previous studies to detect immunoreactive nNOS protein in the $\text{nNOS}^{\Delta/\Delta}$ mice was due to the use of a less potent polyclonal antibody and a less sensitive colorimetric detection system (Huang et al., 1993).

To determine the relationship between the two transcripts identified by RACE-PCR and the two immunoreactive protein bands, COS cells were transfected with constructs corresponding to the truncated forms found in $\text{nNOS}^{\Delta/\Delta}$, that is, 5'a or 5'b spliced to exon 3. Transfection of the 5'a containing construct generated a prominent immunoreactive protein band of 136 kD that comigrated with nNOSb from $\text{nNOS}^{\Delta/\Delta}$ brain (lanes 5b; lanes 3, 4). Transfection of the 5'b containing construct yielded an nNOS band of 125 kD (figure 3-5b, lane 5). Catalytic assays indicated that the 136 kD nNOSb form had activity ~80% that of full length nNOS under these transfection conditions (figure 3-5c). Enzyme activity was fully dependent on calcium / calmodulin and the K_m for arginine was similar to that of full-length nNOS

from brain. By contrast, the activity of the 125 kD nNOSg was ~3% that of nNOS.

NOS activity in wild type brain is highest in cerebellum (Bredt and Snyder, 1990). By contrast, NOS activity in nNOS Δ/Δ is highest in the striatum and lowest in the cerebellum (Huang et al., 1993). The regional distribution of residual nNOS isoforms in nNOS Δ/Δ brain extracts paralleled the pattern of residual nNOS activity previously reported (Huang et al., 1993; figure 3-5 e, f).

nNOS isoforms lacking a PDZ motif do not associate with PSD-95 or brain membranes

nNOS Δ/Δ mice therefore express nNOS isoforms specifically lacking the PDZ motif and can be used as an important tool to determine the functions for this domain *in vivo*. We first evaluated association of residual nNOS isoforms with PSD-95. nNOS proteins purified from wild type and nNOS Δ/Δ mouse forebrain were subjected to pull-down assays as described above. Only full length nNOS protein containing the PDZ motif was retained by G-PSD beads; the alternatively spliced forms in nNOS Δ/Δ did not adhere to G-PSD (figure 3-6 a). We also compared the distribution of nNOS in wild type and nNOS Δ/Δ mouse by subcellular fractionation. Brain homogenates were first extracted with physiological saline, then with buffer containing 1M KCl and 1% triton X-100, leaving a cytoskeletal pellet. nNOS in wild type brain was present in all fractions while residual nNOS isoforms in nNOS Δ/Δ occurred only in the first soluble fraction (figure 3-6 b).

nNOS binds to skeletal muscle syntrophin through PDZ interactions

Interaction of PDZ motifs in nNOS and PSD-95 raised the possibility that the PDZ domain of syntrophin (Adams et al., 1993) represents the binding site for association of nNOS with the dystrophin complex (Brenman et al., 1995). Interaction between the PDZ containing domains of nNOS (amino acids 1-195) and α 1-syntrophin (amino acids 59-166) was first evaluated by the yeast two hybrid system. When co-transfected these constructs reconstituted GAL4 transcriptional activity (table 3-1B).

To biochemically evaluate association, we first conducted "pull-down" assays from skeletal muscle extracts using GST fused to the PDZ domain of nNOS (G-NOS; (Brenman et al., 1995)) α 1-syntrophin was selectively retained by this column but did not associate with a control GST protein column (figure 3-7a; lanes 1, 3, 4). We previously demonstrated that dystrophin is retained by a G-NOS column (Brenman et al., 1995). The absence of dystrophin in *mdx* mouse results in disruption of the dystrophin glycoprotein complex. We therefore evaluated association of skeletal muscle syntrophin from *mdx* mouse with G-NOS. Total α 1-syntrophin levels were decreased ~50% in *mdx* muscle, in agreement with previous findings (Butler et al., 1992). Binding of α 1-syntrophin to G-NOS was unaffected by dystrophin deficiency (figure 3-7a; lanes 2, 5, 6). To demonstrate association of nNOS with syntrophin in muscle extracts, we conducted immunoprecipitation experiments. A polyclonal antibody specific for α 1-syntrophin selectively precipitated nNOS from solubilized skeletal muscle microsomes (figure 3-7b). No precipitation of nNOS occurred with non-immune antibody, and eNOS, was not specifically precipitated by the α 1-syntrophin antibody (figure 3-7c).

To determine the role of the PDZ domain for association of nNOS with sarcolemmal dystrophin complexes *in vivo* we evaluated the subcellular

distribution of nNOS isoforms in nNOS Δ/Δ mouse. Skeletal muscle homogenates were sequentially extracted with physiologic saline, then 500 mM NaCl, and finally 0.5% triton X-100. Following 2'5' ADP agarose purification of these muscle extracts Western blotting indicated that only the nNOSg form is expressed in muscle of nNOS Δ/Δ . This may be due to expression of unique alternative 5' untranslated exons in skeletal muscle (Xie et al., 1995). nNOS in skeletal muscle runs as a doublet due to a 102 bp (34 amino acids) alternative splice near the middle of the gene (Silvagno et al., 1996). Importantly, subcellular fractionation indicated that nNOSg was restricted to soluble fractions of skeletal muscle (figure 3-7e), contrasting with the soluble / particulate distribution of full length nNOS in muscle (figure 3-7d).

Protein overlay assays were performed to evaluate association of nNOS with individual domains of α 1-syntrophin. The three known syntrophins (a1, b1, and b2) have a common domain structure consisting of two pleckstrin homology (PH) domains, a PDZ domain and a carboxy terminal domain unique to syntrophins (SU, syntrophin unique). Figure 3-8A shows the interaction between the nNOS-GST (1-299) fusion protein and the four α 1-syntrophin domain fusion proteins, PH1, PDZ, PH2 and SU. Notably, only PDZ associates with nNOS. Moreover, no prominent bands were detected when the domains were overlayed with GST alone. Syntrophin domain fusion proteins (containing the T7-Tag epitope) were also used to overlay GST and nNOS-GST (Figure 3-8B). Again, only the PDZ domain of α 1-syntrophin bound to nNOS and no binding was observed to GST alone.

Discussion

The PDZ consensus sequence is present in a diverse family of enzymes and structural proteins. Many of these proteins are found concentrated at specialized cell-cell junctions such as, neuronal synapses, epithelial zona occludens, and septate junctions. These observations have motivated suggestions that PDZ domains may participate in protein-protein interactions at the plasma membrane (Cho et al., 1992). Our results support this idea and suggest that PDZ domains may be important elements in interactions required for signal transduction at the membrane.

The demonstration that nNOS directly interacts with the PDZ domain of α 1-syntrophin explains the observed association of nNOS with sarcolemmal dystrophin complexes (Brenman et al., 1995). We also find that nNOS and α 1-syntrophin share a similar distribution in skeletal muscle fast fibers (figure 3-8c.) The association of nNOS and α 1-syntrophin may also account for the absence of nNOS from skeletal muscle sarcolemma in Duchenne muscular dystrophy (Brenman et al., 1995). The three known isoforms of syntrophin (Adams et al., 1993; Ahn et al., 1994; Yang et al., 1994) each have a defined domain structure (Adams et al., 1995). The first PH domain, which begins near the N-terminus, is split by an insert that contains the PDZ domain. A second PH domain is followed by a highly conserved C-terminal domain with apparently no homology to known proteins. Although the binding site for dystrophin has not been definitively identified, recombinant fragments of β 1-syntrophin beginning just downstream of the PDZ domains bind dystrophin and other members of the dystrophin protein family (Ahn and Kunkel, 1995). Since the syntrophins have highly conserved domain structures, the location of the dystrophin binding site is

likely to be the same in all three syntrophins. Thus the binding sites for nNOS and dystrophin appear to lie in distinct regions of $\alpha 1$ -syntrophin. Taken together, these studies define syntrophin as a modular adapter protein linking a signaling enzyme, nNOS, to the sarcolemmal dystrophin complex. A model depicting these interactions is shown in figure 3-8d.

Other proteins containing PDZ domains are likely to be recruited to the dystrophin complex through association with syntrophins. It will be of particular interest to identify proteins that bind to the PDZ domains of $\beta 1$ and $\beta 2$ syntrophin. Since the latter is highly localized to the neuromuscular postsynaptic membrane, it seems likely that proteins specifically associated with the PDZ domain of $\beta 2$ -syntrophin will be important in synaptic signaling. nNOS is restricted to fast twitch muscle fibers in rat while $\alpha 1$ -syntrophin is found in both fast and slow fibers. Thus, depending on the muscle type, only a small fraction of $\alpha 1$ -syntrophin may be associated with nNOS, thus allowing for interactions with other PDZ-containing proteins.

In brain, membrane association of nNOS does not require dystrophin (Brenman et al., 1995). Instead, we find that nNOS binds to the second PDZ repeat of PSD-95, and a related protein PSD-93. nNOS and PSD-95 are co-expressed in numerous populations of developing and mature neurons, and a PSD-95 / nNOS complex can be identified in solubilized cerebellar membranes. Further evidence that the nNOS / PSD-95 interaction is physiological derives from electron micrographic studies showing that 83% of nNOS immunoreactive profiles in cerebral cortex occur in pre- and postsynaptic specializations of axon terminals and over thick postsynaptic densities (Aoki et al., 1993), similar to the distribution of PSD-95 (Cho et al., 1992; Kistner et al., 1993). nNOS also occurs in certain non-neuronal cells including developing chromaffin cells of the adrenal gland and secretory cells

of salivary gland. In these cells nNOS is co-expressed with PSD-93, but not PSD-95. Definitive evidence that nNOS physiologically associates with PSD-93 in these glands will require development of antisera for co-immunoprecipitation studies *in vivo*.

The importance of the PDZ domain of nNOS is further revealed by our studies of nNOS Δ/Δ mouse. This work indicates that the residual NOS activity in brain of these mutants results from alternative splicing that skips the targeted second exon of nNOS. This alternative splicing also occurs at low levels in wild type mice and results in truncated nNOS proteins lacking the PDZ motif encoded in exon 2. nNOS isoforms lacking the PDZ domain have altered subcellular distribution. We find that these isoforms do not adhere to PSD-95 from brain synaptic densities, nor do they associate with the dystrophin complex in skeletal muscle. This demonstrates a primary role for the PDZ domain in mediating subcellular localization of nNOS. Pyloric stenosis in nNOS Δ/Δ mice (Huang et al., 1993) may therefore be due to dyslocalization of nNOS as we find that residual nNOS occurs at 5-10% of normal levels in myenteric neurons of the mutant mice (unpublished). Behavior abnormalities of nNOS Δ/Δ mice (Nelson et al., 1995) may also be due to misexpression rather than lack of nNOS.

Binding interactions between PDZ domains are selective. Though nNOS binds to the PDZ motif in $\alpha 1$ -syntrophin and to the second PDZ motif of PSD-95, nNOS does not associate with the first or third PDZ motifs in PSD-95. Certain PDZ domains are capable of binding to the extreme C-terminus of a family of receptors and ion channels, including NMDA receptors (Kim et al., 1995; Kornau et al., 1995; Sato et al., 1995). These studies identify a C-terminal consensus tSXV that interacts with the second PDZ motif in PSD-95

and with the third PDZ motif of a protein tyrosine phosphatase, FAP-1, but not with the PDZ motif of nNOS (Kornau et al., 1995).

We find that the PDZ containing domain of nNOS and the tSXV motif of NMDA receptors compete for common or nearby binding sites within the second PDZ repeat of a PSD-95 subunit. However, PSD-95 can mediate K⁺ channel clustering and likely occurs as a oligomeric complex (Kim et al., 1995). The binding of nNOS and NMDA receptors to distinct PSD-95 subunits in such a complex may contribute to the well-described functional coupling of NMDA receptors to NOS activity in cerebellum and forebrain (Garthwaite, 1991). Membrane permeant compounds that block association of nNOS with PSD-95 might therefore serve as novel therapeutics, as neuron-derived NO, associated with NMDA receptor activity, mediates brain injury following cerebral ischemia (Huang et al., 1994).

UCSF LIBRARY

Materials and Methods

Antibodies and Western Blotting

The following primary antibodies were used: nNOS1 rabbit polyclonal raised against homogenous nNOS protein purified from rat cerebellum (Bredt et al., 1990), nNOS mouse monoclonal (Transduction Labs), eNOS mouse monoclonal (Transduction Labs), iNOS mouse monoclonal (Transduction Labs), α 1-syntrophin polyclonal (Peters et al., 1994), PSD-95 rabbit polyclonal (Cho et al., 1992), K_v1.4 rabbit polyclonal (Kim et al., 1995), c-myc mouse monoclonal (9E10; BABCO), T7-Tag monoclonal and GST 12 monoclonal (Santa Cruz Biotechnology, Inc.) For western blotting, protein extracts were resolved by SDS / PAGE and transferred to PVDF membranes. Primary antibodies were diluted in block solution containing 3% BSA, 0.1% tween-20 in TBS and incubated with membranes overnight at 4°C. Labeled bands were visualized using ECL (Amersham). All nNOS western blots used the nNOS monoclonal.

***In situ* hybridization**

In situ hybridization used ³⁵S-labeled RNA probes exactly as described (Sassoon and Rosenthal, 1993). Antisense probes to nNOS (nucleotides 4119-5057), PSD-95 (1-1155), PSD 93 (237-927) or sense control PSD-93 (237-927) were synthesized from bluescript vectors.

Immunohistochemistry

UCSF LIBRARY

Rats were perfused with 4% paraformaldehyde, tissues were harvested, post-fixed at 4° C for 3 hours, and cryoprotected in 20% sucrose overnight. Twenty micron sections were cut on a cryostat and melted onto glass slides (Superfrost/Plus, Fisher). Sections were then blocked for one hour in buffer containing 2% goat serum, 0.1% triton X-100 in PBS. Primary antibodies to nNOS (polyclonal nNOS), PSD-95, or α 1-syntrophin were diluted into blocking reagent and incubated with sections overnight. Immunoperoxidase histochemistry was performed using the ABC method (Vector, Burlingame, California). Immunofluorescent staining of rat extensor digitorum longus muscle was done as described (Brenman et al., 1995). Control sections lacking primary antisera were stained in parallel.

Cell Culture and Transfection

Neuronal NOS cDNAs were cloned into the EcoRV and Xba I sites of the mammalian expression vector pcDNA 3 (Invitrogen). 5'a and 5'b containing constructs were amplified by PCR, sequenced, and cloned into the unique Nar I restriction site of nNOS. PSD-95-myc construct (amino acids 1-386 with a C-terminal myc-epitope tag) was amplified by PCR and cloned into the BamHI and EcoRI sites of pcDNAIII. Monkey COS cells were grown and transfected using calcium phosphate as previously described (Bredt et al., 1991). Two days following transfection cells were washed with PBS, harvested in 2 ml of buffer containing 25 mM TrisCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and disrupted using a polytron.

Immunoprecipitations

Rat cerebellum was homogenized in 20 volumes of buffer containing 25 mM TrisCl (pH 7.5), 150 mM NaCl and centrifuged at 100,000x g to yield a cytosol fraction. Cerebellar membranes were solubilized with 1% digitonin and 100 mM NaCl and centrifuged to remove the insoluble cytoskeleton. Three μ l of PSD-95 polyclonal antiserum to PSD-95 or 3 μ l non immune serum were added to 1 ml (500 μ g) of cerebellar cytosol or solubilized membranes. After a 60 minute incubation on ice, 50 μ l of protein A sepharose was added to precipitate antibodies. Protein A pellets were washed 3 times with buffer containing 200 mM NaCl and 1% triton X-100. Immunoprecipitated proteins were denatured with loading buffer and resolved by SDS-PAGE. Heavy microsomes of rat gastrocnemius were prepared and solubilized with 1% triton X-100 as described (Brenman et al., 1995). Five μ g polyclonal antiserum to α 1-syntrophin or non-immune serum were added to 1 ml (500 μ g) solubilized muscle samples. Immunoprecipitations from transfected COS cells used polyclonal antibody to nNOS.

GST Fusion protein affinity chromatography

GST fusion constructs were constructed by PCR and fusion proteins purified as previously described (Brenman et al., 1995). For "pull down" assays, solubilized tissue samples were incubated with control or GST-fusion protein beads for 1 hour. Beads were washed with buffer containing 0.5% triton X-100 + 350 mM NaCl, and proteins eluted SDS loading buffer. NMDA receptor peptide (KLSSIESDV) or control peptide (KPKHAKHPDGHSGNLC) were added where indicated during tissue incubation with the fusion protein.

Generation of α 1-syntrophin fusion proteins and protein overlay assay

cDNAs encoding mouse α 1-syntrophin domains (PH1a domain, amino acids 1-77; PH1b, 162-271; PDZ, 75-170; PH2, 281-402; SU domain 401-499) were amplified by PCR and cloned into pET28a vector (Novagen, Inc.) with the exception of PH1a and PH1b that were ligated together to produce the intact PH1 domain. Clones were sequenced and electroporated into BL21(IDE3)pLysS cells. Overnight cultures were diluted 1:10, incubated 2 hrs. and induced for 3 hrs. with IPTG and expressed proteins, which contain a T7-Tag epitope encoded in the vector, were purified on Nickel columns (Novagen, Inc.). The PH1 and PDZ domains were purified from the soluble fraction; PH2 and SU were purified from urea solubilized inclusions.

Fusion proteins were separated on 15% SDS / PAGE gels, transferred to nitrocellulose membranes, blocked with 5% skim milk in 25 mM Tris (pH 7.5), 150 mM NaCl and 0.1% tween-20 (TBS-Tween) and incubated with purified fusion proteins (20 μ g / ml) in this buffer for 1 hr at 25°C. Blots were washed 3X 10 minutes in TBS-Tween, incubated with primary antibody T7-Tag or GST(12) for 30 min and bands visualized by ECL.

mRNA Isolation and cDNA Analysis

RNA was isolated using the guanidine isothiocyanate / CsCl method and mRNA was selected using oligo dT sepharose. For northern blotting mRNA was separated on a formaldehyde agarose gel and transferred to a Nylon membrane. A random primed probe 32 P probe was generated using the full-length (5057 bp) nNOS cDNA (Bredt et al., 1991) as a template. The filter was washed at high stringency - 68°C, 0.1% SSC, 0.1% SDS and exposed to X-ray film overnight at -70°C.

Thermal RACE-PCR was performed as described (Frohman, 1993). The sequence of the nNOS specific primers in exon 3 were:

Race 1: CCACAGATCATTGAAGACTCG

Race 2: GGGGAATTCCCCGCCCAGGGGCGGAGCTTT

For RT-PCR, mRNA was reverse transcribed with RTth polymerase using random hexamer primers. The sequence of the PCR primers used were:

P1: GTCCCTGCGTATTGATGCA

P2: GGCCGACCTGAGATTCCC

P3: CTCTGCATCTGTCAAGCTGG

P4: CCTTCACCAGGAAGCCCAGA

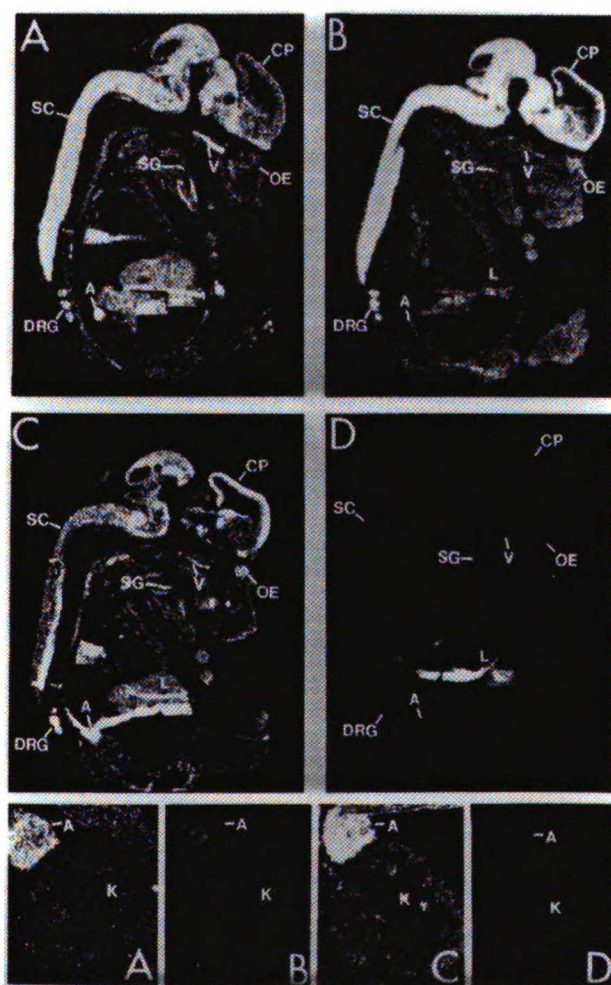
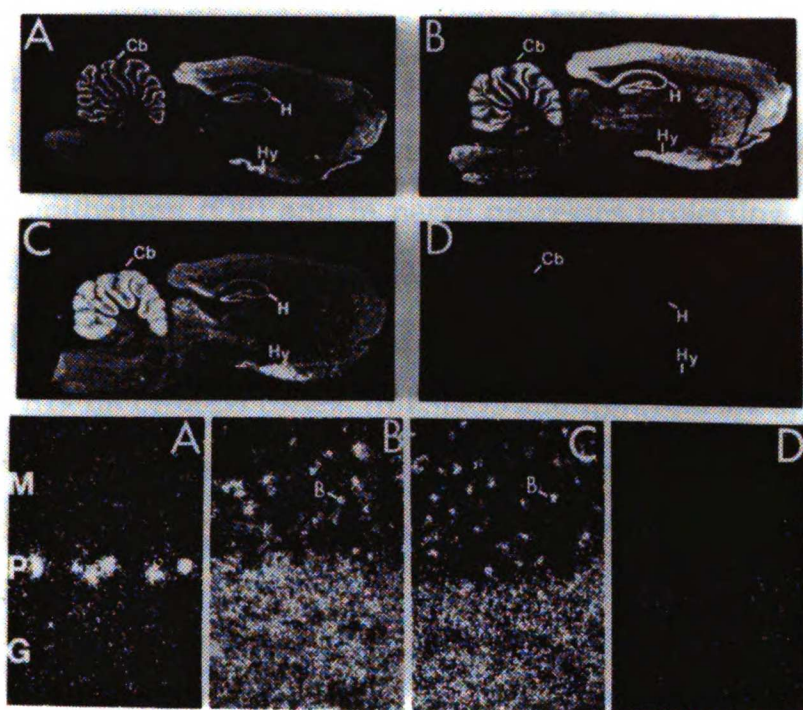
Clones encoding PSD-93 were isolated from a rat brain cDNA library (Stratagene) by plaque hybridization.

nNOS Protein Purification and Catalytic Assays

Solubilized tissue homogenates were incubated with 100 μ l of 2'5'-ADP agarose (Sigma), columns were washed with 5 ml of buffer containing 0.35 M NaCl, and were eluted with 10 mM NADPH. Catalytic NOS activity was quantitated by monitoring the conversion of [3 H]arginine to [3 H]citrulline as described (Bredt and Snyder, 1990).

UCL LIBRARY

Figure 3-1. Expression of PSD-93, PSD-95 and nNOS in rat brain and E15 embryo. *In situ* hybridization was used to localize transcripts for PSD-93 (A), PSD-95 (B), nNOS (C) or sense control (D) in adjacent cryosections. (a) In adult rat brain, PSD-95 occurred only in neurons and was co-expressed with nNOS in certain neurons in hypothalamus, hippocampus and cerebellum. PSD-93 also appeared to be neuron specific, but had a more restricted distribution than did PSD-95. (b) In cerebellum, PSD-95 and nNOS were co-expressed in cerebellar granule cells in the granular layer (G) and basket cells (B) in the molecular layer. By contrast PSD-93 was restricted to Purkinje neurons (P) of cerebellum, which lack nNOS or PSD-95. Double labeling with NADPH diaphorase and *in situ* hybridization identified the PSD-95 and nNOS expressing cells in molecular layer as basket cells (data not shown). (c) In E15 embryo, PSD-95 was found ubiquitously expressed in differentiated central neurons, but not in neuronal precursors. PSD-95 was coexpressed with nNOS in cerebral cortical plate (CP), dorsal root ganglia (DRG) and neurons of the olfactory epithelium (OE). PSD-93 was abundantly expressed in neurons of spinal cord (SC), DRG and trigeminal nerve (V). PSD-93 was specifically co-expressed with nNOS in secretory cells of the submandibular gland (SG) and in (d) chromaffin cells of the developing adrenal gland, which lack PSD-95. The signal in liver (L) seen in all samples including control represents non-specific hybridization to a aberrant fold in the tissue. Abbreviations: K, kidney; A, adrenal gland.



UCL LIBRARY

Figure 3-2. PSD-95 colocalizes with nNOS in developing neurons. Immunohistochemical staining of adjacent sagittal sections of E19 rat fetus indicates that (a, c) PSD-95 and (b, d) nNOS are colocalized in primary olfactory epithelium (OE) and in nerve processes projecting to the olfactory bulb (OB) (a and b, 50X; c and d, 400X). In intestine (Int) of E19 rat, (e, g) PSD-95 and (f, h) nNOS are colocalized in myenteric neurons (MN). (50X, and 200X) In E19 rat cerebral cortex, (i) PSD-95 and (j) nNOS are again colocalized. Both proteins are most concentrated in neuronal processes of the intermediate zone (IZ) and cell bodies of the cortical plate (CP), while the ventricular zone (VZ) is devoid of staining (100X.)

UCL LIBRARY

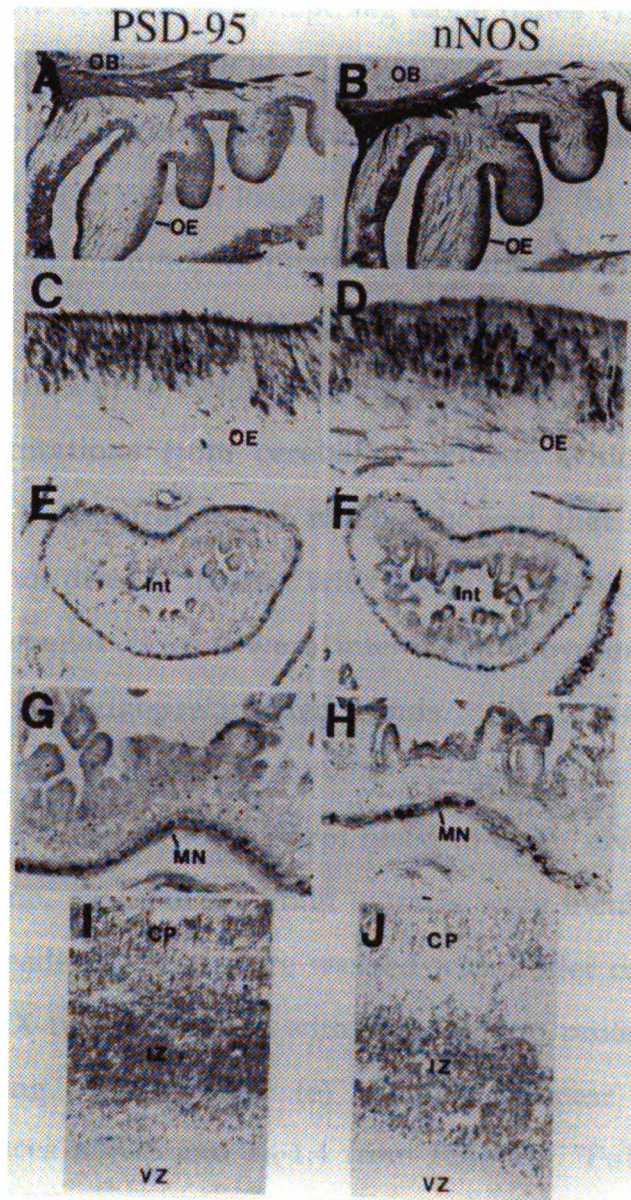


Figure 3-2

Figure 3-3. nNOS binds to PSD-95 through PDZ motif interactions. (a-c) Co-immunoprecipitation of nNOS and PSD-95. (a) COS cells were transfected with an expression construct (PSD-myc) encoding amino acids 1-386 of PSD-95 with a 10 amino acid c-myc epitope tag alone (lanes 1) or were cotransfected with PSD-myc and nNOS (lanes 2). Cell homogenates were immunoprecipitated with nNOS and probed with a monoclonal antibody to c-myc. Input=5% of protein loaded onto columns. (b) Solubilized cerebellar membranes were immunoprecipitated with antibody to PSD-95 (lanes 2) or a non-immune serum (lanes 1). Western blotting shows specific co-immunoprecipitation of nNOS but not eNOS with PSD-95. (c) Identical immunoprecipitations from cerebellar cytosol, which lacks PSD-95 but contains high concentrations of nNOS, showed that the PSD-95 antibody (lane 2) does not directly interact with nNOS. The eNOS blot and the nNOS blot from cerebellar cytosol were intentionally overexposed, but failed to show specific immunoprecipitated bands. (d) Affinity chromatography demonstrates that nNOS is selectively retained by an immobilized PSD-95 protein fragment (amino acids 1-386) fused to GST. eNOS is not retained by the PSD column. Solubilized brain extracts were incubated with G-PSD or control GST beads, columns were washed with buffer containing 0.5 M NaCl and 1% triton X-100, and eluted with SDS. Bound proteins were detected by western blotting. Input=10%. (e) NMDA receptor 2B carboxy terminal peptide displaces nNOS and K_v1.4 from PSD-95. "Pull-down" assays from brain were conducted as above containing 0 (lanes 6, 7), 10 μ M (lanes 4,5) or 30 μ M (lanes 2,3) NMDA receptor peptide or 200 μ M control peptide (lanes 8,9). Input=10%. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

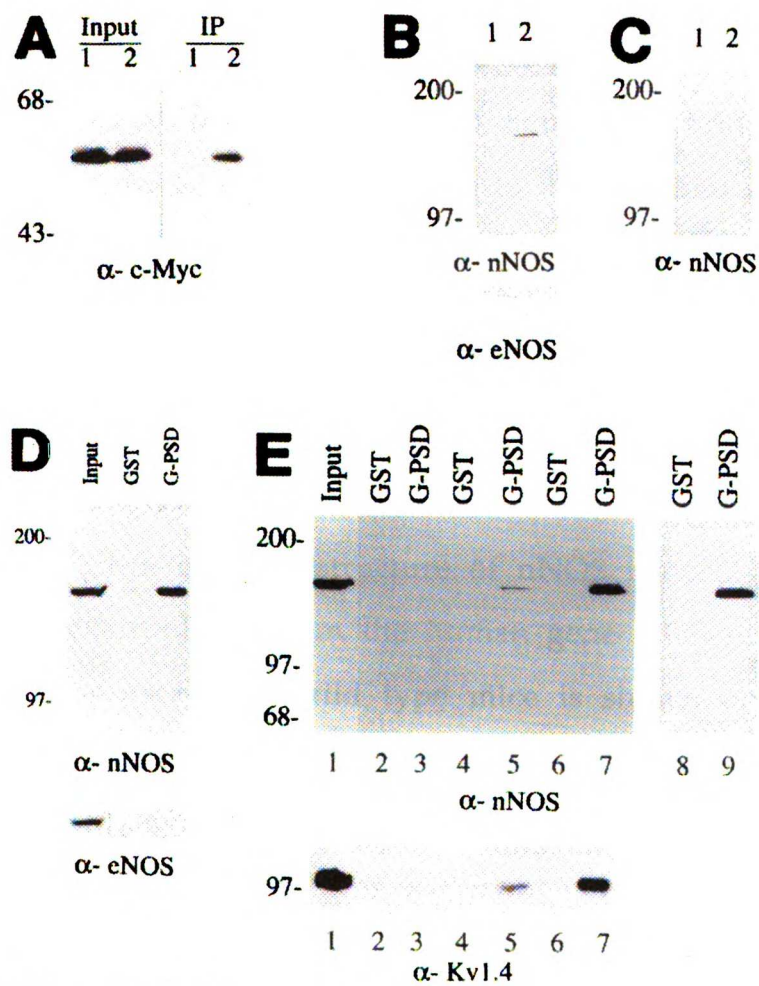


Figure 3-3

Figure 3-4. Exons 1 and 2 of nNOS are alternatively spliced. (a) Northern blot analysis of brain mRNA from wild type (WT) and nNOS Δ/Δ mouse hybridized with a full length nNOS cDNA probe. A broad band of 10.5 kb is recognized in wild type brain and weaker bands of 11 and 9.5 kb are recognized in nNOS Δ/Δ mouse. (b, c) RT-PCR analysis of 5' splicing of nNOS gene. cDNA was amplified with primers 1 and 2 (lanes 1, 2). (b) Ethidium bromide staining shows a band of 1 kb amplified from nNOS Δ/Δ and a band of 2.2 kb from wild type. (c) Southern hybridization with a full length nNOS probe shows hybridization to the ethidium stained bands. A weaker band of 1 kb in amplifications of wild type cDNA is also detected by hybridization (c, lane 2). A similar analysis using primers 3 and 4 confirm that exon 2 sequences are only detected in wild type cDNA (lanes 3, 4.) (d) Nucleotide sequences of 5' RACE-PCR products amplified from nNOS Δ/Δ brain. Predicted CTG starter methionine in 5'a is marked with an asterisk. (e) Schematic diagram of exon structure of nNOS isoforms (exon structure beyond 2 is extrapolated from the human gene (Hall et al., 1994). The predominant transcript in wild type mice is shown on top. The two transcripts detected in nNOS Δ/Δ mice lacking exon 2 are shown below. Positions of PCR primers, intron / exon borders, and methionine start codons are noted. Translated sequences within nNOS are shaded. Unique N-terminal region of nNOSb is hashed. (f) Predicted N-terminal amino acid sequence of nNOS isoforms. Numbers correspond to amino acid positions within mouse nNOS.

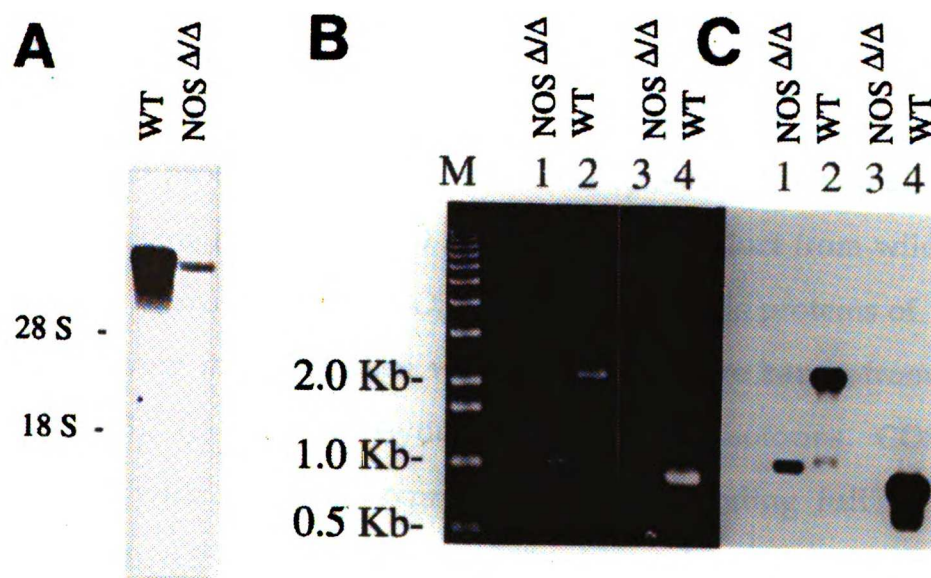


Figure 3-4

Figure 3-5. Catalytically active nNOS isoforms lacking exon 2 are expressed in brain of nNOS Δ/Δ . (a) Western blotting of crude (lanes 1, 2) and 2' 5'-ADP affinity purified (lanes 3, 4) brain extracts indicates that the major nNOS band in wild type brain migrates at 160 kD (lanes 1, 3) while in nNOS Δ/Δ , co-purifying bands of 125 and 136 kD (lanes 2, 4) are recognized. Partial tryptic digestion of 2'5-ADP agarose-purified proteins reveals a similar proteolytic "fingerprint" from wild type (lane 5) and nNOS Δ/Δ (lane 6). Note that 20 fold more protein was loaded from nNOS Δ/Δ (lanes 2, 4, 6) than from wild type samples (lanes 1, 3, 5). (b) cDNA clones encoding nNOS, nNOSb (5'a spliced to exon 3) or nNOSg (5'b spliced to exon 3) were transfected (trx) into COS cells and protein extracts were resolved by SDS / PAGE. Full length NOS (nNOS-trx) comigrates at 160 kD with the major product from wild type brain (lanes 1, 2). Transfection of nNOSb and nNOSg yields proteins of 136 and 125 kD respectively that comigrate with immunoreactive bands from nNOS Δ/Δ (lanes 3, 4, 5). (c) NOS catalytic activity of nNOS isoforms. COS cells were transfected with 10 μ g of expression vector encoding full length nNOS, nNOSb, or nNOSg. NOS activity was measured in cell homogenates three days following transfection in the presence of 200 μ M free calcium. This experiment was replicated twice with similar results. (d) nNOS isoforms are discretely expressed in nNOS Δ/Δ brain. Highest densities of nNOS in wild type (10 μ g / lane) are found in cerebellum (Cb). In nNOS Δ/Δ (100 μ g / lane) highest levels of nNOS isoforms are found in striatum (St) and hippocampus (Hi), lower amounts are found in brainstem (Bs) and cerebral cortex (Cx) while the cerebellum is devoid of nNOS isoforms in nNOS Δ/Δ . (e) eNOS is homogeneously distributed in forebrain (Fb), cerebellum (Cb) as well as the peripheral tissues liver (Li), lung (Lu) and kidney (Ki). All lanes in (c) were

Figure 3-6. nNOS isoforms lacking the PDZ motif do not bind to PSD-95 or to brain membranes. (a) Partially purified nNOS protein from wild type (WT) or nNOS Δ/Δ brain were analyzed by PSD "pull-down" assay. Full length nNOS binds to PSD-95 while the residual isoforms lacking the PDZ motif do not. Input=20%. (b) Residual nNOS isoforms are restricted to cytosol of nNOS Δ/Δ . Brain homogenates, extracted with 100 mM NaCl (lanes 1), 1 M KCl + 1% triton X-100 (lanes 2) or insoluble pellet (lanes 3), from wild type (20 μ g / lane) or nNOS Δ/Δ (200 μ g / lane) were probed by western blotting. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

UNIVERSITY OF CALIFORNIA

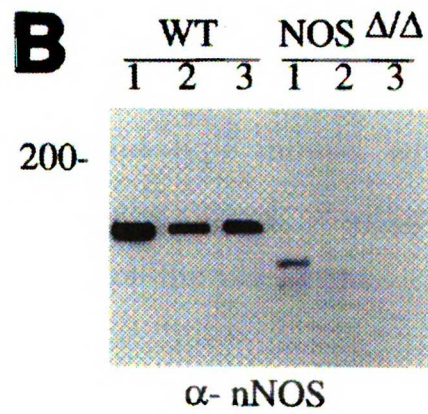
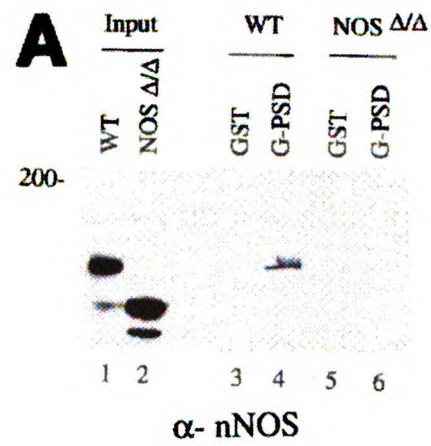


Figure 3-6

Figure 3-7. $\alpha 1$ -Syntrophin binds to the N-terminal PDZ containing domain of nNOS. (a) "Pull-down" assays of solubilized muscle extracts from wild type or *mdx* mouse using an nNOS (amino acids 1-299)-GST fusion protein were done as described in figure 3-5. Western blotting shows that $\alpha 1$ -syntrophin from both wild type and *mdx* mouse is selectively retained by the G-NOS column. Input=20%. (b, c) Immunoprecipitation of solubilized muscle extracts with a polyclonal antibody to $\alpha 1$ -syntrophin shows co-precipitation of (b) nNOS but not (c) eNOS (lanes 2). Control experiments with non-immune serum shows no precipitation of nNOS or eNOS (lanes 1). Bands at 55 kD represent immunoglobulin heavy chains. (d, e) Subcellular fractionation of nNOS is altered nNOS Δ/Δ mouse muscle. Homogenized muscle was sequentially extracted in buffer containing 100 mM NaCl (lanes 1), 500 mM NaCl (lanes 2) and 500 mM NaCl + 1% triton X-100 (lanes 3). These extracts were purified by 2'5'-ADP agarose chromatography and were resolved by SDS / PAGE. (d) nNOS is present in both soluble and membrane associated fractions of wild type mouse, while (e) nNOS isoforms in nNOS Δ/Δ are restricted to the cytosol. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

UNIVERSITY OF TORONTO

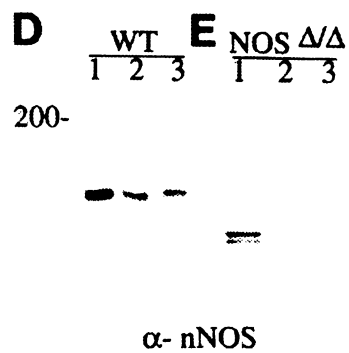
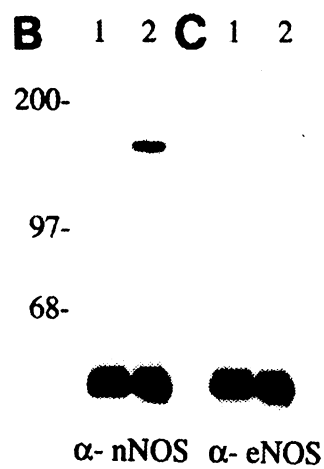
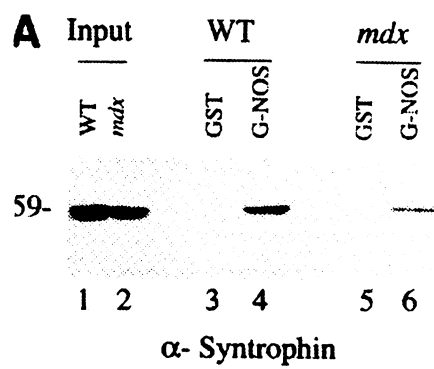


Figure 3-7

Figure 3-8. Direct binding of nNOS to α 1-syntrophin PDZ domain. (a) Purified α 1-syntrophin PH1 (25 kDa, lanes 1), PDZ domain (15kDa; lanes 2), PH2 (18 kDa; lanes 3) and SU domain (16 kDa; lanes 4) fusion proteins were resolved and overlayed with GST or nNOS (1-299)-GST. The position and relative amounts of the α 1-syntrophin domain fusion proteins are indicated by immunoreactivity with a monoclonal against the T7-Tag. Bound GST fusion proteins were detected by blotting with a monoclonal antibody to GST. Only nNOS-GST bound specifically to the PDZ domain of α 1-syntrophin. (b) GST (lanes 1) and nNOS-GST (lanes 2) were separated and overlayed with α 1-syntrophin domain fusion proteins (PH1, PDZ, or PH2) or blotted with a monoclonal antibody to GST. Bound syntrophin fusion proteins were detected with monoclonal antibody to T7-Tag. Of the syntrophin fusion proteins tested, only PDZ bound to nNOS; no binding to GST was detected. (c) Co-localization of nNOS and α 1-syntrophin immunofluorescence at skeletal muscle sarcolemma and neuromuscular junctions (labeled by rhodamine α -bungarotoxin (BGT)). (d) Schematic model showing interaction of nNOS with skeletal muscle α 1-syntrophin (59K syn). The possibility that other enzymes containing a PDZ motif may also bind to syntrophins is indicted at the left with question marks. Abbreviation: DG, dystroglycan. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

UNIVERSITY OF CALIFORNIA

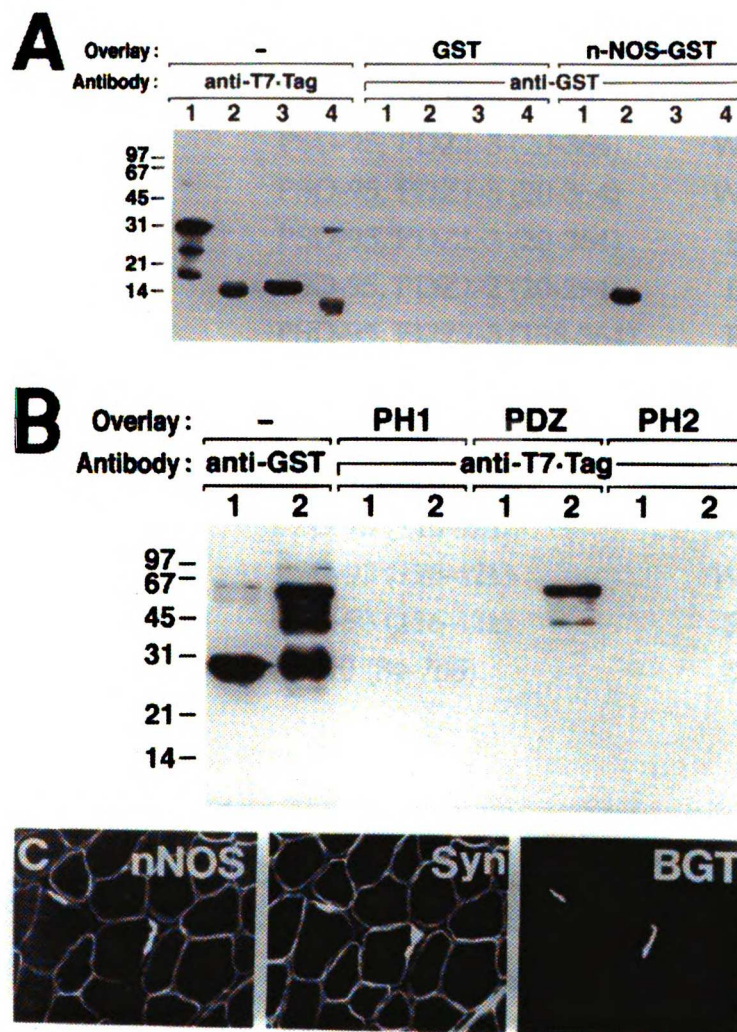


Figure 3-8

TABLE 3-1

A. Interactions between nNOS and PSD-95 or PSD-93

Gal4 DNA Binding Hybrid	Gal4 Activation Hybrid	Colony Color	Growth
nNOS (amino acids 1-195)	SV 40 (amino acids 84-708)	White	-
p53 (72-390)	PSD-95, PDZ1-3 (20-364)	White	-
Lamin C (66-230)	PSD-95, PDZ1-3 (20-364)	White	-
nNOS (1-195)	PSD-95, PDZ1-3 (20-364)	Blue	+
nNOS (1-195)	PSD-95, PDZ1-2 (20-294)	Blue	+
nNOS (1-195)	PSD-95, PDZ2-3 (138-364)	Blue	+
nNOS (1-195)	PSD-95, PDZ1 (20-144)	White	-
nNOS (1-195)	PSD-95, PDZ2 (138-294)	Blue	+
nNOS (1-195)	PSD-95, PDZ3 (291-364)	White	-
p53 (72-390)	PSD-93 (116-421)	White	-
Lamin C (66-230)	PSD-93 (116-421)	White	-
nNOS (1-195)	PSD-93 (116-421)	Blue	+
p53 (72-390)	SV 40 (84-708)	Blue	+

Gal4 DNA Binding Hybrid	Gal4 Activation Hybrid	Colony Color	Growth
nNOS (amino acids 1-195)	SV 40 (amino acids 84-708)	White	-
p53 (72-390)	PSD-95, PDZ1-3 (20-364)	White	-
Lamin C (66-230)	PSD-95, PDZ1-3 (20-364)	White	-
nNOS (1-195)	PSD-95, PDZ1-3 (20-364)	Blue	+
nNOS (1-195)	PSD-95, PDZ1-2 (20-294)	Blue	+
nNOS (1-195)	PSD-95, PDZ2-3 (138-364)	Blue	+
nNOS (1-195)	PSD-95, PDZ1 (20-144)	White	-
nNOS (1-195)	PSD-95, PDZ2 (138-294)	Blue	+
nNOS (1-195)	PSD-95, PDZ3 (291-364)	White	-
p53 (72-390)	PSD-93 (116-421)	White	-
Lamin C (66-230)	PSD-93 (116-421)	White	-
nNOS (1-195)	PSD-93 (116-421)	Blue	+
p53 (72-390)	SV 40 (84-708)	Blue	+

B. Interaction between nNOS and α 1-syntrophin

Gal4 DNA Binding Hybrid	Gal4 Activation Hybrid	Colony Color	Growth
Lamin C (66-230)	α 1-syntrophin (59-166)	White	-
p53 (72-390)	α 1-syntrophin (59-166)	White	-
nNOS (1-195)	α 1-syntrophin (59-166)	Lt. Blue	+

Gal4 DNA Binding Hybrid	Gal4 Activation Hybrid	Colony Color	Growth
Lamin C (66-230)	α 1-syntrophin (59-166)	White	-
p53 (72-390)	α 1-syntrophin (59-166)	White	-
nNOS (1-195)	α 1-syntrophin (59-166)	Lt. Blue	+

Yeast HF7c and Y187 cells were cotransformed with expression vectors encoding various GAL4-binding domain and GAL4 activation domain fusion proteins. Each transformation mixture was plated on two synthetic dextrose plates, one lacking tryptophan and leucine and the other lacking tryptophan, leucine and histidine. Growth was measured on histidine-deficient plates and color was measured by a β -galactosidase colorimetric filter assay (Fields and Song, 1989).

UNIVERSITY OF CALIFORNIA

CHAPTER FOUR

Cloning and characterization of postsynaptic density 93 (PSD-93), a nitric oxide synthase interacting protein

11/11/17 10:00

Abstract

Nitric oxide formation in brain is regulated by the calcium / calmodulin dependence of neuronal nitric oxide synthase (nNOS). Calcium influx through N-methyl-D-aspartate (NMDA) type glutamate receptors is efficiently coupled to nNOS activity while many other intracellular calcium pathways are poorly coupled. To elucidate possible mechanisms responsible for this coupling, we performed yeast two-hybrid screening to identify proteins which interact with nNOS. Two nNOS interacting proteins were identified, the postsynaptic density proteins, PSD-93 and PSD-95. Here, we report the cloning and characterization of PSD-93. PSD-93 is expressed in discrete neuronal populations as well as specific non-neuronal cells and exhibits complex molecular diversity due to tissue specific alternative splicing. PSD-93, like PSD-95, binds to nNOS and to the NMDA receptor 2B. However, PSD-93 is unique among PSD-95 / SAP-90 family members in its expression in Purkinje neuron cell bodies and dendrites. We also demonstrate that the PDZ domain at the N-terminus of nNOS is required, but is not sufficient for interaction with PSD-93/95. Given that PSD-93 and PSD-95 each contain multiple potential binding sites for nNOS and the NMDA receptor, complexes involving oligomers of PSD-93/95 may help account for the functional as well as physical coupling of nNOS to NMDA receptors.

GET LIDIAN!

Recently, NMDA receptor subunits have been shown to bind to a postsynaptic density protein, PSD-95 (Kornau et al., 1995). The physiological significance of NMDA receptor interaction with PSD-95 is unclear but is postulated to play a role in the assembly of multiprotein complexes involved in NMDA receptor mediated synaptic plasticity (Kornau et al., 1995).

To determine which protein-protein interactions participate in coupling NMDA receptors to NO formation in brain, we screened for nNOS binding proteins using the yeast two hybrid system (Fields and Song, 1989). We identified two classes of interacting gene products from a screen of 10^6 plasmids (Brenman et al., 1996). One group encoded fragments of PSD-95 while the other group encoded a novel related protein, PSD-93. Here, we report the cloning and characterization of PSD-93. We find that PSD-93 mRNA is highly enriched in brain where it occurs discretely in specific neuronal populations. Outside the brain, PSD-93 occurs in specific neuronal and non-neuronal cells. Cloning of multiple PSD-93 cDNAs from brain indicates molecular heterogeneity consistent with tissue specific alternative splicing of the mRNA. Immunocytochemistry demonstrates that PSD-93 is probably postsynaptic in cerebellar Purkinje cell dendrites but does not rule out a presynaptic localization elsewhere. Biochemical studies demonstrate that the PDZ motifs within PSD-93 bind to nNOS as well as to the tSXV motif of NMDA receptor 2B. Surprisingly, we find that additional sequence outside the PDZ domain of nNOS is required for binding to PSD-93/95.

RESULTS

Molecular Cloning of PSD-93

To determine the molecular structure of PSD-93 we screened a rat brain library with a fragment of human PSD-93 isolated as a nNOS interacting gene product (Brenman et al., 1996). Ten hybridizing clones were isolated from a screen of 10^6 phage. Clones encoding the full open reading frame were sequenced on both strands and predicted a protein of 834 amino acids and 93 kD (figure 4-1A). Sequence analysis revealed that PSD-93 shares approximately 70% homology with PSD-95 (or SAP-90) and with HDLG (or SAP-97). These three proteins share a common domain structure consisting of three PDZ (or GLGF) protein motifs followed by an SH-3 domain and finally a domain homologous to yeast guanylate kinase. Considerable sequence divergence between these homologous proteins occurs at the N-terminus. In addition, HDLG and PSD-93 have small inserts between the SH3 and guanylate kinase domains. PSD-93 also has a unique larger insert between the second and third PDZ repeats.

Analysis of multiple PSD-93 cDNAs indicated three sites for putative alternative splicing. Certain cDNA clones isolated from the library had either a 102 base pair (34 amino acid) or a 45 base pair (15 amino acid) insert between amino acids K624 and R625 (figure 4-1C). Another example of alternative splicing was found by RT-PCR amplification of rat brain cDNA using primers that flank the large insert that is unique to PSD-93. In these amplifications, we consistently found two bands, one corresponding to the PSD-93 clones isolated from the library, and another which lacked 156 nucleotides (52 amino acids) between PDZ-2 and PDZ-3 (noted as a deletion in figure 4-1D).

The greatest degree of heterogeneity was found at the N-terminus of PSD-93, as we isolated cDNA clones with four distinct sequences (figure 4-1B). The most commonly obtained transcript, 5'b, contained an inframe starter ATG codon which predicted a protein of 93 kD and contained 220 bp of 5' untranslated sequence. The largest transcript, 5'a diverged from 5'b immediately upstream of lysine 14, encoded two potential starter methionines, and contained at least 257 bp of 5' untranslated sequence. The sequence of two other cDNAs isolated from the library diverged from PSD-93 at points further into the translated sequence. 5'c diverged upstream of alanine 69 and contained an inframe starter methionine. 5'd diverged from the common PSD-93 sequence upstream of threonine 78 and did not contain an inframe ATG start codon. Translation of the 5'd transcript may initiate at the first in frame ATG codon, which occurs at amino acid 228, or may initiate at a non-canonical start codon.

Expression of PSD-93 mRNA

We evaluated the overall distribution of PSD-93 by Northern blotting using a probe to sequences that are present in all the alternatively spliced forms. PSD-93 mRNA occurred as a single band of ~7.5 kb in poly A⁺ RNA from brain (figure 4-2A). Under these conditions, PSD-93 could not be detected in poly A⁺ RNA from kidney, spleen, liver, heart, or skeletal muscle. We also evaluated the distribution of PSD-93 in various brain regions. Highest levels were found in cerebral cortex and cerebellum, somewhat lower densities in striatum and hippocampus, and essentially no PSD-93 could be detected in brain stem. The PSD-93 hybridizing bands in forebrain regions

migrated as ~7.5 kb bands, while that in cerebellum migrated just slightly faster (figure 4-2B).

The altered migration of PSD-93 mRNA in cerebellum raised the possibility that the message is alternatively spliced in a tissue specific manner. To evaluate this, we rehybridized the blot with probes corresponding to the two large alternative N-termini. The first probe, 5'a, hybridized to a single band of ~7.5 kb from rat brain RNA and resembled the distribution of the general PSD-93 probe in various brain regions (figure 4-2C). On the other hand, hybridization with 5'b indicated that this transcript occurs selectively in forebrain regions and is absent from cerebellum (figure 4-2D).

We next evaluated the cellular distribution of PSD-93 mRNA by *in situ* hybridization. As previously reported (Brenman et al., 1996), PSD-93 occurred in subpopulations of neurons in the adult brain. In the forebrain, PSD-93 was apparent in cerebral cortex, striatum and hippocampus. Highest densities in hippocampus were found in pyramidal neurons of Ammon's horn and in granule cells of the dentate gyrus (figure 4-3a). In the hindbrain, PSD-93 was restricted to Purkinje neurons of cerebellum (figure 4-3a). PSD-93 was not detected in brain stem structures confirming the regional distribution found by northern analysis.

To determine expression of PSD-93 in peripheral tissues we conducted *in situ* hybridization on E15 rat embryo (figure 4-3b). We found the highest densities of PSD-93 in developing brain and spinal cord. Several neuronal populations in the periphery also showed strong hybridization including myenteric neurons of the intestine and sensory neurons of the dorsal root ganglia and trigeminal ganglia. Unlike PSD-95, certain non-neuronal tissues contained PSD-93. Particularly high levels of PSD-93 were noted in several glands including the adrenal, thymus and submandibular glands.

glutathione-S-transferase protein (G-P93) in *E. coli*. The expressed fragment encoded amino acids 77-453, which represents the first two PDZ domains of PSD-93. We evaluated binding of endogenous nNOS to this fragment of PSD-93 by affinity chromatography (Brenman et al., 1996). Crude solubilized brain extracts were incubated with G-P93 or control. Glutathione sepharose was added and non-specific proteins were removed by washing with buffer containing 300 mM NaCl and 1% Triton X-100. Adherent proteins were eluted with loading buffer and were resolved by SDS-PAGE. Western blotting showed selective retention of nNOS to a PSD-93 column, but not to a control GST column (figure 4-6).

Previous studies have indicated that nNOS interacts selectively with the second PDZ motif of PSD-95 and that binding can be competed away with a peptide corresponding to the 9 terminal amino acids of the NMDA receptor 2B (KLSSIESDV). To determine whether binding of nNOS to PSD-93 displayed similar properties we repeated the "pull-down" assays in the presence of varying concentrations of the NMDA receptor peptide. We found that this 9-mer peptide potently displaced nNOS from PSD-93 ($K_d < 5 \mu\text{M}$). Displacement by the NMDA receptor peptide was specific as a control peptide (KLSSIEADA) with only point mutations of the consensus serine and valine residues to alanines had no effect on binding even at higher concentrations (figure 4-6).

The PDZ domain of nNOS is contained within the first 100 amino acids, while the first 195 amino acids of nNOS were originally used to identify PSD-93 in a two-hybrid screen. We therefore asked whether the PDZ domain of nNOS was sufficient for interaction with PSD-93. To address this question, we engineered overlapping constructs spanning the amino terminal 195 amino acids of nNOS and screened for interaction with PSD-93 by the yeast

two-hybrid system (table 4-1). We found that constructs containing the first 150 amino acids of nNOS were necessary and sufficient for association with PSD-95. Deletion of sequences on either side of this 150 amino acid domain produced fusions that failed to interact with PSD-93. Importantly, an N-terminal 100 amino acid construct encoding the entire consensus PDZ domain of nNOS did not associate with PSD-93.

To verify that the PDZ domain of nNOS is not sufficient for interaction with PSD-93, we biochemically evaluated binding using affinity chromatography. GST fusion proteins encoding amino acids 1-100, 1-150 and 1-195 were linked to columns of glutathione-sepharose beads and were incubated with solubilized brain extracts (figure 4-7). After washing the columns, retained proteins were identified by Western blotting. Again, we found that amino acids 1-150 of nNOS potently interacted with PSD-93 but that amino acids 1-100 are not sufficient for association. In parallel experiments, we found that amino acids 1-150 of nNOS are necessary and sufficient for interaction with PSD-95 (data not shown).

UNIVERSITY OF CALIFORNIA

DISCUSSION

These studies identify PSD-93 as a novel member of a growing family of proteins with both PDZ motifs and homology to guanylate kinases. These proteins appear to organize signaling molecules at membrane junctions. The PDZ motif contains ~100 amino acids and is present in a diverse family of enzymes and structural proteins. The first identified member of this group, discs-large (*dlg*) tumor suppressor protein of *Drosophila* is localized to septate junctions of epithelial cells in *Drosophila* larvae. The absence of *dlg* in mutant flies results in disruption of the septate junctions, loss of cell-cell adhesion and neoplastic growth of epithelial cells in the larval imaginal discs (Woods and Bryant, 1991). Mammalian homologues of *dlg* have also been localized to sites of cell-cell contact. For example, zona occludens 1 and 2 (ZO1 and ZO2) occur at tight junctions of epithelial cells (Jesaitis and Goodenough, 1994; Stevenson et al., 1986; Willott et al., 1993). PSD-95 / SAP-90 and HDLG / SAP-97 are enriched in presynaptic compartments of certain synapses (Kistner et al., 1993; Lue et al., 1994; Muller et al., 1995) and postsynaptic densities of forebrain neurons (Hunt et al., 1996). The high densities of PSD-93 mRNA identified here in specific neuronal populations in brain and periphery suggest an analogous role for PSD-93 in synaptic function. The dramatic enrichment of PSD-93 in cerebellar Purkinje cells, which lack other members of this gene family, may indicate an important role for PSD-93 in synaptogenesis for Purkinje cells as well as postsynaptic function in cerebellum. Targeted disruption of PSD-93 *in vivo* should help clarify this question.

PSD-95 / SAP-90 was independently identified by two laboratories as an abundant and detergent insoluble protein enriched in brain synaptosomal

fractions (Cho et al., 1992; Kistner et al., 1993). Specific functions for PSD-95 remain uncertain and no catalytic activity has yet been demonstrated for the guanylate kinase domain. Yeast two hybrid analysis, however, demonstrated that PSD-95 can bind to NMDA receptor subunits and to certain K⁺ channels (Kim et al., 1995; Kornau et al., 1995). PSD-95 and NMDA receptor 2B have been shown to cluster together at synaptic sites in hippocampal neurons in culture (Kornau et al., 1995). In addition, coexpression of PSD-95 with the K⁺ channel Kv1.4 results in redistribution and clustering of the channels in cell surface patches (Kim et al., 1995). These studies identified that the first two PDZ repeats of PSD-95 can participate in a domain interaction with ion channels that contain a C-terminal tSXV motif (Kim et al., 1995; Kornau et al., 1995). We similarly find that PSD-93, expressed as a GST-fusion protein interacts with K_v 1.4, which contains the tSXV motif (data not shown). PSD-93 may, therefore, also play a role in clustering of synaptic receptors in certain neurons.

In brain, nNOS is enriched in membrane fractions (Brenman et al., 1996; Hecker et al., 1994). Electron micrographic studies demonstrate that membrane-associated nNOS is concentrated in axon terminals and over thick postsynaptic densities (Aoki et al., 1993). This membrane association of nNOS in neurons is mediated by the PDZ containing domain, as nNOS isoforms lacking this domain occur only in soluble fractions of brain extracts (Brenman et al., 1996). Membrane association of nNOS in brain may be mediated by interaction with PDZ repeats in PSD-95 and PSD-93. These PDZ domain interactions are specific as nNOS interacts selectively with the second PDZ motif of PSD-93 / 95 (Brenman et al., 1996). Because the first and third PDZ domain of PSD-95 can independently interact with tSXV domains of certain ion channels (Doyle et al., 1996; Kim et al., 1995), PSD-95 and PSD-93 may

serve as molecular bridges linking ion channels to nNOS. This compartmentalization of nNOS with ion channels may account for the selective coupling of specific calcium influx pathways to NO formation (Garthwaite, 1991; Kiedrowski et al., 1992; Raiteri et al., 1991).

In central neurons NO formation is functionally coupled to NMDA receptor activity and nNOS protein is colocalized with PSD-95 in some, but not all, neuronal populations (Brenman et al., 1996). In cerebellum an nNOS / PSD-95 complex was identified by immunoprecipitation (Brenman et al., 1996). We have not yet been able to evaluate possible formation of an nNOS / PSD-93 complex in brain membranes because, following affinity purification, our antiserum does not immunoprecipitate native PSD-93 protein from brain extracts. Outside the brain, nNOS also occurs in certain neuronal and non-neuronal cell populations and in many of these peripheral tissues nNOS occurs together with PSD-93. For example, we find that PSD-93 mRNA is enriched in neurons of the myenteric plexus. nNOS also occurs at high densities in myenteric neurons and NO formed in these cells mediates non-adrenergic-non-cholinergic relaxation of the intestines (Bult et al., 1990). NMDA receptor activity does not regulate nNOS in the enteric nervous system. Instead, NO formation appears to be linked to calcium influx through w-conotoxin sensitive channels (Daniel et al., 1994). It will now be important to determine whether these channels interact with PSD-93 and nNOS in myenteric neurons.

PDZ domains appear to be modular motifs capable of diverse modes of protein-protein interaction. One class of ligands for PDZ domains is the terminal tSXV motif that occurs in certain ion channels and receptor. Recent X-ray crystallography studies of a single PDZ domain demonstrate that the terminal carboxylate group of the tSXV is cradled by the main chain amides as

well as a arginine side chain of the PDZ domain (Doyle et al., 1996). In the crystal, the PDZ domain forms a dimer in which the surface of the peptide-binding domain of one PDZ subunit interacts with residues in β -strands from the other subunit (Cabral et al., 1996). This binding topology of PDZ domains may explain why the tSXV peptide of NR2B potently blocks nNOS binding to PSD-93.

Other studies have shown that PDZ domains can interact with transmembrane proteins, which lack entirely lack tSXV motifs (Shieh and Zhu, 1996). Interaction of nNOS with PSD-93 / 95 shows certain unique characteristics. nNOS is the first peripheral membrane protein found to interact with a PDZ domain. Also, the PDZ domain of nNOS is involved in binding to the PDZ domain of PSD-93 / 95. Future structural studies of the amino terminal domain of nNOS should identify determinants and confer binding of nNOS to PSD-93 / 95. Interestingly, we report here that the PDZ domain of nNOS is not alone sufficient for association with PSD-95. It will be important to determine whether interaction of PDZ domains and adjacent sequences is a common mechanism for organization of signaling machinery.

Cloning of PSD-93 identifies a significant heterogeneity of transcripts. Our molecular studies identified six examples of putative alternative splicing and it seems likely that additional heterogeneity exists. Alternative splicing within the open reading frame regulates the expression of inserted sequences between the second and third PDZ motifs and between the SH3 and guanylate kinase domains. These alternative splicing events may modify the protein-binding properties of the PDZ and SH3 domains. A more complex level of alternative splicing occurs at the N-terminus of PSD-93, where the alternative 5' regions are expressed in a tissue specific manner. Thus the PSD-93 gene may contain multiple promoter regions that are differentially active in

MATERIALS AND METHODS

Generation of PSD-93 and PSD-95 antiserum

Glutathione-S-transferase fusion proteins encoding amino acids 77-453 of PSD-93 and amino acids 1-386 of PSD-95 were expressed and purified from *E. coli*. Guinea pigs were immunized with the PSD-93 fusion and rabbits were immunized with the PSD-95 fusion. Antigens were emulsified in complete and incomplete Freund's adjuvant. Serum bleeds were evaluated by ELISA.

cDNA cloning and analysis

A human PSD-93 cDNA encoding amino acids 116-421 isolated as a nNOS interacting fragment was used to screen a rat brain cDNA library (Stratagene). Ten hybridizing cDNAs were isolated from a screen of 10^6 phage. Clones were sequenced on both strands using the dideoxy chain termination method (US Biologicals). The Genbank accession number for PSD-93 is U50717.

mRNA Isolation and Northern Blot Analysis

RNA was isolated using the guanidine isothiocyanate / CsCl method and mRNA was selected using oligo dT sepharose. For northern blotting, mRNA was separated on a formaldehyde agarose gel and transferred to a Nylon membrane. The filter was sequentially hybridized with random primed ^{32}P probes which were generated using PSD-93 cDNAs as template. The common probe corresponded to nucleotides 237-927 of PSD-93. Transcript specific probes, 5'a and 5'b, were generated by the polymerase chain reaction and corresponded to the unique regions 5' of lysine14 (see figure 4-1 B). After each hybridization, the membrane was washed at high stringency, 68°C , 0.1% SSC, 0.1% SDS and exposed to X-ray film at -70°C .

In situ Hybridization

Adult rats were perfused with 4% paraformaldehyde, brains were harvested, post-fixed at 4^o C for 3 hours, and cryoprotected in 20% sucrose overnight. Twenty micron sections were cut on a cryostat and melted onto glass slides (Plus, Fisher). Embryonic day 15 rats were delivered by Cesarean section, fixed in paraformaldehyde, and processed in parallel with adult brain. *In situ* hybridization used ³⁵S-labeled RNA probes as described (Sassoon and Rosenthal, 1993). Antisense probe to PSD 93 (nucleotides 237-927) and sense control were synthesized from bluescript vectors. Tissue sections were exposed to X-ray film for 4 days and then were dipped into photographic emulsion (Kodak NTB-2) and exposed for 14 days.

Immunohistochemistry

Sprague-Dawley rats were anesthetized with pentobarbital and perfused with 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer. Tissues were removed and post-fixed in paraformaldehyde for 1 hr at 4^oC. Tissues were cryoprotected overnight in 20 % sucrose. Free floating sections (40 µm) were cut on a sliding microtome. Sections were blocked for 1 hr in phosphate buffered saline containing 1.5% normal goat serum and then incubated overnight in the same buffer containing diluted antiserum. For indirect immunofluorescence, secondary goat anti-rabbit FITC and donkey anti-guinea pig Cy-3, conjugated antibodies were used according to the manufacturer's specifications (Jackson Laboratories, 1:200).

Fusion Protein Affinity Chromatography

Fusion protein of GST fused to amino acids 77-453 of PSD-93 or amino acids 1-100, 1-150 or 1-195 of nNOS were expressed in *Escherichia coli* and purified

Figure 4-1. Predicted amino acid sequence and alternative splicing of PSD-93. (A) Alignment of PSD-93 with PSD-95, and HDLG-SAP97 indicates an overall amino acid identity of ~70%. Identical amino acids are indicated by black boxes, and conserved amino acid changes are shaded by gray boxes. (B) The N-terminus of PSD-93 is alternatively splice prone. Sequences of the four alternative N-terminal transcripts (5'a-d) are indicated. Black triangles denote the sites at which the sequences diverge from PSD-93. The amino acid numbers in bold refer to that predicted using 5'b of PSD-93. In-frame starter methionines or stop codons are indicated. (C) Two different alternatively spliced inserts occur between amino acids K624 and R625. The arrow indicates the location at which the two isoform insertions occur. The sequences of the alternative insertions are also shown. (D) A schematic model showing the domain structure of PSD-93. Identified sites of putative alternative splicing are indicated.

B 5'a
 TCCCCCACCCTCGTCTCTTGGGAAAGACACCCCGAGACGACGTACTTTCGGGA
 GCAGTGCCTGCACAGGCTCGGGATGGCTGCACACGGGCGAGGGGACGACATCACAGCTGCTGGTCTAGGCTGCTAGGCAAGCTATGCTGCG
 AGCACTGGGCGAGCACCGAGCTCGGTTCCCTCAGCAAGCTGCACCTGCCGACGCCAAAAGAAAGTCCCTGTCTTCAAGGACCTGACGCTTTACACCA
 ATG ATT TGC CAC TGC AAA GTT GGT TGC ACC AAC AAC ACT TGG TCG CTG ATG TTT GGA TGC AAG AAG TAT CGA TAC...
 M I C K C K V A C T H N T L E L N F G C E K Y R Y ...
 14

5'b
 CCAATGAAATCTGGGCAATAGCTAT
 ATATGCAATGAGAGATCTGAGCTCTACTCAGTGCCTCAAGACATGCTGTTTGTAGTACAGGAAACAGGGATCAATGATTTTCAACCTGACAGAG
 GAAGGATGCTCTCTTACGACACATGCCAGGATCGTGTGTGTGGGCTTGGCTGTGCTGAGAAAGCTGATACCGGCCCATATGCTCTTATTCTAGTGA
 ATG TTT TGC GCA TGC TAT TGT GCA CTC CGG ACT AAC GTG AAG AAG TAT CGA TAC...
 M F F A C Y C A L R T H V K K Y R Y ...
 14

5'c
 GAGTCTCTCTCTAAACCAATGTGAAGAAGCT ATG CAA CAT GCG TTC ATT CCG GGT AGC CCG GCT CCG ATG ATT GTC AAC...
 N Q H A P I P A S P A P I I V N ...
 69

5'd
 GGACGAGCTCTCTCACATGTTT TAG AGA AGC AAT TGT ATC ACA CAG AAG CTG ATA ATT CTC AAG ACA GAC ACT CTG ...
 PROG R S N C I P Q K L I I L S T D T L ...
 78

C

GCA AAA CCG GGT GTG ATT GAT TCC AAA CGA GGA CAA GAA GAC CTC ATT CTC TCC...
 A K P G V I D S K R G Q E D L T L S ...
 616

Insertion 1
 GGG TCA TTC AAT GAC AAG CGT AAA AAG AGC TTC ATC TTT TCA CGA AAA TCC CAA
 G S F M D K R K K S F I F S R K F P
 TTC TAC AAG AAC AAG GAG CAG AGT GAG CAG GAA ACC AGT GAT CCG GAA
 P Y K N K S Q S R Q S T S D P E

Insertion 2
 GGG ACA TCC CGG TTA GGT GAC GAC GGT TAT GGA ACA AAG ACT CTG
 G T S R L G D D G Y G T K T L

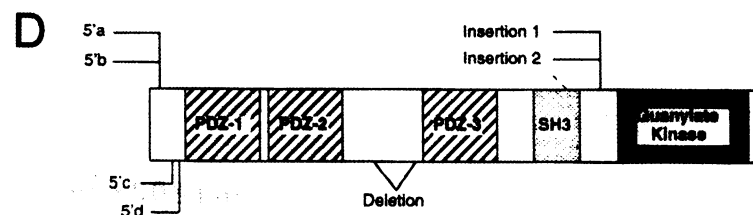


Figure 4-2. Tissue expression pattern and alternative splicing of PSD-93.

(A) Northern blotting of adult rat tissues indicates that PSD-93 is expressed as an ~7.5 kb transcript that occurs in brain (Br), but not in kidney (Ki), spleen (Sp), liver (Li), heart (He), or skeletal muscle (Sk). (C) Northern blotting of brain regions demonstrates that PSD-93 is present in cerebellum (Cb), cortex (Cx), hippocampus (Hi) and striatum (St), but is absent from brainstem (BS). Note that the band in cerebellum migrates slightly faster than that in other brain regions. For A and B, a probe common to all the alternatively spliced forms of PSD-93 was used. (D, E) The blot in B was sequentially rehybridized with probes corresponding to two of the alternatively spliced N-terminal regions of PSD-93. (C) Probing with 5'a reveals that the regional distribution of this splice variant is similar to that of PSD-93. (D) 5'b, however, is selectively absent from cerebellum. (B, F) Duplicate samples of mRNA were probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to demonstrate loading and integrity of the mRNA for the above blots. Positions of molecular size markers are indicated in kilobases on the y-ordinate as is the 18S ribosomal subunit (18 S).

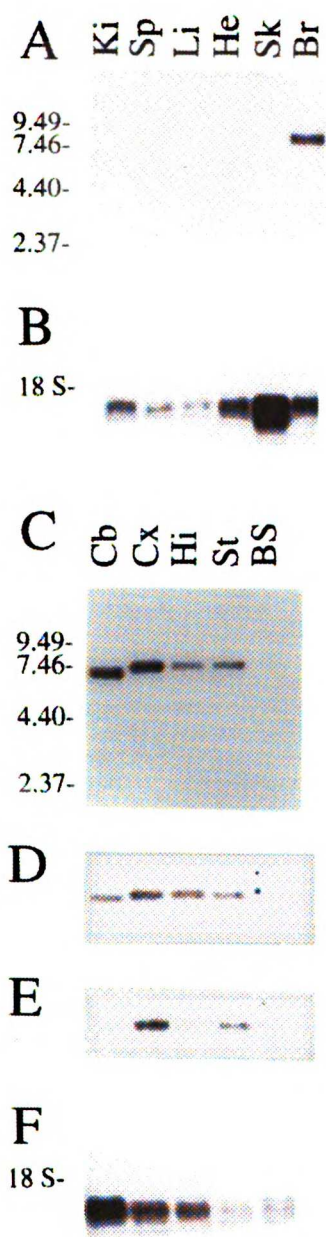


Figure 4-2

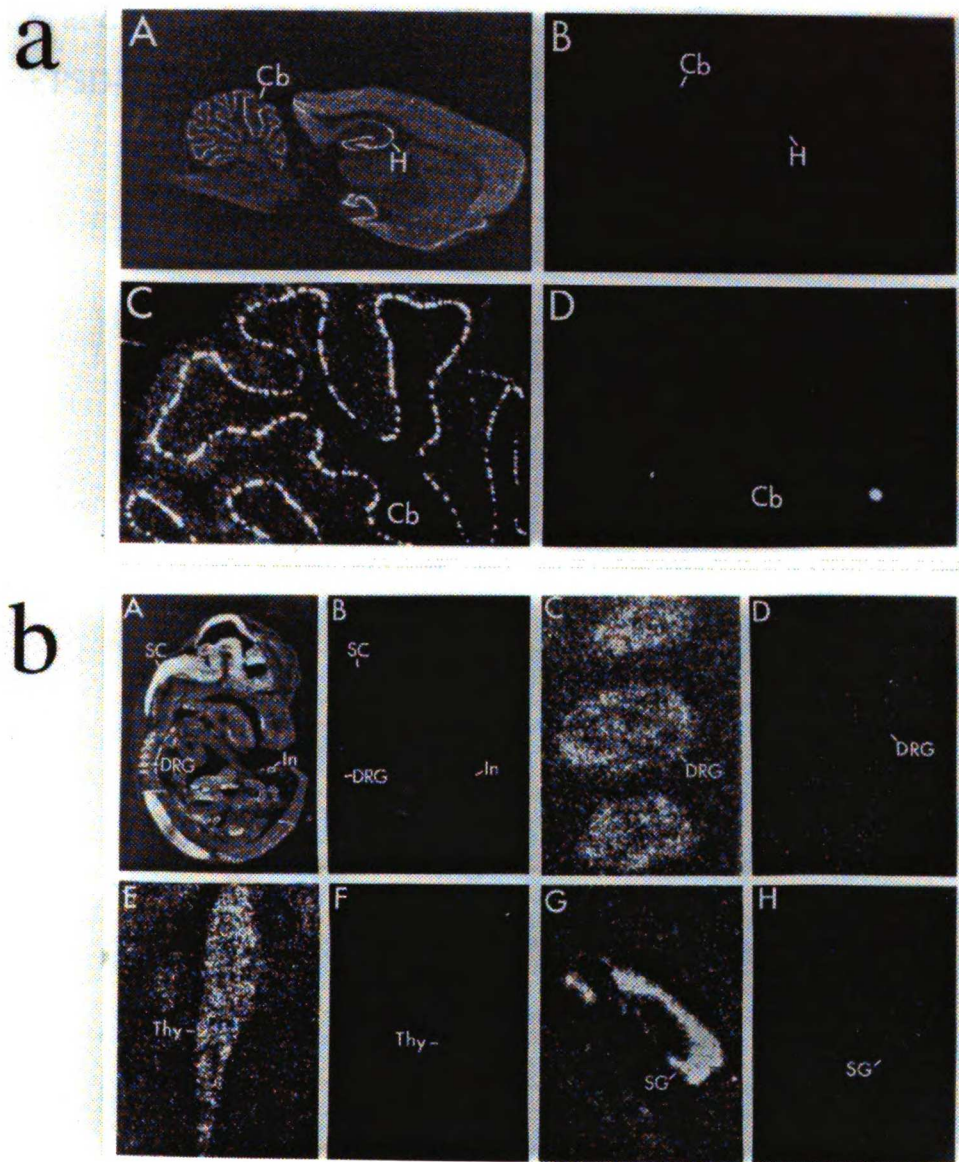


Figure 4-3

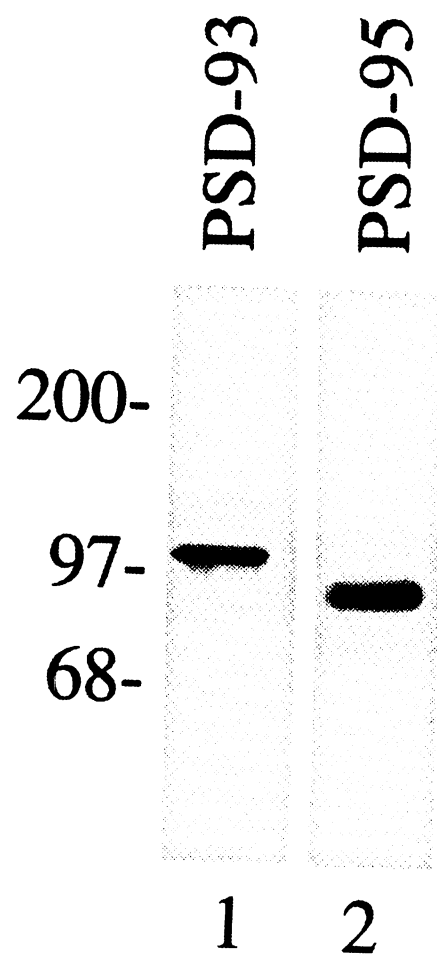


Figure 4-4

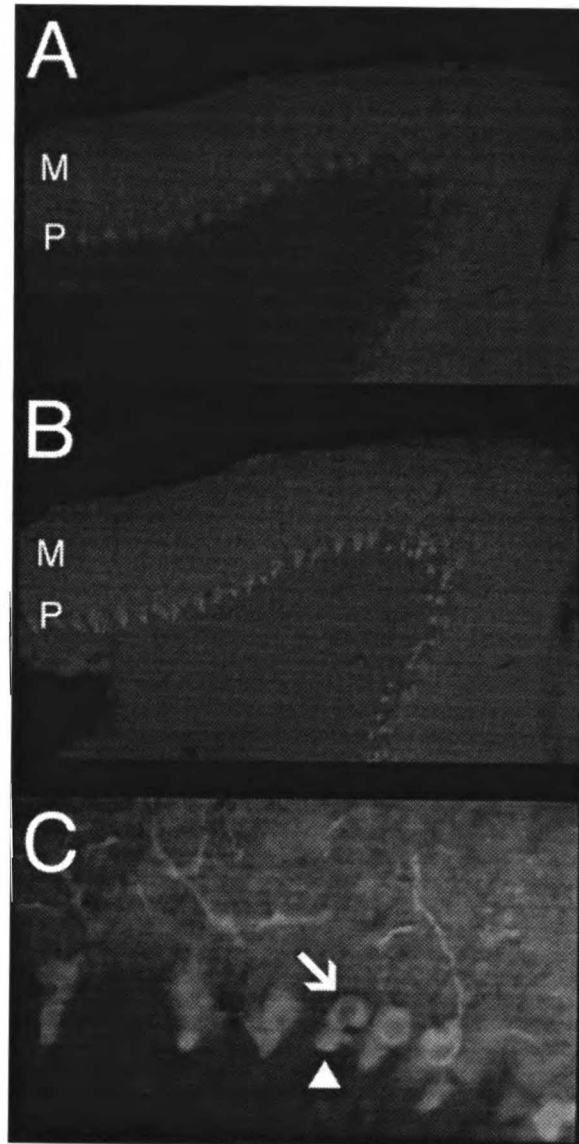


Figure 4-5

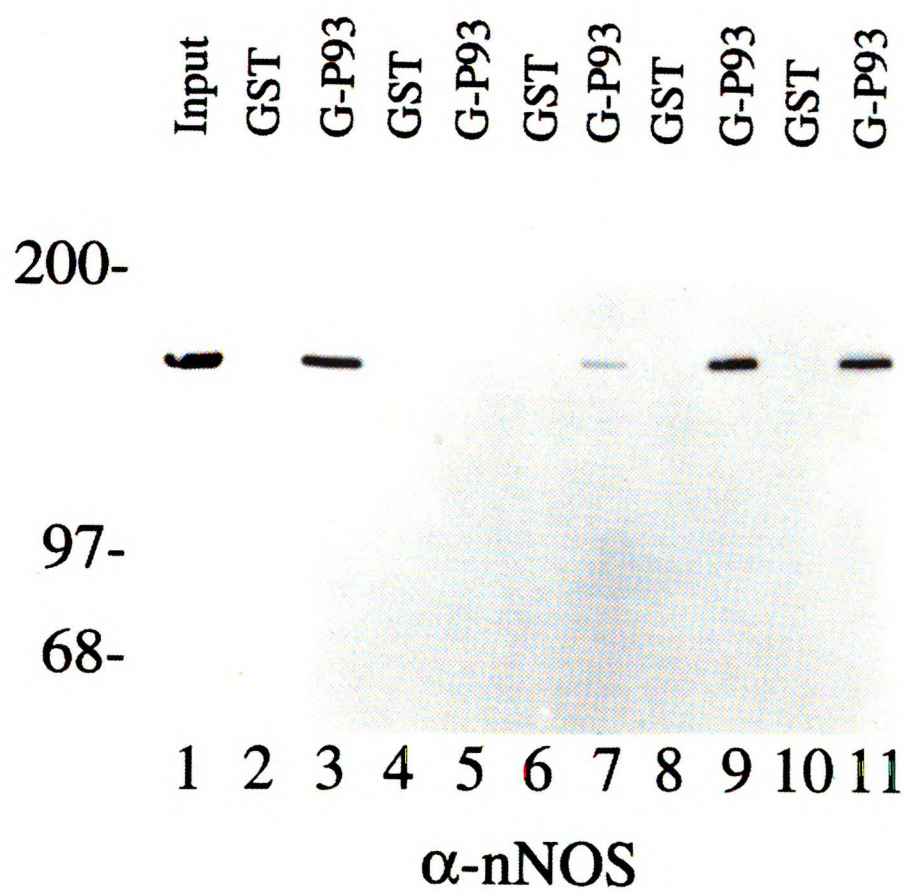


Figure 4-6



Figure 4-7

Table 4-1. Interactions of nNOS and PSD-93

<u>Gal4 DNA Binding Hybrid</u>	<u>Gal4 Activation Hybrid</u>	<u>β-galactosidase units</u>
nNOS (amino acids 1-100)	PSD-93 (116-421)	1.23
nNOS (40-150)	PSD-93 (116-421)	1.64
nNOS (40-195)	PSD-93 (116-421)	1.51
nNOS (100-195)	PSD-93 (116-421)	1.06
nNOS (1-150)	PSD-93 (116-421)	14.7
nNOS (1-195)	PSD-93 (116-421)	17.2
Lamin C (66-230)	PSD-93 (116-421)	0.430

Yeast Y187 cells were cotransformed with expression vectors encoding various GAL4 DNA binding domain and Gal4 activation domain fusion proteins. Each transformation mixture was plated on synthetic dextrose plates lacking tryptophan and leucine. Interaction was measured by the liquid culture b-galactosidase assay as described (Fields and Song, 1989). Values are representative of experiments repeated twice with similar results.

bioRxiv preprint doi: <https://doi.org/10.1101/000000>; this version posted January 1, 2014. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

CHAPTER FIVE

BINDING OF THE INWARD RECTIFIER K⁺ CHANNEL Kir 2.3 TO PSD-95 IS REGULATED BY PROTEIN KINASE A PHOSPHORYLATION

UNIVERSITY OF MICHIGAN

Abstract

Dynamic regulation of ion channel interactions with the cytoskeleton mediates aspects of synaptic plasticity, yet mechanisms for this process are largely unknown. Here, we report that two inwardly rectifying K⁺ channels, Kir 2.1 and 2.3, bind to PSD-95, a cytoskeletal protein of postsynaptic densities that clusters NMDA receptors and voltage-dependent K⁺ channels. Kir 2.3 colocalizes with PSD-95 in neuronal populations in forebrain, and a PSD-95 / Kir 2.3 complex occurs in hippocampus. Within the C-terminal tail of Kir 2.3, a serine residue critical for interaction with PSD-95, is also a substrate for phosphorylation by protein kinase A (PKA). Stimulation of PKA in intact cells causes rapid dissociation of the channel from PSD-95. This work identifies a physiological mechanism for regulating ion channel interactions with the postsynaptic density.

Introduction

The inward rectifying K⁺ current (Kir) is the predominant conductance of a resting cell (Hille, 1992). Unlike the voltage gated K⁺ channels (Kv) which are activated by membrane depolarization, conductance through Kir channels is increased by hyperpolarization to potentials negative of the reversal potential for potassium (E_K). At potentials slightly positive to E_K, Kir channels conduct a small outward potassium current, which is regulated through a voltage-dependent block by intracellular Mg⁺⁺ (Lu and MacKinnon, 1994; Matsuda, 1991; Wible et al., 1994) and polyamines (Fakler et al., 1995; Ficker et al., 1994; Lopatin et al., 1994) resulting in the inward rectification property. These asymmetrical conductances stabilize the resting membrane potential around E_K, while closure of Kir at depolarized potentials allows for prolonged action potentials. Modulation of Kir conductances can therefore control both the membrane potential and the excitability of a neuron.

Molecular cloning of Kir channels identifies at least six subfamilies which display distinct mechanisms for regulation (Chandy and Gutman, 1993; Dascal et al., 1993; Ho et al., 1993; Kubo et al., 1993). Kir 1.0 channels (ROMK) are ATP sensitive and display mild rectification. Kir 2.0 channels (IRK) display strong inward rectification, while Kir 3.0 channels (GIRK) are activated by G proteins. Sequence analysis predicts that all Kir channels contain two transmembrane domains and that both the amino and carboxy-termini are on the cytoplasmic side of the membrane. Determinants within the large C-terminal domains appear to participate in the intrinsic rectification properties of these channels and to confer regulation of channel activity by Mg²⁺, polyamines, G-proteins and protein kinases (Fakler et al.,

1994; Huang et al., 1995; Kunkel and Peralta, 1995; Taglialatela et al., 1994; Takao et al., 1994; Wischmeyer and Karschin, 1996).

Many ion channels are also regulated by interactions with the cytoskeleton (reviewed in Froehner, 1993) which can regulate channel activity (Rosenmund and Westbrook, 1993) and mediate the spatial sorting of surface proteins. Examples include: (1) high densities of the voltage gated Na⁺ channel at the node of Ranvier in myelinated axons through interaction with the cytoskeletal linker protein ankyrin (Kordeli et al., 1990; Srinivasan et al., 1988); (2) clustering of the glycine receptor at the postsynaptic membrane through the linker protein gephyrin (Kuhse et al., 1995); and (3) localization of the nicotinic acetylcholine receptor at the neuromuscular junction by interaction with the 43K protein rapsyn (Froehner et al., 1990; Phillips et al., 1991).

Targeting of ion channels and enzymes to discrete plasma membrane sites is a dynamic process often regulated by protein phosphorylation. During development nicotinic acetylcholine receptors are clustered at neuromuscular junctions in response to neural agrin release (Gautam et al., 1996; McMahan, 1990). Agrin and other growth factor mediated signal transduction pathways are regulated by receptor protein tyrosine kinases (Glass et al., 1996; Wallace, 1992). Serine / threonine protein kinases also play critical roles in modulating synaptic transmission. Cyclic nucleotide and calmodulin regulated kinases (CamK) phosphorylate ionotropic glutamate receptors and K⁺ channels and alter channel activity (Jonas and Kaczmarek, 1996; Raymond et al., 1993). PKA and CamK mediate synaptic plasticity (Kandel and Abel, 1995; Nicoll and Malenka, 1995) perhaps by causing a redistribution of ion channels at the postsynaptic density of "silent synapses" (Isaac et al., 1995; Liao et al., 1995). However, mechanisms for regulating ion channel interactions

with the cytoskeleton by serine / threonine protein kinases are not well characterized.

Recently, postsynaptic density-95 (PSD-95) a prominent cytoskeletal protein component of postsynaptic densities (Cho et al., 1992; Kistner et al., 1993), has been demonstrated to bind the C-terminal domain of NMDA receptors (Kornau et al., 1995) as well as voltage gated K⁺ channels (Kim et al., 1995). These interactions are mediated by a conserved C-terminal sequence, designated tSXV, which is present in numerous receptors and channels (Kornau et al., 1995). The region of PSD-95 that binds tSXV constitutes another consensus sequence, the PDZ domain, named for the first three proteins initially identified to contain the sequence: PSD-95, discs-large protein of Drosophila septate junctions (Woods and Bryant, 1991), and zona occludens-1 (ZO1) protein of epithelial tight junctions (Willott et al., 1993).

We, and others, previously reported the cloning and characterization of a K⁺ channel, Kir 2.3, which displays strong inward rectification and is predominately localized to neuronal systems in the forebrain (Bredt et al., 1995; Falk et al., 1995; Morishige et al., 1993; Perier et al., 1994). Kir 2.3 terminates with the amino acid sequence RRESAI which contains the RRXS consensus sequence for cAMP dependent protein kinase (PKA) phosphorylation as well as demonstrating loose homology to the tSXV sequence.

In the present study, we find that Kir 2.3 and PSD-95 occur together in a protein complex in hippocampal membranes. We demonstrate that Kir 2.3 directly binds to PSD-95 in a manner that is dependent upon the terminal 6 amino acids of the channel and that mutation of the serine in this region abolishes this interaction. We further show that this serine is phosphorylated by PKA and that this phosphorylation abolishes the interaction with PSD-95.

Results

Phosphorylation of Kir 2.3 by PKA

To evaluate whether Kir 2.3 can be phosphorylated in intact cells, we performed two dimensional (2D) phosphopeptide analysis on the channel following stimulation of transfected cells with the kinase activators forskolin, the phorbol ester TPA (phorbol-12-myristate-13-acetate), and the calcium ionophore A23187. In all treatments a poorly resolved series of phosphopeptides was observed. Only forskolin treatment resulted in the addition of a prominent phosphopeptide (Figure 5-1A). This implies that forskolin, which activates adenylyl cyclase, led to cyclic AMP dependent phosphorylation by PKA of a peptide sequence within Kir 2.3. Coincident phosphoamino acid analysis revealed that both in basal and in forskolin stimulated cells phosphorylation occurs exclusively on serine moieties (Figure 5-1B).

Kir 2.3 contains 29 serines of which 23 are intracellular. The best PKA consensus sequence occurs in the very distal C-terminal region, RRES (AA437-440). To examine whether this serine, S-440, is phosphorylated we performed site directed mutagenesis. A serine to alanine mutation abolished the forskolin stimulated phosphopeptide (Figure 5-1C).

To quantify the identified Kir 2.3 sequence ISYRRESAI as a PKA substrate, we compared its *in vitro* phosphorylation with Kemptide (LRRASLG), a classic PKA substrate. Both peptides demonstrated robust phosphorylation with K_m values of 30 and 10 μ M for the Kir 2.3 peptide and Kemptide, respectively. Stoichiometric analysis indicated a single phosphate per molecule for each of the peptides.

Kir 2.3 Binds to PSD-95

Recently, four ion channel subunits (NR2A, NR2B and NR2D of the NMDA receptor and Kv1.4 of the delayed rectifier voltage dependent K⁺ channel) have been demonstrated to interact with the cytoskeletal protein PSD-95 (Kim et al., 1995; Kornau et al., 1995). This binding is mediated by the carboxy terminus of the channel proteins interacting with the either the first or second of the three PDZ domains of PSD-95. At the C-terminus of the channels, a three amino acid consensus sequence for binding has been proposed to consist of tSXV, where t/S denotes either a threonine or a serine at the -2 position, X denotes any amino acid at the -1 position, and V is a terminal valine at the 0 position. In addition to the tSXV motif, at the -3 position, all these interacting proteins possess a glutamate which may also play a role in specific binding to the PDZ motif (Doyle et al., 1996) (Figure 5-2). Moreover a peptide with an arginine at the -4 position, preceding the glutamate (-3), also binds to PSD-95 (Kim et al., 1995). Since isoleucine and valine are structurally similar, the C-terminus sequence of Kir 2.3 (RESAI) could fulfill the requirements for a tSXV motif and bind to PSD-95.

To investigate the possible interaction between PSD-95 and Kir 2.3, HEK293 cells were transfected with a mammalian expression vector containing an N-terminal myc-epitope fused to the C-terminal 271 amino acids of the channel (AA171-442). The binding capacity of these transfected cell lysates was examined by use of a fusion protein consisting of GST (glutathione S-transferase) and the second PDZ domain of PSD-95 bound to glutathione agarose beads. After incubation of the lysates with the beads and extensive washing, the bound material was resolved by SDS-PAGE,

transferred to PVDF and subjected to western blot analysis utilizing an anti-myc antibody. These experiments demonstrated that the intact C-terminus of Kir 2.3 (wt), associated specifically with the second PDZ domain of PSD-95 (figure 5-3A, left panel). Deletion of the last 6 amino acids of the C-terminus containing the tSXV-like motif or mutagenesis of serine 440 to alanine, aspartate, or glutamate abolished this binding (figure 5-3A, right panel). Since Kir 2.3 does not terminate in a valine (0), nor is an aspartate or glutamate in the penultimate position (-1), the suggested tSXV motif now contains additional members (t/S-X(D/E/A)-V/I) and this consensus may continue to expand. We therefore propose a simpler term "C-TAG" indicating C-Terminal Association with GLGF. GLGF is a conserved tetra-peptide found within many proteins of the PSD-95/SAP-90 family.

To determine whether this interaction occurs in a cellular environment, co-immunoprecipitation experiments were performed. COS-7 cells were transfected with an N-terminal myc-epitope tagged Kir 2.3, with or without PSD-95. Cell lysates were immunoprecipitated with an antibody to PSD-95 and processed for western blotting with an anti-myc antibody. Only in those cells transfected with PSD-95 and the wild type Kir 2.3 was the channel co-immunoprecipitated. Similar to the *in vitro* binding (figure 5-3A), mutagenesis of serine-440 to aspartate abolished the co-immunoprecipitation (Figure 5-3B).

Kir 2.3 associates with PSD-95 in synaptic membranes

If Kir 2.3 and PSD-95 interact *in vivo*, overlapping localization should occur in brain. Comparing the cellular localizations of mRNA for Kir 2.3 and PSD-95 in adjacent sagittal sections illustrates that although PSD-95 is more

widely distributed than Kir 2.3, there are areas of substantial overlap (Figure 5-4A, B). For example, the dentate gyrus of the hippocampus is greatly enriched in both mRNAs (Figure 5-4C, D) as are areas of the cerebral cortex. In contrast, PSD-95 is concentrated in the molecular and granule cell layers of the cerebellum and diffusely in the brainstem, whereas Kir 2.3 is absent in these regions (Figure 5-4A, B).

Subcellular fractionation of forebrain membranes revealed that Kir 2.3 occurred together with PSD-95 in postsynaptic density fractions (Figure 5-4E). Like PSD-95, Kir 2.3 was found in Triton X-100 and sarkosyl extracted pellet fractions. In contrast, endothelial nitric oxide synthase (eNOS), which is membrane bound due to N-terminal myristoylation, was not found in these fractions.

Based on areas of overlapping localization, interactions between native Kir 2.3, and the second PDZ domain of PSD-95 were examined in brain membranes. Detergent solubilized extracts of several brain regions were incubated with the GST-PDZ fusion protein attached to agarose beads. After extensive washing, bound material was subjected to SDS-PAGE and analyzed with an anti-Kir 2.3 antibody. Highest binding of the endogenous channel to the PDZ domain was evident in the olfactory bulb, which is greatly enriched in Kir 2.3, and lower levels were found in the cerebral cortex and hippocampus. No band was detectable in the cerebellum, which lacks Kir 2.3 (Figure 5-5A).

Confirmation of this interaction was evaluated by co-immunoprecipitation. Previous studies demonstrate that 2% SDS does not disrupt association of ion channels with PSD-95-like proteins (Lau et al., 1996; Muller et al., 1996). We therefore used SDS-solubilized postsynaptic density fractions for immunoprecipitation experiments. PSD-95 was co-

immunoprecipitated with Kir 2.3 from hippocampal extracts but not from cerebellar extracts (Figure 5-5B). Thus, although PSD-95 is abundant in cerebellum (Cho et al., 1992; Kistner et al., 1993), immunoprecipitation was not observed there due to lack of Kir 2.3 expression. Also, no immunoprecipitation of PSD-95 was observed using preimmune serum or Kir 2.3 antiserum preabsorbed with the immunogen (Figure 5-5C).

PKA Phosphorylation of Kir 2.3 Inhibits Binding to PSD-95

Overlap of the PKA phosphorylation site with the C-TAG, together with the knowledge that mutation of the serine in the C-TAG disrupts the interaction with PSD-95 suggests that PKA phosphorylation might modulate the interaction of Kir2.3 and PSD-95. To study this potential modulatory mechanism, we treated HEK293 cells transfected with N-terminal myc tagged Kir2.3 (AA171-442), with forskolin. The binding of these cell lysates to the GST-PSD-95 fusion protein was evaluated by western blotting. In lysates from untreated cells, but not from forskolin treated cells, robust binding of Kir 2.3 to the PSD-95 fusion protein was detected (Figure 5-6A). To ascertain whether the effect of forskolin involved phosphorylation, we incubated the forskolin treated lysates with calf intestinal phosphatase, which restored binding (Figure 5-6A). Thus, PKA-dependent phosphorylation appears to disrupt the binding of Kir 2.3 to PSD-95.

To confirm the ability of the PDZ domain to selectively bind non-phosphorylated Kir 2.3 C-TAG, two petri dishes of HEK-293 cells were transfected with the carboxy cytoplasmic region of Kir2.3 and were incubated with [³²P]orthophosphate. Prior to lysis one dish was treated with forskolin for 20 minutes while the other was treated with vehicle (DMSO). Equal

volumes of treated and untreated cell lysates were mixed and incubated with the GST-PSD-95 attached to agarose beads. After one hour the unbound fraction (flowthrough) was collected. Following extensive washing of the beads the bound fraction was eluted with 1% SDS. The preload, flowthrough and eluate fractions were immunoprecipitated and the labeled Kir 2.3 subjected to 2D phosphopeptide analysis. The S-440 phosphorylated peptide was prominent in the load and flowthrough fractions but was absent in the eluate demonstrating the ability of the PDZ domain to selectively bind the unphosphorylated C-TAG (Figure 5-6B).

Further confirmation that PKA phosphorylation blocks the interaction of PSD-95 and Kir 2.3 was observed in COS-7 cells, co-transfected with myc-Kir 2.3 and PSD-95. An anti-PSD-95 antibody co-immunoprecipitated myc-Kir 2.3 to a much greater extent from untreated than cells treated for 20 minutes with forskolin (Figure 5-6C).

Kir 2.1 and Kir 2.3 but not Kir 3.1 Bind to PSD-95

Whether other members of the Kir family of channels interact with PSD-95 poses an interesting question. Kir 2.1 possesses a C-TAG motif, RRESEI, very similar to Kir 2.3, where the C-terminal valine of the tSXV domain is replaced by isoleucine. Kir 3.1 lacks a C-TAG motif (NSDRFT) (Figure 5-2). To explore potential specificity of binding of these channels to PSD-95, HEK293 cells were transfected with N-terminal myc-tagged carboxy tails of these channels and binding to GST-PSD-95 was examined. As expected, Kir 2.1 and Kir 2.3 but not Kir 3.1 bound to PSD-95, confirming the importance of the C-TAG motif for interactions with PDZ domains (Figure 5-7).

Discussion

The present study provides evidence that Kir 2.3 associates physiologically with PSD-95 and that phosphorylation of serine 440 of Kir 2.3 by PKA disrupts this interaction. Both the specific binding and phosphorylation are dependent upon the amino acid sequence (C-TAG) at the extreme C-terminus of the channel. Another member of the Kir 2.0 family with a similar C-TAG domain, Kir 2.1, also binds to PSD-95. Of the cloned Kir subfamilies, the Kir 1.0, 3.0, 5.0 and 6.0 lack the C-TAG motif. Kir 4.0 possesses this motif with SNV as the three final amino acids.

What might be the functional consequences of Kir channels binding to PSD-95? The large C-terminal domain of all Kir channels is predicted to be cytoplasmic. Mutagenesis studies demonstrate that the C-termini of Kir 2.0 and 3.0 channels contribute to rectification properties and may form an acidic particle that physically blocks the pore (Pessia et al., 1995; Taglialatela et al., 1994; Yang et al., 1995). This would be analogous to the ball-and-chain model for the gating of the Shaker K⁺ channel (Zagotta et al., 1990). It is conceivable that anchoring of this intracellular domain to PSD-95 would maintain an open state. Testing this model by heterologous co-expression of Kir channels and PSD-95 in oocytes or mammalian cells may be complicated by the presence of endogenous PDZ containing proteins in these cells.

What might be the electrophysiological consequences of serine phosphorylation in the C-TAG domain? Since the C-termini of Kir channels contain residues that appear to interact with the pore (Pessia et al., 1995; Taglialatela et al., 1994; Yang et al., 1995), one might predict that phosphorylation of the C-terminus would dissociate the C-terminal tail from PSD-95 and leave it free to block the channel. Indeed, Wischmeyer and

Karschin (Wischmeyer and Karschin, 1996) recently demonstrated that PKA treatment of Kir 2.1 inhibits its conductance, an effect which is abolished in COS-7 cells transfected with the channel in which serine 425 (analogous to serine 440 of Kir 2.3) has been mutated to an asparagine. These authors also found that serotonin, acting via cotransfected 5-HT_{1A} receptors, inhibits Kir 2.1 conductance presumably by stimulating PKA dependent phosphorylation. Because of similar phosphorylation sequences, Kir 2.3 phosphorylation at serine 440 may also lead to conductance inhibition. Interestingly, when expressed in oocytes, Kir 2.1 channel activity is not blocked by PKA but rather is enhanced (Fakler et al., 1994). This discrepancy may reflect differences in endogenous PDZ proteins expressed in oocytes and COS-7 cells. Endogenous Kir currents have also been shown to be alternatively suppressed (Koumi et al., 1995) or activated (Wang and Giebisch, 1991) by PKA activity.

Rather than regulating channel activity, interaction with PSD-95 may primarily function to target Kir to specific synaptic sites. PSD-95 has a modular domain structure consisting of three N-terminal PDZ domains, followed by an src homology-3 (SH3) motif often involved in binding to cytoskeleton and a C-terminal region homologous to guanylate kinase (Cho et al., 1992; Kistner et al., 1993). These multiple protein-protein interaction domains, and the fact that PSD-95 and related proteins form oligomers (Kim et al., 1996), suggests a scaffolding role for PSD-95 in organizing signaling cascades at the postsynaptic density (Brenman et al., 1996; Gomperts, 1996). For example, neuronal nitric oxide synthase (nNOS), which itself displays a PDZ domain (Brenman et al., 1995), binds to PSD-95 (Brenman et al., 1996), and this interaction likely accounts for the presence of nNOS at postsynaptic densities in proximity to NMDA receptors (Aoki et al., 1993). The potential association of Kir with NMDA receptors at postsynaptic densities fits with the

known voltage-dependent Mg^{2+} block of NMDA receptor currents (Mayer et al., 1984; Nowak et al., 1984). At rest, Kir maintains dendrites in a hyperpolarized state, silencing the NMDA receptor. Closure of Kir during synaptic stimulation would allow prolonged depolarization, which is required for NMDA receptor activity. Development of antisera to Kir 2.3 that allow quantitative electron micrographic analysis will be necessary to evaluate this model in detail.

A recent X-ray crystallographic structure of a peptide-PDZ domain complex suggests a structural relationship for the C-TAG (Doyle et al., 1996). This structure shows that the main chain atoms of the C-TAG peptide participate in hydrogen bonding with backbone atoms of the signature GLGF tetrapeptide of the PDZ domain. Specific contacts formed between side chains of the C-TAG and PDZ explain how phosphorylation of the S(-2) position disrupts Kir binding to PSD-95. Thus, the hydroxyl side chain of the T/S forms a critical hydrogen bond with his-372 of PSD-95 which would be abolished by phosphorylation of this T/S residue. The crystal structure also suggests that a valine to isoleucine substitution at the terminal position would be tolerated and not disrupt the binding of Kir 2.0 channels to PDZ (Doyle et al., 1996). Taken together these studies emphasize that numerous channels associate with PSD-95 and related proteins. Dynamic regulation of these interactions by physiological stimuli, such as protein phosphorylation, may underlie certain aspects of synaptic plasticity.

Materials and Methods

Generation of Antisera to Kir 2.3 and PSD-95 and Western Blotting

An N-terminally acetylated 14 amino acid peptide (CRMQAATLPLDNISY), corresponding to amino acids 424-436 of Kir 2.3 with a carboxy-terminal cysteine was synthesized and coupled to BSA. A glutathione-S-transferase (GST) fusion protein encoding amino acids 1-386 of PSD-95 was expressed and purified from *E. coli*. These antigens were emulsified in complete and incomplete Freund's adjuvant and used to immunize sets of rabbits. Serum bleeds were affinity-purified. Specificity of the polyclonal antibody to PSD-95 is described elsewhere (Brenman et al., 1996). Membrane fractions from rat brain and detergent-extracted postsynaptic density (PSD) fractions were prepared as described (Cho et al., 1992). For western blotting, protein extracts were resolved by SDS / PAGE and transferred to PVDF membranes. Primary antibodies were diluted in block solution containing 3% BSA, 0.1% tween-20 in TrisHCl buffered saline and incubated with membranes overnight at 4°C. Labeled bands were visualized using enhanced chemiluminescence (Amersham).

Cell Culture and Transfection

Wild type and mutant Kir 2.3 cDNAs were cloned into a mammalian expression vector pcDNA3 (Invitrogen) which had been modified to include the c-myc sequence EQKLESEEDL adjacent to the 5' cloning site. The carboxy cytoplasmic regions of Kir 2.1 (amino acids 182-427), Kir 2.3 (171-442) and Kir 3.1 (183-501) were constructed by PCR amplification and subcloned into the

myc-tagged pcDNA3 vector. PSD-95 was cloned into GW1-CM (British Biotechnology). HEK-293 or COS-7 cells were grown and transfected using calcium phosphate as previously described (Brenman et al., 1996).

Phosphopeptide Mapping and Phosphoamino Acid Analysis of Kir2.3 Expressed in HEK-293 Cells

HEK-293 cells transiently transfected with wild type or mutant / truncated forms of Kir 2.3 were prelabeled with [^{32}P]orthophosphate (5 mCi/ml) for 3-4 hour in phosphate-free DMEM (Gibco). Cells were treated with forskolin (20 μM), TPA (100 nM) or A23187 (1 μM) for 30 minutes. Cells were then harvested and Kir 2.3 was immunoprecipitated as described (Roche et al., 1996). Kir 2.3 immunoprecipitates were processed for trypsin digestion and were separated in two dimensions on thin layer chromatography (TLC) cellulose plates as described previously (Roche et al., 1996) with the following modification: the ascending chromatography buffer was isobutyric acid/butanol/pyridine/acetic acids/ H_2O , 66:2:5:3:30 (vol/vol). The TLC plate was visualized using a Phosphoimager Analysis System (Molecular Dynamics). For phosphoamino acid analysis, [^{32}P] labeled tryptic fragments of Kir 2.3 were resuspended in 6N HCl and heated to 105 $^{\circ}$ C under vacuum for 1 hour. Samples were concentrated and spotted on a TLC plate. Phosphoamino acids were resolved by electrophoresis in formic acid / acetic acid / H_2O (1:10:89) buffer pH 1.9, followed by electrophoresis at pH 3.5. The TLC plate was visualized by autoradiography.

Fusion Protein Affinity Chromatography

Fusion proteins of GST fused to amino acids 1-386 (PDZ1-3) or amino acids 157-248 (PDZ2) of PSD-95 were expressed in *E. coli* and purified on glutathione sepharose beads as described (Brenman et al., 1995). Transfected HEK293 cells or dissected brain tissues were homogenized and solubilized in lysis buffer [5 mM EDTA, 5 mM EGTA, 100 mM NaAl, 1 mM Na₃VO₃, 50 mM NaF, 10 mM sodium pyrophosphate, 1mM PMSF and protease inhibitors (2 mg/L chymostatin, 2 mg/L pepstatin, 2 mg/L antipain, 4 mg/L leupeptin) in 10 mM sodium phosphate buffer pH 7.4 containing 1% Triton X-100] and centrifuged for 15 minutes at 4°C, 16,000 × g. These solubilized homogenates were incubated for 60 minutes with 2 µg purified fusion protein bound to 20µl of glutathione agarose. Samples were loaded into disposable columns, which were washed with 50 volumes of binding buffer supplemented to 500 mM NaCl. Retained proteins were eluted with 150 µl of loading buffer and were resolved by SDS / PAGE.

***In situ* Hybridization**

In situ hybridization used ³⁵S-labeled RNA probes as described (Sassoon and Rosenthal, 1993). Antisense probes to PSD-95 (1-1155), Kir 2.3 (1-1301) or sense control Kir 2.3 (1-1301) were synthesized from bluescript vectors.

Co-immunoprecipitation of PSD-95 and Kir 2.3

Transfected HEK293 cells were harvested, homogenized and solubilized in lysis buffer as described above. Synaptic plasma membranes from brain were prepared and solubilized with SDS as described (Lau et al., 1996; Muller et al., 1996). Briefly, membranes were solubilized in lysis buffer supplemented with

2% SDS, incubated for 30 minutes, and then diluted with five volumes of ice cold lysis buffer containing 2% Triton X-100. Solubilized extracts from HEK293 cells or brain membranes (~400 µg) were incubated with polyclonal antibodies (8 µg / ml) to Kir2.3, PSD-95 or preimmune control at 4° C. After a 2 hour incubation, 50 µl of protein A sepharose was added to precipitate antibodies. Protein A pellets were washed 3 times with buffer containing 200 mM NaCl and 1% triton X-100. Immunoprecipitated proteins were denatured with loading buffer and resolved by SDS-PAGE.

Figure 5-1. PKA Phosphorylates Serine 440 of Kir2.3.

HEK293 cells transfected with wild type Kir2.3 (A) or the S440A mutant (C, bottom panel) were prelabeled with 5 mCi/ml [32P]-orthophosphate and then treated with either 20 μ M forskolin and 100 μ M IBMX, or 100 nM TPA, or 200 nM A23187. Labeled channel was immunoprecipitated, resolved by SDS-PAGE, excised from the gel, and then digested with trypsin. Tryptic fragments were either hydrolyzed with HCl for phosphoamino acid analysis (B) or resolved in two dimensions on TLC plates and visualized by autoradiography (A, C). Treatment with forskolin but not TPA or A23187 produces a prominent autoradiographic spot (A, C arrow). Forskolin does not stimulate phosphorylation of this peptide in the S440A mutant (C, bottom panel).

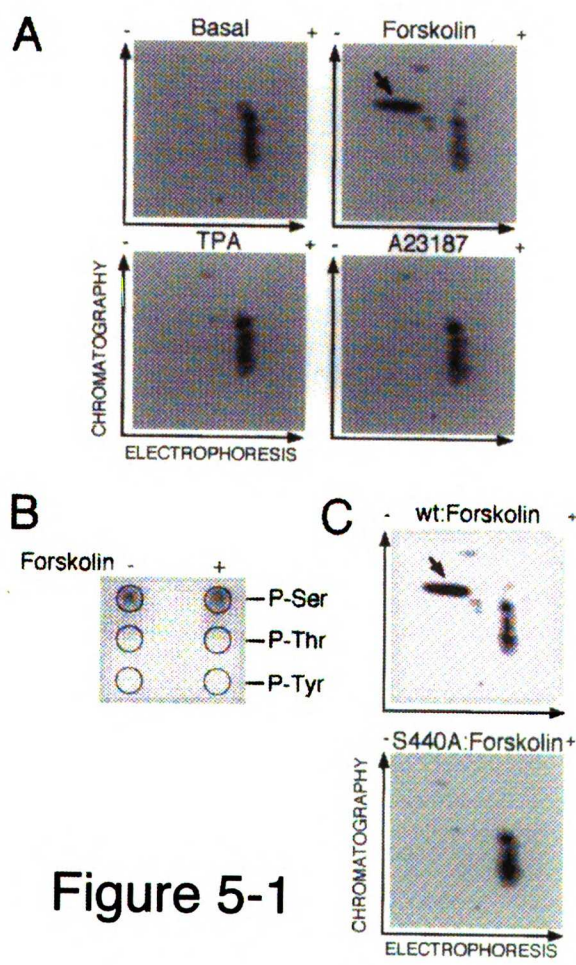


Figure 5-1

Figure 5-2. Inward Rectifying K⁺ Channels with Homology to the tSXV Motif.

Alignment of the terminal 6 amino acids from NMDAR2 and shaker type K⁺ channels demonstrated to interact with PSD-95 along with the terminal 6 amino acids of the inward rectifying K⁺ channels.

NR2A	S I E S D V
NR2B	S I E S D V
NR2C	S L E S D V
NR2D	S L E S D V
ShA	S V E T D V
ShB	S V E T D V
Kv1.4	A V E T D V
Kir1.1a-e	T D D T Q M
Kir2.1	R R E S E I
Kir2.2	R R E S E I
Kir2.3	R R E S A I
Kir3.1	N S D R F T
Kir3.2	L T E R N G
Kir3.3	R C G R W S
Kir3.4	A T R G S M
Kir4.1	V R I S N V
Kir5.1	C H G E Q L
Kir6.1	Q C P S E S
Kir6.2	S P D S L S

Figure 5-2

Figure 5-3. Kir 2.3 interacts with PSD-95.

(A) Lysates of HEK293 cells transfected with the cytoplasmic carboxy tail of Kir 2.3 (AA 171-442) or C-terminal mutant were evaluated for binding to the second PDZ domain of PSD-95 by GST fusion protein affinity chromatography as described in "Experimental Procedures". Left panel demonstrates specific interaction of Kir 2.3 with the second PDZ domain of PSD-95 (GST-PSD-95) and not GST. Right panel demonstrates deletion of the terminal 6 amino acids of the channel or mutation of serine 440 abolishes the interaction. Input (I) represents 10% of the total extract that was applied to the affinity columns.

(B) COS-7 cells were transiently transfected with an N-terminal myc-tagged Kir 2.3 (wt) or the S440D mutant, with (+) or without (-) PSD-95. Following immunoprecipitation with an anti-PSD-95 antibody, association of Kir 2.3 was evaluated by western blotting using an antibody to the myc epitope. Left side illustrates similar levels of expression of wt and S440D Kir 2.3 transfected with or without PSD-95. Right side demonstrates specific interaction of wt Kir 2.3 but not S440D Kir 2.3 with PSD-95. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

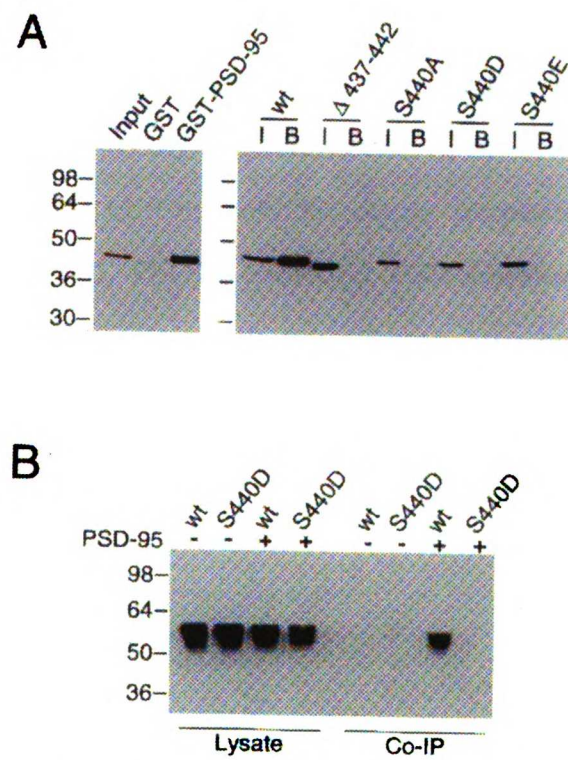


Figure 5-3

Figure 5-4. Co-localization of Kir 2.3 and PSD-95.

In situ mRNA analysis demonstrates overlapping distribution of Kir 2.3 and PSD-95. (A) Dark field image of a parasagittal section of a rat brain demonstrates expression of Kir 2.3 in discrete neuronal populations in the forebrain including hippocampus (Hp), cortex (CTX), and striatum (Str). No expression of Kir 2.3 is detected in the cerebellum (Cb). (B) PSD-95 is also enriched in the hippocampus, cortex, and striatum, but is also present in cerebellum and brainstem. Both Kir 2.3 (C) and PSD-95 (D) mRNA are specifically enriched in granule cells of the dentate gyrus (Dg) region of the hippocampus. (E) Subcellular fractionation of rat brain extracts indicates that Kir 2.3 and PSD-95, but not eNOS, occur in postsynaptic density (PSD) fractions; PSDI, PSDII, PSDIII (purified PSD fractions after extraction with Triton X-100 once (I) twice (II) or with Triton followed by sarkosyl (III)). To show relative fractionation equal percentages were loaded.

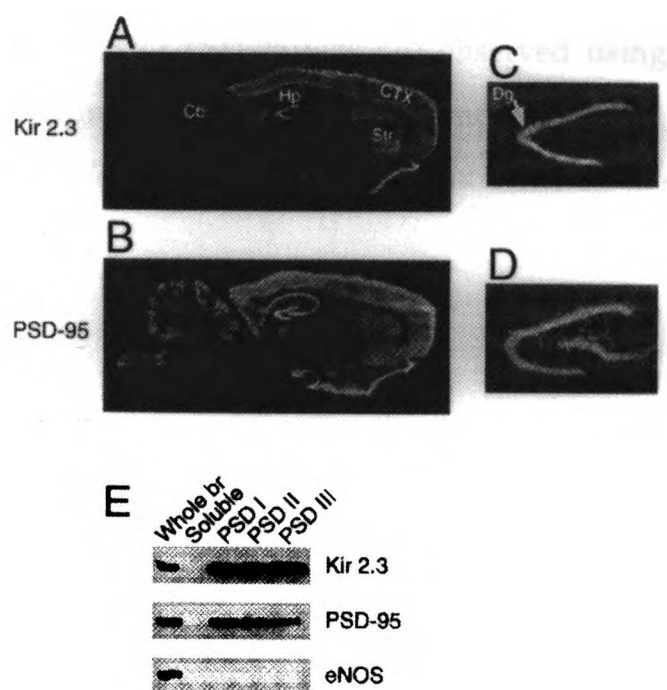


Figure 5-4

Figure 5-5. Kir 2.3 Associates With PSD-95 in Brain.

(A) Detergent solubilized brain membranes from olfactory bulb (Ob), cortex (CTX), hippocampus (Hp) and cerebellum (Cb) were incubated with a GST fusion protein containing the second PDZ domain of PSD-95 (P) or GST control and "pull-down" assays were done as described in figure 5-3. Endogenous Kir 2.3 was specifically retained by PSD-95 but not by GST coated beads. (B) An antiserum to Kir 2.3 co-immunoprecipitated PSD-95 from hippocampal but not cerebellar synaptic membranes. (C) Immunoprecipitation of PSD-95 was not observed using preimmune serum or Kir 2.3 antiserum presorbed with immunogen (10 μ g / ml). Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

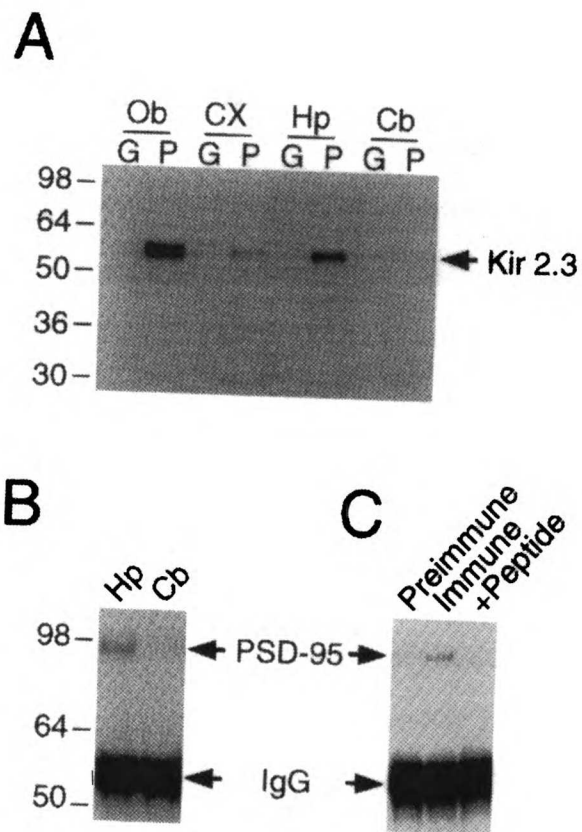


Figure 5-5

Figure 5-6. Phosphorylation by PKA Inhibits the Kir 2.3 / PSD-95 Interaction.

(A) HEK293 cells transfected with the cytoplasmic carboxy tail of Kir 2.3 were treated with or without forskolin 20 μ M and IBMX 100 μ M for 20 minutes and then homogenized in lysis buffer. A portion of the forskolin treated lysate was then incubated with calf intestinal phosphatase (250 units / ml). These lysates were evaluated for binding to the second PDZ domain of PSD-95 as described in figure 5-3. Forskolin treatment abolishes binding of Kir 2.3 to PSD-95 and treatment with phosphatase restores this interaction. Input = 10%. (B) Two dishes of HEK293 cells transfected with Kir 2.3 were prelabeled with [32 P]-orthophosphate and one was incubated with forskolin (20 μ M) and IBMX (100 μ M). Lysates from the two dishes were mixed together and incubated with the GST-PDZ fusion protein attached to agarose beads and the column was washed and eluted as described above. Immunoprecipitation and phosphopeptide analysis were performed on preload, flowthrough, and eluate fractions from the PDZ domain GST fusion protein column. Kir 2.3 phosphorylated at serine 440 (arrow) specifically flowed through the PDZ column while Kir 2.3 that was not phosphorylated at serine 440 was retained. (C) COS-7 cells were co-transfected with myc-Kir 2.3 and PSD-95 and incubated with or without forskolin (20 μ M) and IBMX (100 μ M). Solubilized membranes were then immunoprecipitated with antiserum to PSD-95. Forskolin treatment diminished the amount of Kir 2.3 that co-immunoprecipitated with PSD-95. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

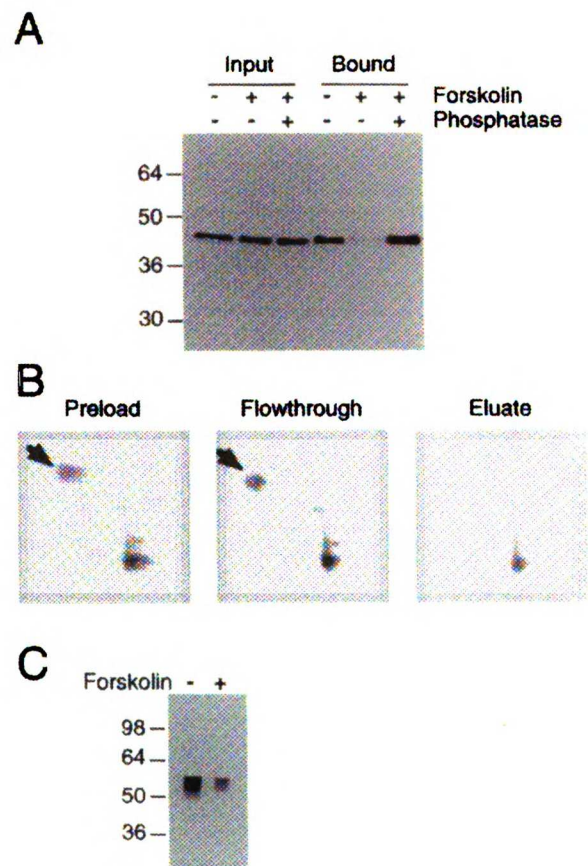


Figure 5-6

Figure 5-7. Kir 2.1 and 2.3 but not 3.1 Interact with the Second PDZ Domain of PSD-95.

Lysates of HEK293 cells transfected with myc-tagged carboxy terminal regions of Kir 2.1, Kir 2.3, or Kir 3.1 were incubated with a GST fusion protein containing the second PDZ domain of PSD-95 bound to agarose. Bound (B) proteins retained by the column were detected by western blotting with an anti-myc antibody. Input (I) = 10%. Positions of molecular size markers are indicated in kilodaltons on the y-ordinate.

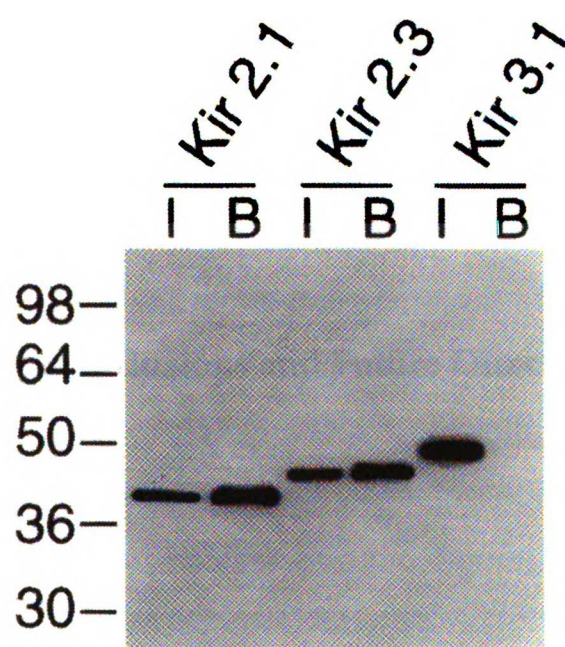


Figure 5-7

CHAPTER SIX

Conclusions and Future Directions

Summary

Neuronal nitric oxide synthase (nNOS) activity is regulated by calcium. Increases in intracellular calcium lead to calmodulin mediated activation of nNOS and the subsequent production of nitric oxide (NO) (Garthwaite et al., 1988; Bredt and Snyder, 1989). In cerebellar granule cells it has been demonstrated that glutamate mediated calcium influx through NMDA-type receptors is effectively coupled to NO production (Bredt and Snyder, 1989; Garthwaite et al., 1989), while calcium influx through other channels is only poorly coupled to NO production (Kiedrowski et al., 1992). Possible mechanisms to explain this functional coupling was completely unknown.

In both brain and skeletal muscle extracts, nNOS is predominantly particulate (Hecker et al., 1994), even though highly similar NOS proteins are not. Endothelial NOS (eNOS) is 60% identical at the amino acid level to nNOS and both proteins display virtually identical enzymatic activities. Although eNOS is only loosely associated with membranes due to N-terminal myristoylation (Robinson et al., 1995), it is unclear why nNOS is predominantly particulate. nNOS lacks any known modifications or protein sequences that would render it particulate. The major difference between eNOS and nNOS lies in the amino terminus of nNOS which contains a PDZ domain. It is with these questions and insights that I began my thesis.

The PDZ domain of nNOS is most similar in amino acid sequence to the PDZ domain in syntrophin. Interestingly, both of these proteins are colocalized at the skeletal muscle membrane (sarcolemma) with dystrophin (Kobzik et al., 1994; Adams et al., 1995). In chapter two, we demonstrate, that in fact nNOS is associated with the Dystrophin complex in skeletal muscle. This nNOS subcellular targeting is mediated by its PDZ domain, which allows association with the dystrophin complex. Dystrophin, which is thought to

form head-to-tail dimers, links the cytoskeleton to the extracellular matrix (reviewed in Tinsley et al., 1994). Dystrophin contains an actin binding motif intracellularly and binds to a complex of proteins at the sarcolemma (figure 6-1). Consistent with an association with the dystrophin complex, we find that in either mouse mutants lacking dystrophin (*mdx* mice), or human patients with Duchenne muscular dystrophy, nNOS immunoreactivity is completely absent from the sarcolemma. This absence from the sarcolemma in dystrophin abnormalities, is similar to other known dystrophin binding proteins, such as syntrophin. We also find biochemical evidence that nNOS localization is altered in *mdx* mice. nNOS and dystrophin both copurify on wheat germ agglutinin columns from wild type but not *mdx* mice. Wheat germ agglutinin affinity chromatography is a standard means of purifying dystrophin from muscle (Ervasti et al., 1991). Additionally, we find that nNOS is displaced from particulate fractions of *mdx* muscle and instead enriched in the soluble fractions.

In chapter three, we identify the direct molecular targets of the nNOS PDZ domain. These molecular targets serve to localize nNOS to its appropriate cellular milieu. Two-hybrid screening of a brain cDNA library revealed that the PDZ domain of nNOS interacts with the PDZ domains of two related proteins PSD-93 and PSD-95. nNOS and these PSD proteins are coexpressed in numerous neuronal populations in the adult and embryo and a PSD-95/nNOS complex can be identified *in vivo* by coimmunoprecipitation. Recently, the NMDA receptor has been shown to bind to PSD-95 (Kornau et al., 1995). Given that PSD-93 and PSD-95 each contain multiple binding sites for the NMDA receptor and nNOS, complexes of nNOS, the NMDA receptor, and PSD-95, could explain the functional coupling of nNOS to NMDA receptors (figure 6-2). In skeletal muscle, we

find that PDZ interactions also mediate the association with syntrophin, a protein which directly binds dystrophin. Syntrophin and nNOS can be coimmunoprecipitated in muscle, and the PDZ domains of syntrophin will directly bind the PDZ domains of nNOS. nNOS protein lacking a PDZ domain does not associate with PSD-95 from brain nor does it associate with muscle membranes. This is the first demonstration of a PDZ-PDZ interface mediating protein complex formation *in vivo*.

In chapter four, we characterize in detail the cloning of a novel membrane associated guanylate kinase (MAGUK) family member, we termed PSD-93. PSD-93 was originally identified as an nNOS interacting protein that is coexpressed in development with nNOS in spinal cord, dorsal root ganglia, and the adrenal gland. PSD-93 reflects the nomenclature of a known homolog, PSD-95, as well as the predicted protein mass of the major cDNA product. PSD-93 is neuronally enriched and expressed most abundantly by both RNA and protein levels in Purkinje neurons in the cerebellum. PSD-93 exhibits complex molecular heterogeneity due to tissue specific alternative splicing producing at least six alternatively spliced forms. PSD-93 is unique among known PSD family members in its expression in Purkinje neurons. PSD-93 is heavily enriched in cell bodies and dendrites of Purkinje neurons.

Dynamic regulation of ion channel interactions with the cytoskeleton is thought to underlie certain aspects of synaptic plasticity (Rosenmund and Westbrook, 1993; reviewed by Froehner, 1993). In chapter five, we extend observations that cytoskeletal PSD family members can bind to certain ion channels that end C-terminally in the loose consensus sequence threonine/serine - "X" - valine (T/S X V) (Kornau et al., 1995; Kim et al., 1995). We find that PSD-95 can bind to the potassium channel Kir 2.3, which ends in arginine - arginine - glutamate - serine - alanine - isoleucine

(RRESAI). Isoleucine being similar to valine seems to fulfill the C-terminal hydrophobic requirement for binding to PSD-95, but more interestingly, the critical serine residue is also a potent protein kinase A (PKA) phosphorylation site. Kir 2.3 and PSD-95 can be coimmunoprecipitated *in vivo*, and PKA induced phosphorylation by addition of forskolin decreases the amount that coimmunoprecipitates. Stimulation of PKA in intact cells is demonstrated to lead to phosphorylation of Kir 2.3 and thus its dissociation from PSD-95. This work demonstrates a mechanism for regulating an ion channel interaction with a cytoskeletal protein, PSD-95.

When neuronal nitric oxide synthase (nNOS) was molecularly cloned (Bredt et al., 1991), it was not appreciated that the amino-terminus of this protein contained a conserved protein-protein interaction motif. This motif, known as a PDZ domain, is named for three proteins containing amino acid sequence homology to this motif (postsynaptic density - 95, discs large, and zona occludens). Since the cloning of nNOS, when there were only a handful of known PDZ containing proteins, there are now at least 50 PDZ containing proteins (Tsunoda et al., 1997) (Table 6-1). Most PDZ containing proteins also possess additional protein motifs. In fact, the largest single family of PDZ domain containing proteins is the membrane associated guanylate kinase (MAGUK) family. MAGUKs contain 1 to 3 PDZ domains, followed by an SH3 domain, followed by a region with homology to yeast guanylate kinase (figure 6-3). However, this kinase is thought to be non-functional due to the absence of amino acids critical for binding ATP. Some MAGUKs also contain additional protein motifs at the amino terminus. For example, vertebrate CASK and its *C. elegans* homolog, *lin-2*, are MAGUK family proteins with an N-terminal domain homologous to calcium / calmodulin dependent (CAM) kinase (Hoskins et al., 1996; Hata et al., 1996). While *in vivo* functions for

MAGUKs are not completely clear in vertebrates, it is becoming apparent that in invertebrates, PDZ domains serve as protein tethers to target a protein or proteins to specific microenvironments within the cell.

Discussion

Future Directions

While *in vitro* evidence supports the role of PDZ domains and MAGUKs in clustering receptors and tethering signaling molecules to the cytoskeletal/membrane interface, *in vivo* functional roles for PDZ domains are lacking for vertebrates. Experiments in transfected heterologous cells demonstrate that when overexpressed, PSD-95 can cluster voltage gated potassium channels and the NMDA receptor (Kornau et al., 1995; Kim et al., 1995). However, the *in vivo* relevance of this phenomenon has yet to be demonstrated. Similarly, while it is clear that the PDZ domain of nNOS targets it to subcellular locations, it is unclear what affect this localization has on NO signaling. Experiments are underway, in this laboratory and others, to try to determine the *in vivo* functional importance of PDZ domains.

In this laboratory, recent results indicate that a protein complex of the NMDA receptor, PSD-95, and nNOS can be reconstituted *in vitro* (personal communication Karen Christopherson). Others in the laboratory are trying to perturb the localization of nNOS to determine what affects this might have on NO signaling. Two basic approaches to perturb NO signaling are being utilized. Primary neuronal cultures are infected with adenovirus (Hardy et al., 1997) encoding nNOS with and without a PDZ domain to determine affects on both subcellular localization and enzymatic activity. Conveniently, nNOS "knockout" mice provide the neurons to be infected and lack endogenous nNOS. However, experiments with primary neurons so far have proven to be technically difficult. In addition, the small number of glia get infected at nearly 100% efficiency and produce large amounts of protein, while neurons are infected more sporadically and produce less protein,

complicating interpretation of results (personal communication David Morton).

Primary neurons are also being perturbed with peptides that *in vitro* block the association of PSD-93/95 with nNOS and the NMDA receptor to measure the effects on nitric oxide signaling. Various means of peptide delivery are currently being tested and optimized (personal communication Karen Christopherson).

Another approach for deducing *in vivo* functions for molecules is through transgenic and targeted disruption mouse models. Targeted disruption and transgenic mouse models of relevant PDZ domain containing molecules may help reveal *in vivo* functions as well as provide testable model systems for current hypotheses. Surprisingly, to date none of the MAGUKs have been tested in either "knockout" or transgenic models. However, in this laboratory, targeted disruption of PSD-93 in mice is currently underway and in the later stages of completion. This should prove to be a useful model system to test the various hypothesis about MAGUK function in cells. PSD-93 knockouts may be particularly useful because Purkinje neurons are to date the only neurons known to contain a single MAGUK member (figure 6-4). Additionally, Purkinje neurons are extremely large and elaborate making structural studies more feasible. Finally, other laboratories are expressing the T/S - X - V consensus sequence on various proteins that normally lack them in transgenic models in order to determine the affect this might have on protein localization.

PDZ Domains in Genetic organisms

Perhaps the best examples of *in vivo* functions for PDZ domain containing proteins occurs in *Drosophila* and *C. elegans* where they were identified by forward genetics. In *Drosophila* the *inaD* mutant refers to a visual phototransduction mutant that was initially identified by its "apparent" inability to maintain a postdepolarization after potential (PDA) on electroretinograms (ERGs) recorded from photoreceptor cells (Pak, 1979; Shieh and Niemeyer, 1995). In *Drosophila*, photoreceptor cells are highly specialized with large microvillar foldings (rhabdomeres) that contain the phototransduction machinery (figure 6-5). Phototransduction involves light mediated conversion of rhodopsin which activates a G protein that activates a phospholipase C, which leads to generation of IP₃ and calcium influx.

Cloning of the *inaD* gene revealed that it was a cytoplasmic protein containing five PDZ domains with no other protein motifs. It was later shown that *in vivo* InaD protein is localized to regions within rhabdomeres. InaD has been demonstrated to function in localizing visual signal transduction molecules to the plasma membrane near the site of the initial signaling cascade (figure 6-6). Initial mutations identified in *inaD* disrupt the binding of a known ligand, the Trp calcium channel, and cause a mislocalization of Trp (Shieh and Zhu, 1996). Trp is no longer localized within the rhabdomeres and instead diffuses into the plasma membrane. Trp has been identified to bind to PDZ3, while other components in the signaling pathway, the protein kinase C and the phospholipase C, bind to PDZs 4 and 5 respectively (Tsunoda et al., 1997). Recently, a null allele of *inaD* has been generated and other identified PDZ ligands, the phospholipase C (NORAP - *no receptor activation potential*) and the protein kinase C (eye-PKC), were

also shown to be mislocalized. These authors also re-evaluated the *inaD* mutant electrophysiologically, and determined that the mislocalization of the signaling machinery leads to a latency defect that only appears to be a deactivation defect (Tsunoda et al., 1997). This example demonstrates, that PDZ domains function *in vivo* to localize signaling molecules to limited microenvironments to generate robust and rapid responses.

PDZ Domain Function in *C. elegans*

In *C. elegans*, there is a well characterized lineage determination process for forming the vulva of hermaphrodites (reviewed in Eisenmann and Kim, 1994). Six vulval squamous epithelial precursor cells divide and differentiate to form the mature vulva (figure 6-7). All six precursor cells can express one of three cell fates termed 1^o, 2^o, or 3^o. It is thought that the anchor cell induces the nearest cell termed P6.p, to express the 1^o cell fate. This induction has been shown to be mediated by a receptor tyrosine kinase / ras mediated pathway. Recent discoveries have molecularly characterized PDZ components necessary for proper signaling in this pathway (Hoskins et al., 1996; Simske et al., 1996).

The anchor cell secretes an EGF-like molecule (Lin-3) which binds to its EGF-like receptor (Let-23) on P6.p. Lack of receptor tyrosine kinase signaling leads to a vulvaless phenotype. Mutations in two genes termed *lin-2* and *lin-7*, which both contain PDZ domains leads to a vulvaless phenotype. Mutations in either *Lin-2* or *Lin-7* can cause mislocalization of *Let-23*. *Let-23* encodes the receptor tyrosine kinase, *Lin-7* actually binds to *let-23* and is thought to physically localize *let-23* (Simske et al., 1996). *Lin-2* is a PDZ containing MAGUK family member necessary for *Let-23* localization but the

mechanism by which Lin-2 mediates this localization is unknown. Let-23, lin-2, and lin-7 normally colocalize at the apical cellular tight junction, and just above it basally, towards the anchor cell. In *lin-2* or *lin-7* mutants, Let-23 is mislocalized instead to the entire apical membrane. It is thought that the apical side of the epithelial junction does not have access to Lin-3 (the ligand) and when Let-23 (the receptor) diffuses apically, it is less potent at signaling. Alternatively, it could be that concentrating a dimerizing receptor tyrosine kinase in a small area could lead to more efficient activation of the signaling cascade, necessary to avoid a vulvaless phenotype.

Dissecting PDZ signaling pathways in genetically tractable organisms is proving to be a powerful approach, that complements functions for PDZ domain proteins in vertebrates when they may be unknown. For instance, both *trp* and *lin-2* have homologs in vertebrates and in the case of *lin-2*, its vertebrate homolog, CASK, has no known function (Hata et al., 1996). Now, experiments for CASK can be designed knowing that in *C. elegans*, it is critical to localize an EGF-like receptor.

Figure 6-1. Schematic model demonstrating the interaction of neuronal nitric oxide synthase (nNOS) with α -1 syntrophin (Syn) in skeletal muscle. The presence of a question mark indicates other PDZ containing proteins may also bind to syntrophin. Head-to-tail dimers of dystrophin are thought to link the actin cytoskeleton to the extracellular matrix. Note glycosylation of dystrophin complex proteins α -dystroglycan (α -DG), β -dystroglycan (β -DG), and adhalin (AD). It is this glycosylation of dystrophin associated proteins that allows dystrophin purification by means of wheat germ agglutinin affinity chromatography. AchR, acetylcholine receptor; 43K, rapsyn.

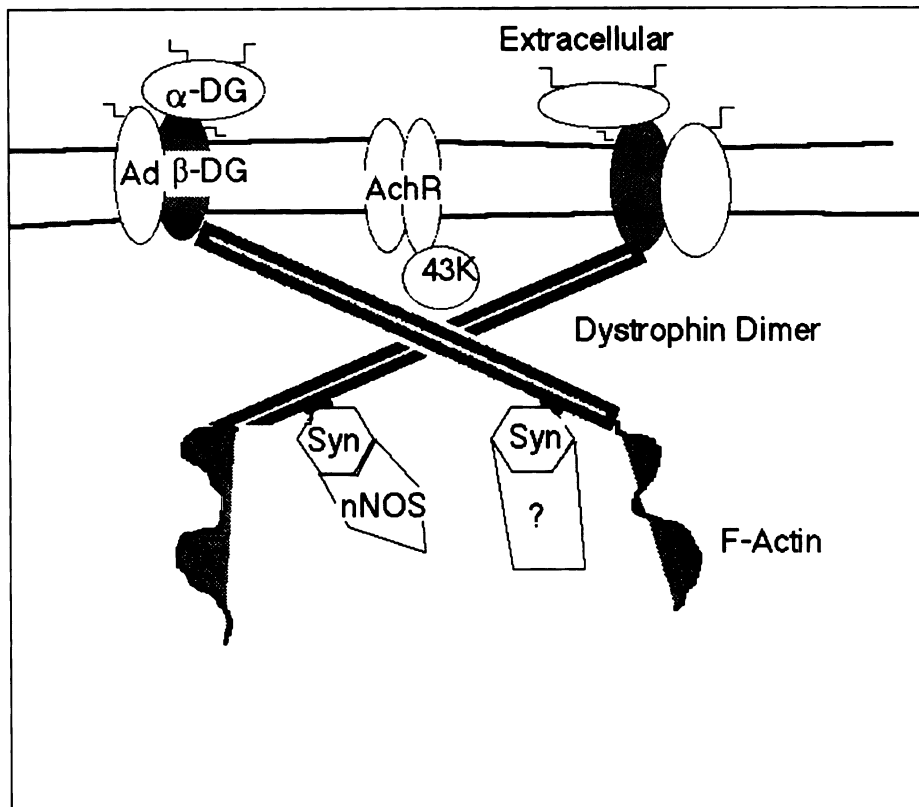


Figure 6-1

Figure 6-2. Schematic model demonstrating PSD-95 linking nNOS to the N-methyl-D-aspartate receptor (NMDA R). Functional coupling of nNOS to the NMDA receptor may be mediated by a physical coupling in which PSD-95 localizes nNOS directly to the NMDA receptor. PDZ1 of PSD-95 can bind directly to the NMDA receptor carboxy tail, while PDZ2 of PSD-95 can bind directly to nNOS. In this way, a calcium dependent enzyme, nNOS, is brought directly to a calcium source, the NMDA receptor. CaM, calmodulin. Hexagons represent PDZ domains.

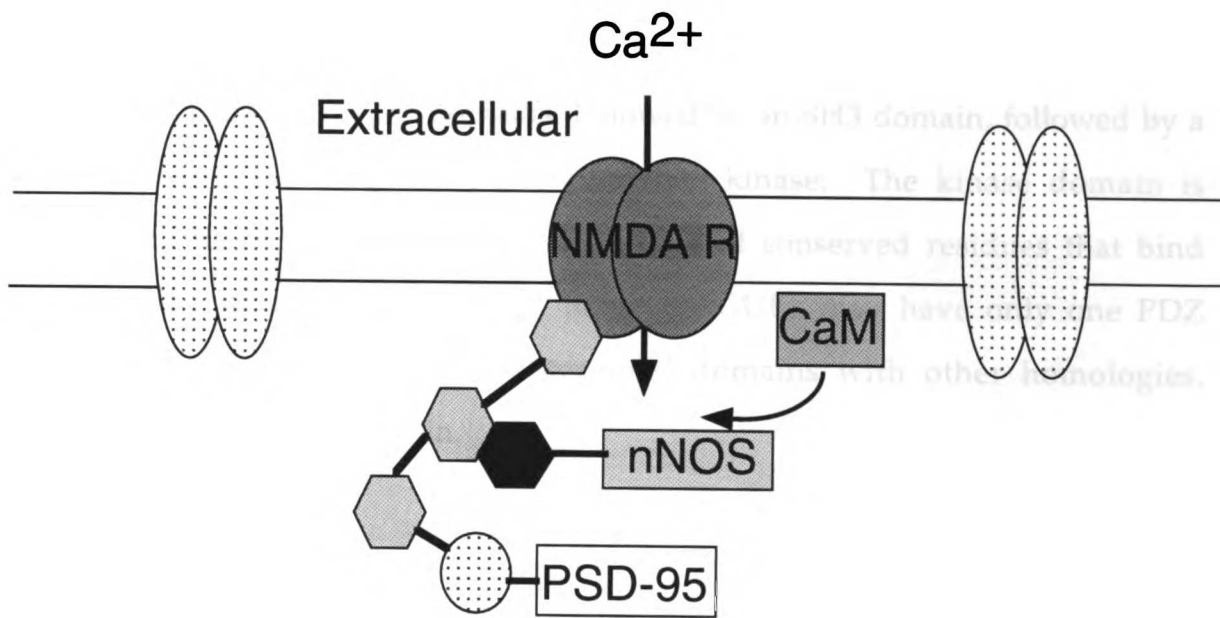


Figure 6-2

Figure 6-3. Schematic model illustrating the prototypical membrane associated guanylate kinase (MAGUK) protein structure. The vast majority of MAGUKs contain 3 PDZ domains, followed by an SH3 domain, followed by a region with homology to yeast guanylate kinase. The kinase domain is believed to be inactive due to the absence of conserved residues that bind adenosine trisphosphate (ATP). Some MAGUKs may have only one PDZ domain and may also have N-terminal domains with other homologies. Cam, calcium/calmodulin.

MAGUK Protein Structure

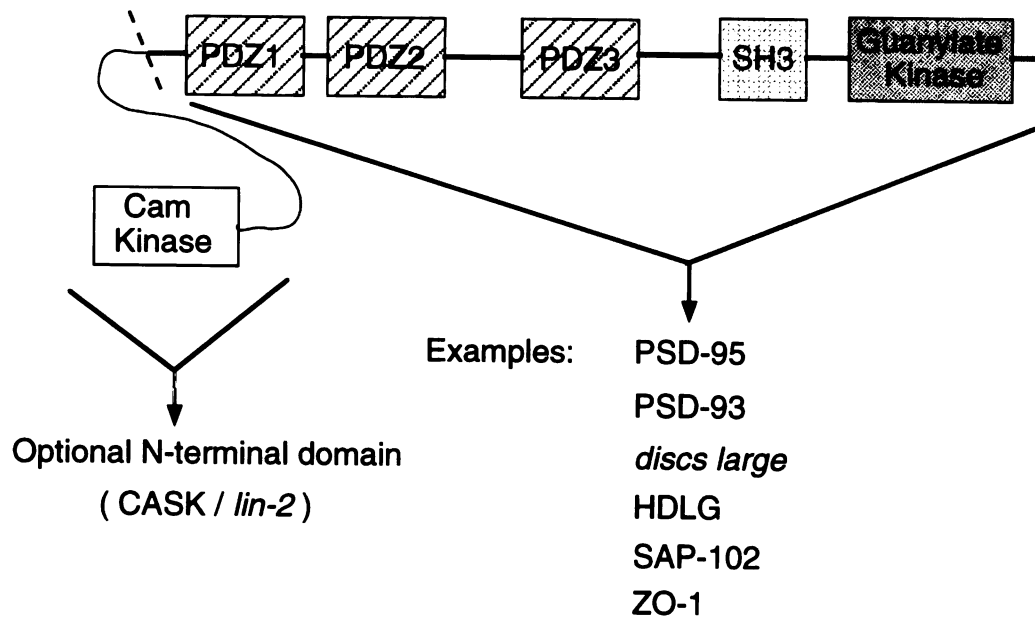


Figure 6-3

Figure 6-4. PSD-93 immunostaining robustly labels Purkinje neuron soma and dendrites in cerebellum. Indirect immunoperoxidase/diaminobenzadine detection reveals staining of cell bodies (arrowhead) in the Purkinje cell layer (P) and dendritic proximal shafts (arrow) in the molecular layer (M) (400X). PSD-93 is the sole known MAGUK in Purkinje neurons.

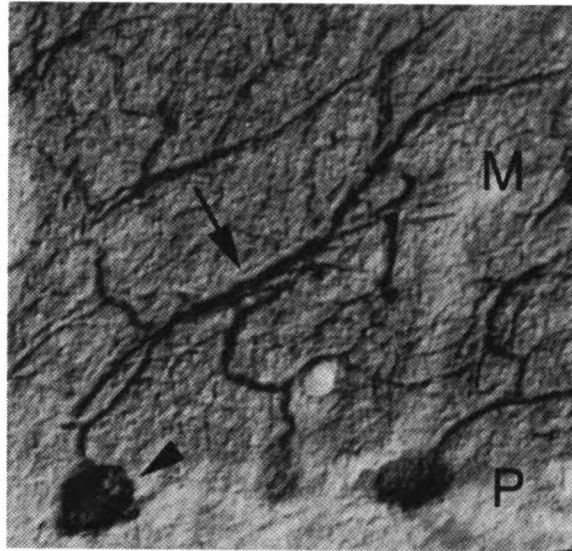


Figure 6-4

Figure 6-5. (A) Schematic drawing illustrating highly folded and specialized regions of *Drosophila* photoreceptor cells which constitute the rhabdomeres. (B) Schematic electroretinogram (ERG) recording of wild type or *inaD* mutant flies. A 6 second pulse of intense blue light triggers a 30 milivolt prolonged depolarizing afterpotential (PDA) in wild type but not *inaD* mutant flies. *inaD*, inactivation no afterpotential.

Drosophila Photoreceptor Cell

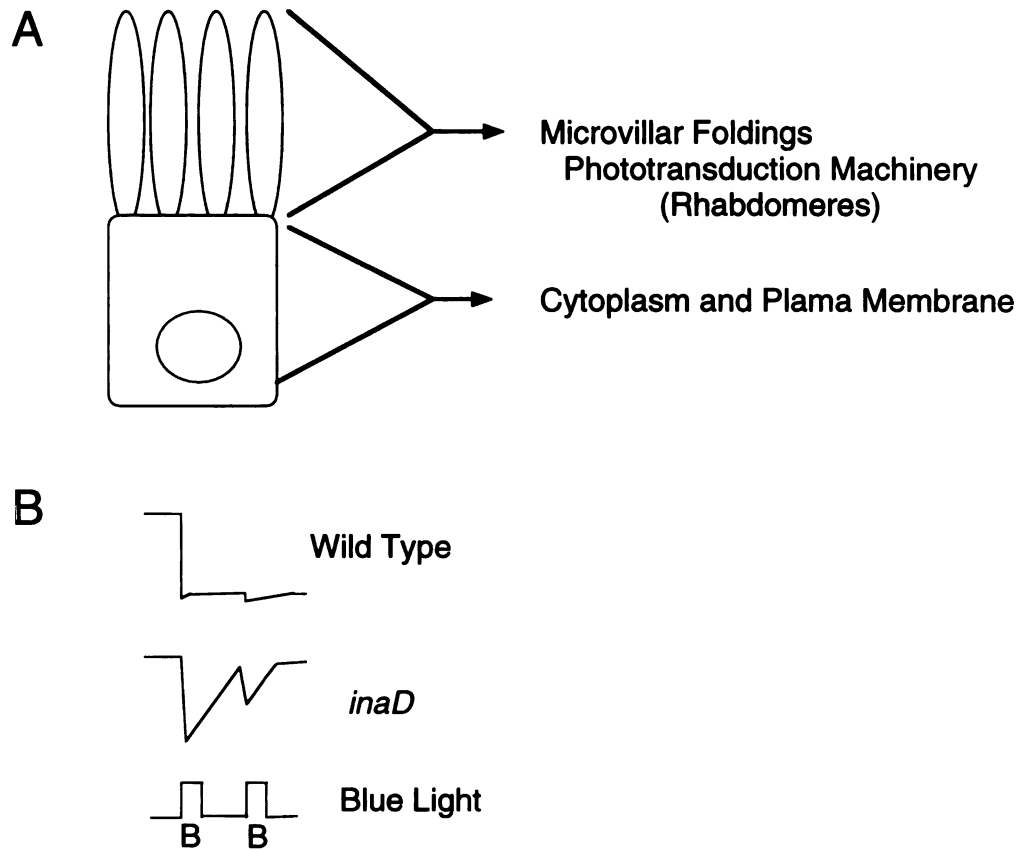


Figure 6-5

Figure 6-6. Schematic diagram illustrating that mutations in *inaD* lead to mislocalization of the calcium transient receptor potential (TRP) channel. Normally, the PDZ domains of InaD maintain the TRP channel within specific regions of the rhabdomeres. However, a mutation in PDZ domain 3 of InaD abolishes InaD association with TRP *in vivo* and *in vitro*. TRP is no longer localized and instead, is found diffusely throughout the outer membrane, particularly along the plasma membrane of the cell body. Inset illustrates that PDZ domains 4 and 5 of InaD bind to protein kinase C and phospholipase C respectively, which are also maintained within rhabdomeres by InaD.

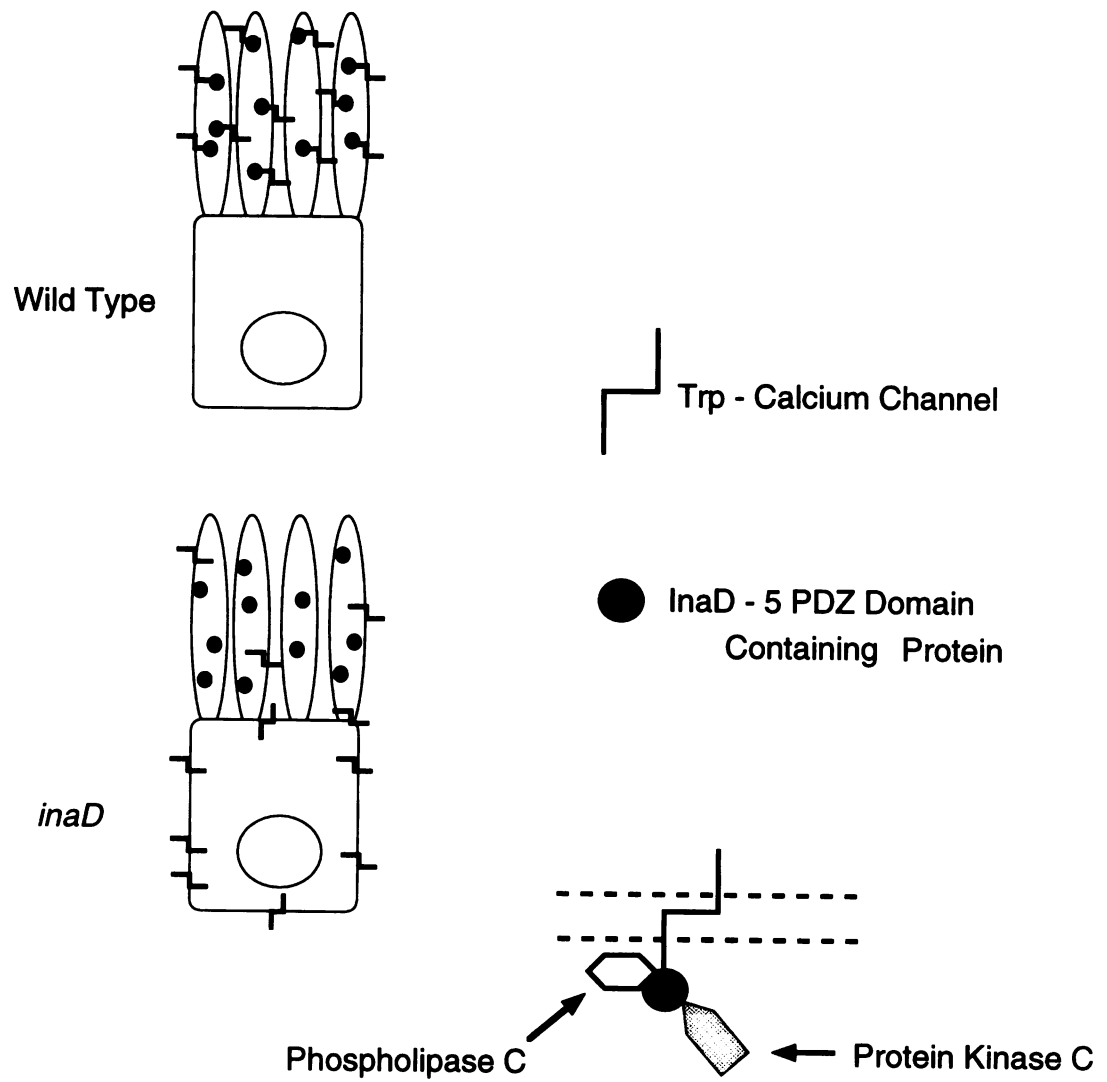


Figure 6-6

Figure 6-7. Schematic diagram illustrating vulval lineage development in *C. elegans*. The anchor cell normally induces the P6.p cell to acquire the 1^o cell fate through an EGF receptor-like tyrosine kinase signaling pathway. Normally, the receptor (Let-23) is localized at and just basally toward the anchor cell at tight junctions, where it is highly concentrated and has access to ligand (Lin-3). In either *lin-2* or *lin-7* mutants, Let-23 is no longer localized and instead diffuses apically, where it has less access to ligand. Identified mutations in *lin-2* and *lin-7*, both PDZ containing proteins, result in a vulvaless phenotype.

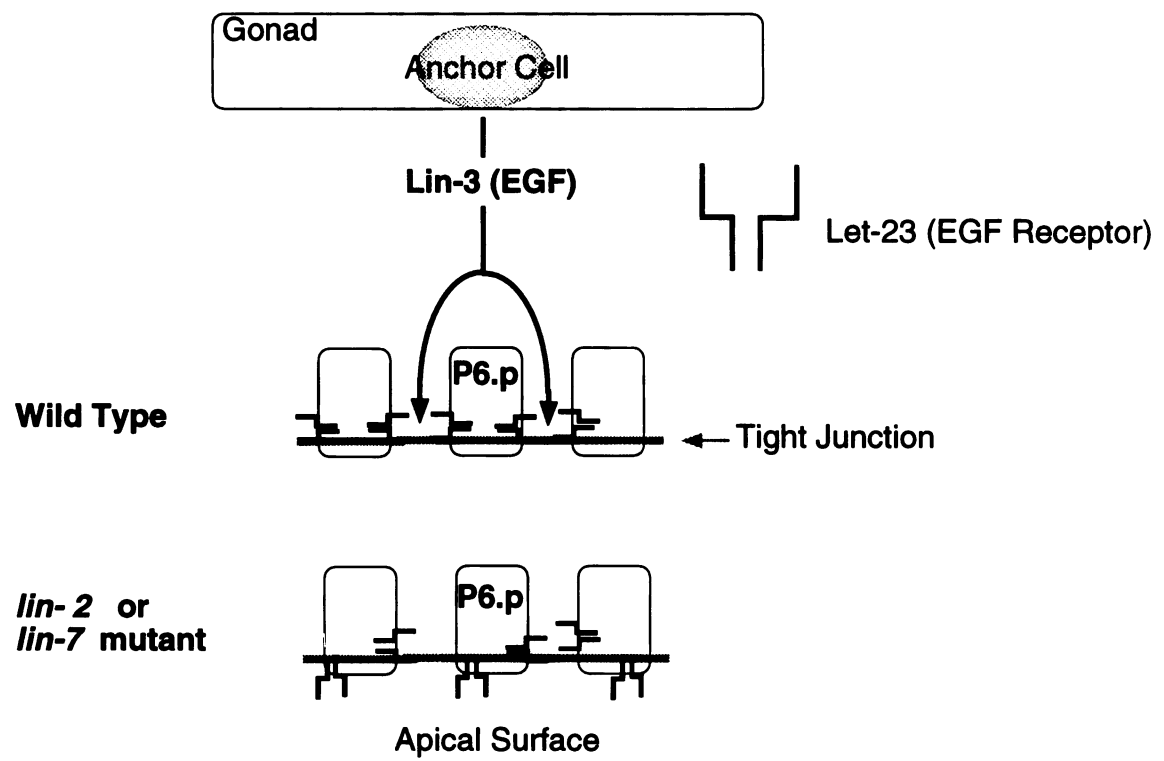


Figure 6-7

Table 6-1: A Sampling of PDZ Containing Proteins

<u>Vertebrates</u>	<u>Drosophila</u>	<u>C. elegans</u>
ALP	<i>canoe</i>	<i>lin-2</i>
CASK	<i>discs large</i>	<i>lin-7</i>
Densin-180	<i>dishevelled</i>	(not included -
enigma	<i>inaD</i>	genome project
FAP1		hypothetical
GRIP		proteins)
HDLG/SAP-97		
Homer		
nNOS		
p55		
PSD-93		
PSD-95		
SAP-102		
α -1 syntrophin		
β -1 syntrophin		
β -2 syntrophin		
Tiam-1		
TKA-1		
ZO-1		
ZO-2		

REFERENCES

- Adams, M. E., Butler, M. H., Dwyer, T. M., Peters, M. F., Murnane, A. A., and Froehner, S. C. (1993). Two forms of mouse syntrophin, a 58 kd dystrophin-associated protein, differ in primary structure and tissue distribution. *Neuron* 11, 531-40.
- Adams, M. E., Dwyer, T. M., Dowler, L. L., White, R. A., and Froehner, S. C. (1995). Mouse α 1- and β 2-Syntrophin Gene Structure, Chromosome Localization, and Homology with a Discs Large Domain. *Journal of Biological Chemistry* 270, 25859-25865.
- Ahn, A. H., and Kunkel, L. M. (1995). Syntrophin binds to an alternatively spliced exon of dystrophin. *J Cell Biol* 128, 363-71.
- Ahn, A. H., Yoshida, M., Anderson, M. S., Feener, C. A., Selig, S., Hagiwara, Y., Ozawa, E., and Kunkel, L. M. (1994). Cloning of human basic A1, a distinct 59-kDa dystrophin-associated protein encoded on chromosome 8q23-24. *Proc Natl Acad Sci U S A* 91, 4446-50.
- Aoki, C., Fenstermaker, S., Lubin, M., and Go, C. G. (1993). Nitric oxide synthase in the visual cortex of monocular monkeys as revealed by light and electron microscopic immunocytochemistry. *Brain Res* 620, 97-113.
- Bewick, G. S., Nicholson, L. V., Young, C., and Slater, C. R. (1993). Relationship of a dystrophin-associated glycoprotein to junctional acetylcholine receptor clusters in rat skeletal muscle. *Neuromuscul Disord* 3, 503-6.
- Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351, 714-8.
- Bredt, D. S., Hwang, P. M., and Snyder, S. H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 347, 768-70.

- Bredt, D. S., and Snyder, S. H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci U S A* 87, 682-5.
- Bredt, D. S., and Snyder, S. H. (1989). Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc Natl Acad Sci U S A* 86, 9030-3.
- Bredt, D. S., and Snyder, S. H. (1992). Nitric oxide, a novel neuronal messenger. *Neuron* 8, 3-11.
- Bredt, D. S., and Snyder, S. H. (1994). Nitric Oxide: A Physiologic Messenger Molecule. *Ann. Rev. Biochem.* 63, 175-195.
- Bredt, D. S., and Snyder, S. H. (1994). Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. *Neuron* 13, 301-13.
- Bredt, D. S., Wang, T. L., Cohen, N. A., Guggino, W. B., and Snyder, S. H. (1995). Cloning and expression of two brain-specific inwardly rectifying potassium channels. *Proc Natl Acad Sci U S A* 92, 6753-7.
- Brenman, J. E., Chao, D., S., Xia, H., Aldape, K., and Bredt, D., S. (1995). Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 82, 743-752.
- Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Bredt, D. S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α -1 syntrophin mediated by PDZ motifs. *Cell* 84, 757-767.
- Brenman, J. E., Christopherson, K. S., Craven, S. E., McGee, A. W., and Bredt, D. S. (1996). Cloning and characterization of postsynaptic density 93 (PSD-93), a nitric oxide synthase interacting protein. *Journal of Neuroscience* 16, 7407-7415.
- Bryant, P. J., and Woods, D. F. (1992). A major palmitoylated membrane protein of human erythrocytes shows homology to yeast guanylate kinase and to the product of a *Drosophila* tumor suppressor gene [letter]. *Cell* 68, 621-2.

- Bulfield, G., Siller, W. G., Wight, P. A., and Moore, K. J. (1984). X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc Natl Acad Sci U S A* *81*, 1189-92.
- Bult, H., Boeckxstaens, G. E., Pelckmans, P. A., Jordaens, F. H., Van Maercke, Y. M., and Herman, A. G. (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter [see comments]. *Nature* *345*, 346-7.
- Busconi, L., and Michel, T. (1993). Endothelial nitric oxide synthase. N-terminal myristoylation determines subcellular localization. *J Biol Chem* *268*, 8410-3.
- Butler, M. H., Douville, K., Murnane, A. A., Kramarcy, N. R., Cohen, J. B., Sealock, R., and Froehner, S. C. (1992). Association of the Mr 58,000 postsynaptic protein of electric tissue with Torpedo dystrophin and the Mr 87,000 postsynaptic protein. *J Biol Chem* *267*, 6213-8.
- Cabral, J. H. M., Petosa, C., Sutcliffe, M. J., S., R., Byron, O., Poy, F., Marfatia, S. M., Chishti, A. H., and Liddington, R. C. (1996). Crystal structure of a PDZ domain. *Nature* *382*, 649-652.
- Campanelli, J. T., Roberds, S. L., Campbell, K. P., and Scheller, R. H. (1994). A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. *Cell* *77*, 663-74.
- Campbell, K. P. (1995). Three Muscular Dystrophies: Loss of Cytoskeleton-Extracellular Matrix Linkage. *Cell* *80*, 675-679.
- Chandy, K. G., and Gutman, G. A. (1993). Nomenclature for mammalian potassium channel genes. *Trends Pharmacol Sci* *14*, 434.
- Chao, D. S., Gorospe, R. M., Brenman, J. E., Rafael, J. A., Peters, M. F., Froehner, S. C., Hoffman, E. P., Chamberlain, J. S., and Bredt, D. S. (1996). Selective loss of sarcolemmal nitric oxide synthase in Becker Muscular Dystrophy. *Journal of Experimental Medicine* *184*, 609-618.
- Chao, D. S., Silvagnano, F., Cornwell, T. L., Lincoln, T. M., and Bredt, D. S. (1997). Nitric oxide synthase and cyclic GMP-dependent

protein kinase concentrated at neuromuscular endplates. *Neuroscience* 76, 665-672.

Chevesich, J., Kreuz, A. J., and Montell, C. (1997). Requirement for the PDZ domain protein InaD, for localization of the TRP store-operated channel to a signaling complex. *Neuron* 18, 95-105.

Cho, H. J., Xie, Q. W., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., and Nathan, C. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J Exp Med* 176, 599-604.

Cho, K. O., Hunt, C. A., and Kennedy, M. B. (1992). The rat brain postsynaptic density fraction contains a homolog of the *Drosophila* discs-large tumor suppressor protein. *Neuron* 9, 929-42.

Choi, D. W. (1994). Glutamate receptors and the induction of excitotoxic neuronal death. *Prog Brain Res* 100, 47-51.

Cohen, N. A., Brenman, J. E., Snyder, S. H., and Brecht, D. S. (1996). Binding of the inward rectifier K⁺ channel Kir 2.3 to PSD-95 is regulated by protein kinase A phosphorylation. *Neuron* 17, 759-767.

Daniel, E. E., Haugh, C., Woskowska, Z., Cipris, S., Jury, J., and Fox-Threlkeld, J. E. (1994). Role of nitric oxide-related inhibition in intestinal function: relation to vasoactive intestinal polypeptide. *Am J Physiol* 266, G31-9.

Dascal, N., Schreibmayer, W., Lim, N. F., Wang, W., Chavkin, C., DiMagno, L., Labarca, C., Kieffer, B. L., Gaveriaux-Ruff, C., Trollinger, D., and et al. (1993). Atrial G protein-activated K⁺ channel: expression cloning and molecular properties. *Proc Natl Acad Sci U S A* 90, 10235-9.

Davison, A., Tibbits, G., Shi, Z. G., and Moon, J. (1988). Active oxygen in neuromuscular disorders. *Mol Cell Biochem* 84, 199-216.

Dawson, T. M., Dawson, V. L., and Snyder, S. H. (1992). A novel neuronal messenger molecule in brain: the free radical, nitric oxide [see comments]. *Ann Neurol* 32, 297-311.

- Dawson, V. L., Dawson, T. M., London, E. D., Brecht, D. S., and Snyder, S. H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci U S A* 88, 6368-71.
- Dawson, V. L., Kizushi, V., M., Huang, P. L., Snyder, S. H., and Dawson, T. M. (1996). Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. *J. Neuroscience* 16, 2479-2487.
- Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996). Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. *Cell* 85, 1067-1076.
- Eisenmann, D. M., and Kim, S. K. (1994). Signal transduction and cell fate specification during *Caenorhabditis elegans* vulval development. *Current Biology* 4, 508-516.
- Ervasti, J. M., and Campbell, K. P. (1993). Dystrophin and the membrane skeleton. *Curr Opin Cell Biol* 5, 82-7.
- Ervasti, J. M., Kahl, S. D., and Campbell, K. P. (1991). Purification of dystrophin from skeletal muscle. *J Biol Chem* 266, 9161-5.
- Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G., and Campbell, K. P. (1990). Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* 345, 315-9.
- Fakler, B., Brandle, U., Glowatzki, E., Weidemann, S., Zenner, H. P., and Ruppersberg, J. P. (1995). Strong voltage-dependent inward rectification of inward rectifier K⁺ channels is caused by intracellular spermine. *Cell* 80, 149-54.
- Fakler, B., Brandle, U., Glowatzki, E., Zenner, H. P., and Ruppersberg, J. P. (1994). Kir2.1 inward rectifier K⁺ channels are regulated independently by protein kinases and ATP hydrolysis. *Neuron* 13, 1413-20.
- Falk, T., Meyerhof, W., Corrette, B. J., Schafer, J., Bauer, C. K., Schwarz, J. R., and Richter, D. (1995). Cloning, functional expression and mRNA distribution of an inwardly rectifying potassium channel protein. *FEBS Lett* 367, 127-31.

- Ferrendelli, J. A., Chang, M. M., and Kinscherf, D. A. (1974). Elevation of cyclic GMP levels in central nervous system by excitatory and inhibitory amino acids. *Journal of Neurochemistry* 22, 535-540.
- Ficker, E., Taglialatela, M., Wible, B. A., Henley, C. M., and Brown, A. M. (1994). Spermine and spermidine as gating molecules for inward rectifier K⁺ channels. *Science* 266, 1068-72.
- Fields, S., and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* 340, 245-6.
- Froehner, S. C., Luetje, C. W., Scotland, P. B., and Patrick, J. (1990). The postsynaptic 43K protein clusters muscle nicotinic acetylcholine receptors in *Xenopus* oocytes. *Neuron* 5, 403-10.
- Frohman, M. A. (1993). Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: thermal RACE. *Methods Enzymol* 218, 340-56.
- Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288, 373-6.
- Garthwaite, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci* 14, 60-7.
- Garthwaite, J., and Boulton, C. L. (1995). Nitric oxide signaling in the central nervous system. *Annu Rev Physiol* 57, 683-706.
- Garthwaite, J., Charles, S. L., and Chess-Williams, R. (1988). Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 336, 385-8.
- Garthwaite, J., Garthwaite, G., Palmer, R. M., and Moncada, S. (1989). NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *Eur J Pharmacol* 172, 413-6.
- Gautam, M., Noakes, P. G., Moscoso, L., Rupp, F., Scheller, R. H., Merlie, J. P., and Sanes, J. R. (1996). Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85, 525-536.

- Gee, S. H., Montanaro, F., Lindenbaum, M. H., and Carbonetto, S. (1994). Dystroglycan- α , a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell* 77, 675-86.
- Glass, D. J., Bowen, D. C., Stitt, T. N., Radziejewski, C., Bruno, J., Ryan, T. E., Gies, D. R., Shah, S., Mattson, K., Burden, S. J., Distefano, P. S., Valenzuela, D. M., DeChiara, T. M., and Yoncopoulos, G. D. (1996). Agrin acts via a MuSK receptor complex. *Cell* 85, 513-524.
- Gomperts, S. N. (1996). Clustering membrane proteins: It's all coming together with the PSD-95/SAP90 protein family. *Cell* 84, 659-62.
- Hall, A. V., Antoniou, H., Wang, Y., Cheung, A. H., Arbus, A. M., Olson, S. L., Lu, W. C., Kau, C. L., and Marsden, P. A. (1994). Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J Biol Chem* 269, 33082-90.
- Hantraye, P., Brouillet, E., Ferrante, R., Palfi, S., Dolan, R., Matthews, R. T., and Beal, M. F. (1996). Inhibition of neuronal nitric oxide synthase prevents MPTP-induced parkinsonism in baboons. *Nature Medicine* 2, 1017-1021.
- Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., and Phipps, M. L. (1997). Construction of Adenovirus vectors through Cre-*lox* recombination. *J. Virology* 71, 1842-1849.
- Hata, Y., Butz, S., and Sudhof, T. C. (1996). CASK: A novel *dlg*/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. *J. Neuroscience* 16, 2488-2494.
- Hecker, M., Mulsch, A., and Busse, R. (1994). Subcellular localization and characterization of neuronal nitric oxide synthase. *J Neurochem* 62, 1524-9.
- Hibbs, J. B., Jr., Taintor, R. R., and Vavrin, Z. (1987). Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* 235, 473-6.
- Hibbs, J. B., Jr., Vavrin, Z., and Taintor, R. R. (1987). L-arginine is required for expression of the activated macrophage effector

- mechanism causing selective metabolic inhibition in target cells. *J Immunol* *138*, 550-65.
- Hille, B. (1992). *Ionic Channels of Excitable Membranes* (Sunderland, MA: Sinauer).
- Ho, K., Nichols, C. G., Lederer, W. J., Lytton, J., Vassilev, P. M., Kanazirska, M. V., and Hebert, S. C. (1993). Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature* *362*, 31-8.
- Hoskins, R., Hajnal, A. F., Harp, S. A., and Kim, S. K., (1996). The *C. elegans* vulval induction gene *lin-2* encodes a member of the MAGUK family of cell junction proteins. *Development* *122*, 97-111.
- Huang, C. L., Slesinger, P. A., Casey, P. J., Jan, Y. N., and Jan, L. Y. (1995). Evidence that direct binding of G beta gamma to the GIRK1 G protein-gated inwardly rectifying K⁺ channel is important for channel activation. *Neuron* *15*, 1133-43.
- Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H., and Fishman, M. C. (1993). Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* *75*, 1273-86.
- Huang, Z., Huang, P. L., Ma, J., Meng, W., Ayata, C., Fishman, M. C., and Moskowitz, M. A. (1996). Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J Cereb Blood Flow Metab* *16*, 981-7.
- Huang, Z., Huang, P. L., Panahian, N., Dalkara, T., Fishman, M. C., and Moskowitz, M. A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* *265*, 1883-5.
- Huber, A., Sander, P., Gobert, A., Bahner, M., Hermann, R., and Paulsen, R. (1996). The transient receptor potential protein (Trp), a putative store-operated calcium channel essential for phosphoinositide-mediated photoreception, forms a signaling complex with NorpaA, InaC and InaD. *The EMBO J.* *15*, 7036-7045.

- Hunt, A. C., Schenker, L. J., and Kennedy, M. B. (1996). PSD-95 is associated with the postsynaptic density and not with the presynaptic membrane at forebrain synapses. *Journal of Neuroscience* 16, 1380-1388.
- Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W., and Campbell, K. P. (1992). Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* 355, 696-702.
- Isaac, J. T., Nicoll, R. A., and Malenka, R. C. (1995). Evidence for silent synapses: implications for the expression of LTP. *Neuron* 15, 427-34.
- Jaffrey, S. R., and Snyder, S. H. (1996). PIN: An associated protein inhibitor of neuronal nitric oxide synthase. *Science* 274, 774-777.
- Jesaitis, L. A., and Goodenough, D. A. (1994). Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the *Drosophila* discs-large tumor suppressor protein. *J Cell Biol* 124, 949-61.
- Jonas, A. A., and Kaczmarek, L. K. (1996). Regulation of potassium channels by protein kinases. *Current Opinion in Neurobiology* 6, 318-323.
- Kandel, E., and Abel, T. (1995). Neuropeptides, adenylyl cyclase, and memory storage. *Science* 268, 825-6.
- Kiedrowski, L., Costa, E., and Wroblewski, J. T. (1992). Glutamate receptor agonists stimulate nitric oxide synthase in primary cultures of cerebellar granule cells. *J Neurochem* 58, 335-41.
- Kim, E., Cho, K.-O., Rothschild, A., and Sheng, M. (1996). Heteromultimerization and NMDA receptor clustering activity of chapsyn-110, a novel member of the PSD-95 family of synaptic proteins. *Neuron* 17, 103-113.
- Kim, E., Niethammer, M., Rothschild, A., N., J. Y., and Sheng, M. (1995). Clustering of Shaker-type K⁺ channels by direct interaction with the PSD-95/SAP90 family of membrane-associated guanylate kinases. *Nature* 378, 85-88.

- Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appeltauer, U., Voss, B., Gundelfinger, E. D., and Garner, C. C. (1993). SAP90, a rat presynaptic protein related to the product of the *Drosophila* tumor suppressor gene *dlg-A*. *J Biol Chem* 268, 4580-3.
- Knowles, R. G., Palacios, M., Palmer, R. M., and Moncada, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc Natl Acad Sci U S A* 86, 5159-62.
- Kobzik, L., Reid, M. B., Brecht, D. S., and Stamler, J. S. (1994). Nitric Oxide in Skeletal Muscle. *Nature* 372, 546-548.
- Koenig, M., Monaco, A. P., and Kunkel, L. M. (1988). The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* 53, 219-26.
- Kordeli, E., Davis, J., Trapp, B., and Bennett, V. (1990). An isoform of ankyrin is localized at nodes of Ranvier in myelinated axons of central and peripheral nerves. *J Cell Biol* 110, 1341-52.
- Kornau, H.-C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* 269, 1737-1740.
- Koumi, S., Wasserstrom, J. A., and Ten Eick, R. E. (1995). Beta-adrenergic and cholinergic modulation of inward rectifier K⁺ channel function and phosphorylation in guinea-pig ventricle. *J Physiol (Lond)* 486, 661-78.
- Kubo, Y., Baldwin, T. J., Jan, Y. N., and Jan, L. Y. (1993). Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature* 362, 127-33.
- Kuhse, J., Betz, H., and Kirsch, J. (1995). The inhibitory glycine receptor: architecture, synaptic localization and molecular pathology of a postsynaptic ion-channel complex. *Curr Opin Neurobiol* 5, 318-23.

- Kunkel, M. T., and Peralta, E. G. (1995). Identification of domains conferring G protein regulation on inward rectifier potassium channels. *Cell* 83, 443-9.
- Kuzin, B., Roberts, I., Peunova, N., and Enikolopov, G. (1996). Nitric oxide regulates cell proliferation during drosophila development. *Cell* 87, 639-649.
- Lau, L. F., Mammen, A., Ehlers, M. D., Kindler, S., Chung, W. J., Garner, C. C., and Huganir, R. L. (1996). Interaction of the N-methyl-D-aspartate receptor complex with a novel synapse-associated protein, SAP102. *Jour. Biol. Chem.*, 21622-8.
- Lee, K. H., Baek, M. Y., Moon, K. Y., Song, W. K., Chung, C. H., Ha, D. B., and Kang, M. S. (1994). Nitric oxide as a messenger molecule for myoblast fusion. *J Biol Chem* 269, 14371-4.
- Liao, D., Hessler, N. A., and Malinow, R. (1995). Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* 375, 400-4.
- Lopatin, A. N., Makhina, E. N., and Nichols, C. G. (1994). Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature* 372, 366-9.
- Lu, Z., and MacKinnon, R. (1994). Electrostatic tuning of Mg²⁺ affinity in an inward-rectifier K⁺ channel. *Nature* 371, 243-6.
- Lue, R. A., Marfatia, S. M., Branton, D., and Chishti, A. H. (1994). Cloning and characterization of hdlg: the human homologue of the Drosophila discs large tumor suppressor binds to protein 4.1. *Proc Natl Acad Sci U S A* 91, 9818-22.
- Luo, D., Knezevich, S., and Vincent, S. R. (1993). N-methyl-D-aspartate-induced nitric oxide release: an in vivo microdialysis study. *Neuroscience* 57, 897-900.
- Marfatia, S. M., Lue, R. A., Branton, D., and Chishti, A. H. (1994). In vitro binding studies suggest a membrane-associated complex between erythroid p55, protein 4.1, and glycophorin C. *J Biol Chem* 269, 8631-4.

- Mashimo, H., He, X. D., Huang, P. L., Fishman, M. C., and Goyal, R. K. (1996). Neuronal constitutive nitric oxide synthase is involved in murine enteric inhibitory neurotransmission. *J Clin Invest* 98, 8-13.
- Matsuda, H. (1991). Magnesium gating of the inwardly rectifying K⁺ channel. *Annu Rev Physiol* 53, 289-98.
- Matsumura, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. D., and Campbell, K. P. (1992). Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. *Nature* 360, 588-91.
- Mayer, M. L., Westbrook, G. L., and Guthrie, P. B. (1984). Voltage-dependent block by Mg²⁺ of NMDA responses in spinal cord neurones. *Nature* 309, 261-3.
- McMahan, U. J. (1990). The agrin hypothesis. *Cold Spring Harbor Symp. Quant. Biol.* 55, 407-418.
- Meffert, M. K., Calakos, N. C., Scheller, R. H., and Schulman, H. (1996). Nitric oxide modulates synaptic vesicle docking fusion reactions. *Neuron* 16, 1229-36.
- Mitchell, R. D., Palade, P., and Fleischer, S. (1983). Purification of morphologically intact triad structures from skeletal muscle. *J Cell Biol* 96, 1008-16.
- Monaco, A. P., Neve, R. L., Colletti-Feener, C., Bertelson, C. J., Kurnit, D. M., and Kunkel, L. M. (1986). Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. *Nature* 323, 646-50.
- Moncada, S., and Higgs, A. (1993). The L-arginine-nitric oxide pathway. *N Engl J Med* 329, 2002-12.
- Morishige, K., Takahashi, N., Findlay, I., Koyama, H., Zanelli, J. S., Peterson, C., Jenkins, N. A., Copeland, N. G., Mori, N., and Kurachi, Y. (1993). Molecular cloning, functional expression and localization of an inward rectifier potassium channel in the mouse brain. *FEBS Lett* 336, 375-80.

- Muller, B. M., Kistner, U., Kindler, S., Chung, W. K., Kuhlendahl, S., Fenster, S. D., Lau, L.-F., Veh, R. W., Huganir, R. L., Gundelfinger, E. D., and Garner, C. C. (1996). SAP102, a novel postsynaptic protein that interacts with NMDA receptor complexes in vivo. *Neuron* 17, 255-265.
- Muller, B. M., Kistner, U., Veh, R. W., Cases-Langhoff, C., Becker, B., Gundelfinger, E. D., and Garner, C. C. (1995). Molecular characterization and spatial distribution of SAP97, a novel presynaptic protein homologous to SAP90 and the Drosophila discs-large tumor suppressor protein. *J Neurosci* 15, 2354-66.
- Nakane, M., Schmidt, H. H., Pollock, J. S., Forstermann, U., and Murad, F. (1993). Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* 316, 175-80.
- Nathan, C., and Xie, Q. W. (1994). Regulation of biosynthesis of nitric oxide. *J Biol Chem* 269, 13725-8.
- Nelson, R. J., Demas, G. E., Huang, P. L., Fishman, M. C., Dawson, V. L., Dawson, T. M., and Snyder, S. H. (1995). Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. *Nature* 378, 383-386.
- Nicoll, R. A., and Malenka, R. C. (1995). Contrasting properties of two forms of long-term potentiation in the hippocampus. *Nature* 377, 115-8.
- Nitkin, R. M., Smith, M. A., Magill, C., Fallon, J. R., Yao, Y. M., Wallace, B. G., and McMahan, U. J. (1987). Identification of agrin, a synaptic organizing protein from Torpedo electric organ. *J Cell Biol* 105, 2471-8.
- Nowak, L., Bregestovski, P., Ascher, P., Herbet, A., and Prochiantz, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* 307, 462-5.
- O'Dell, T. J., Huang, P. L., Dawson, T. M., Dinerman, J. L., Snyder, S. H., Kandel, E. R., and Fishman, M. C. (1994). Endothelial NOS and the blockade of LTP by NOS inhibitors in mice lacking neuronal NOS. *Science* 265, 542-6.

- Ohlendieck, K., and Campbell, K. P. (1991). Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. *J Cell Biol* 115, 1685-94.
- Ohlendieck, K., Ervasti, J. M., Snook, J. B., and Campbell, K. P. (1991). Dystrophin-glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. *J Cell Biol* 112, 135-48.
- Pak, W. L. (1979). Study of photoreceptor function using *Drosophila* mutants. *Neurogenetics: Genetic approaches to the Nervous System*, X. O. Breakfield, ed. (New York: Elsevier), pp. 67-99.
- Perier, F., Radeke, C. M., and Vandenberg, C. A. (1994). Primary structure and characterization of a small-conductance inwardly rectifying potassium channel from human hippocampus. *Proc Natl Acad Sci U S A* 91, 6240-4.
- Pessia, M., Bond, C. T., Kavanaugh, M. P., and Adelman, J. P. (1995). Contributions of the C-terminal domain to gating properties of inward rectifier potassium channels. *Neuron* 14, 1039-45.
- Peters, M. F., Kramarcy, N. R., Sealock, R., and Froehner, S. C. (1994). beta 2-Syntrophin: localization at the neuromuscular junction in skeletal muscle. *Neuroreport* 5, 1577-80.
- Phillips, W. D., Kopta, C., Blount, P., Gardner, P. D., Steinbach, J. H., and Merlie, J. P. (1991). ACh receptor-rich membrane domains organized in fibroblasts by recombinant 43-kildalton protein. *Science* 251, 568-70.
- Ponting, C. P., and Phillips, C. (1995). DHR domains in syntrophins, neuronal NO synthases and other intracellular proteins. *Trends in biological sciences* 20, 102-3.
- Przedborski, S., Jackson-Lewis, V., Yokoyama, R., Shibata, T., Dawson, V. L., and Dawson, T. M. (1996). Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. *Proc Natl Acad Sci U S A* 93, 4565-71.
- Rafael, J. A., Sunada, Y., Cole, N. M., Campbell, K. P., Faulkner, J. A., and Chamberlain, J. S. (1994). Prevention of dystrophic pathology in

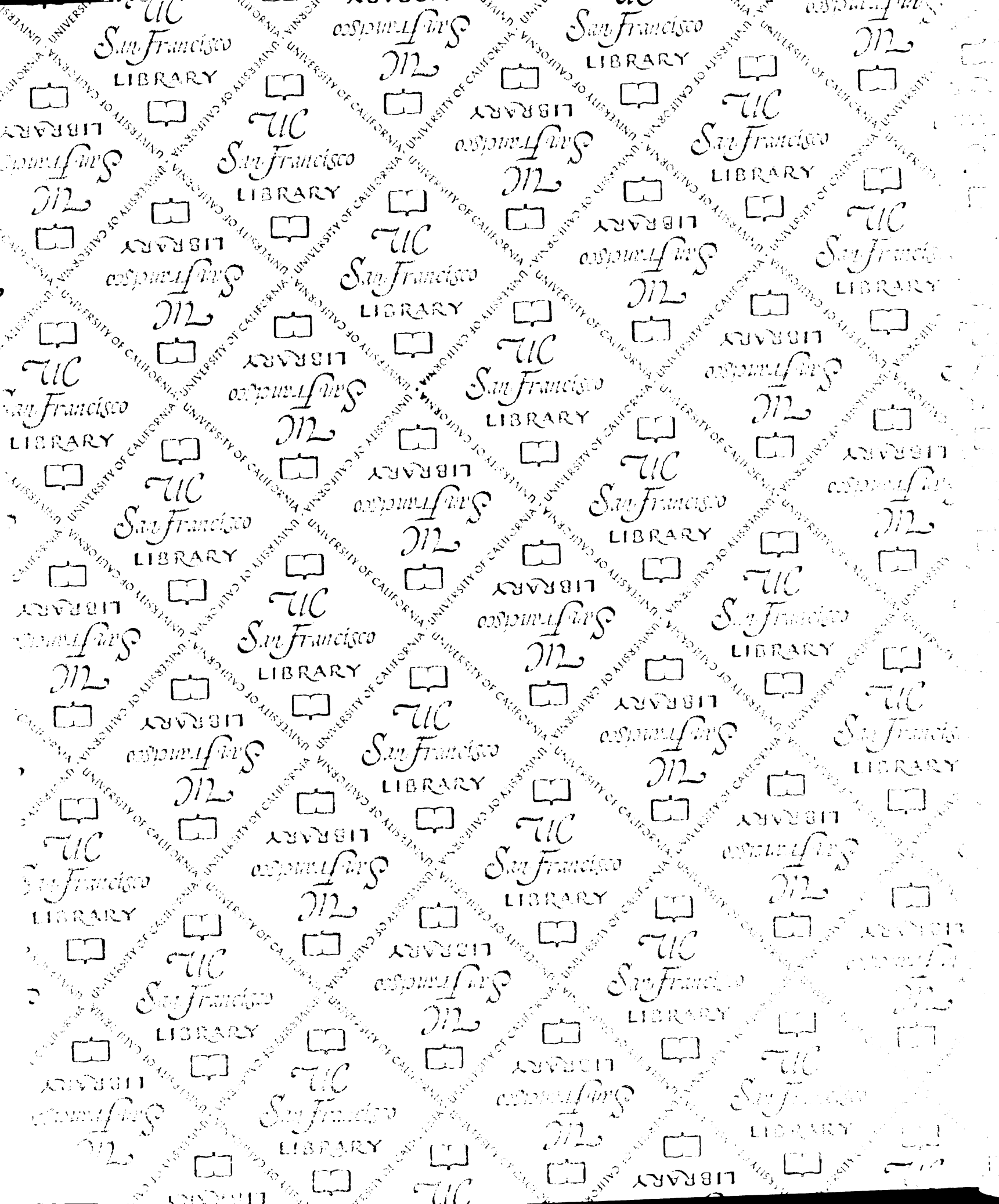
- mdx mice by a truncated dystrophin isoform. *Hum Mol Genet* 3, 1725-33.
- Raiteri, M., Maura, G., and Barzizza, A. (1991). Activation of presynaptic 5-hydroxytryptamine₁-like receptors on glutamatergic terminals inhibits N-methyl-D-aspartate-induced cyclic GMP production in rat cerebellar slices. *J Pharmacol Exp Ther* 257, 1184-8.
- Raymond, L. A., Blackstone, C. D., and Huganir, R. L. (1993). Phosphorylation of amino acid neurotransmitter receptors in synaptic plasticity. *Trends Neurosci* 16, 147-53.
- Reid, M. B., Haack, K. E., Franchek, K. M., Valberg, P. A., Kobzik, L., and West, M. S. (1992). Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue in vitro. *J Appl Physiol* 73, 1797-804.
- Reid, M. B., Shoji, T., Moody, M. R., and Entman, M. L. (1992). Reactive oxygen in skeletal muscle. II. Extracellular release of free radicals. *J Appl Physiol* 73, 1805-9.
- Robinson, L. J., Busconi, L., and Michel, T. (1995). Agonist-modulated palmitoylation of endothelial nitric oxide synthase. *J Biol Chem* 270, 995-8.
- Roche, W. K., O'Brien, R. J., Mammen, A. L., Bernhardt, J., and Huganir, R. L. (1996). Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* 16, 1179-1188.
- Rosenmund, C., and Westbrook, G. L. (1993). Calcium-induced actin depolymerization reduces NMDA channel activity. *Neuron* 10, 805-14.
- Sassoon, D., and Rosenthal, N. (1993). Detection of messenger RNA by in situ hybridization. *Methods Enzymol* 225, 384-404.
- Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995). FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science* 268, 411-5.
- Schmidt, H. H., Gagne, G. D., Nakane, M., Pollock, J. S., Miller, M. F., and Murad, F. (1992). Mapping of neural nitric oxide synthase in

- the rat suggests frequent co-localization with NADPH diaphorase but not with soluble guanylyl cyclase, and novel paraneural functions for nitrinergic signal transduction. *J Histochem Cytochem* 40, 1439-56.
- Schulz, J. B., Huang, P. L., Matthews, R. T., Passov, D., Fishman, M. C., and Beal, M. F. (1996). Striatal malonate lesions are attenuated in neuronal nitric oxide synthase knockout mice. *J Neurochem* 67, 430-3.
- Schuman, E. M., and Madison, D. V. (1994). Nitric oxide and synaptic function. *Annu Rev Neurosci* 17, 153-83.
- Sealock, R., Butler, M. H., Kramarcy, N. R., Gao, K. X., Murnane, A. A., Douville, K., and Froehner, S. C. (1991). Localization of dystrophin relative to acetylcholine receptor domains in electric tissue and adult and cultured skeletal muscle. *J Cell Biol* 113, 1133-44.
- Shieh, B. H., and Zhu, M. Y. (1996). Regulation of the TRP Ca²⁺ channel by INAD in *Drosophila* photoreceptors. *Neuron* 16, 991-8.
- Sicinski, P., Geng, Y., Ryder-Cook, A. S., Barnard, E. A., Darlison, M. G., and Barnard, P. J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* 244, 1578-80.
- Simske, J. S., Kaech, S. M., Harp, S. A., and Kim, S. K. (1996). LET-23 receptor localization by the cell junction protein Lin-7 during *C. elegans* vulval induction. *Cell* 85, 195-204.
- Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67, 31-40.
- Son, H., Hawkins, R. D., Martin, K., Kiebler, M., Huang, P. L., Fishman, M. C., and Kandel, E. R. (1996). Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell* 87, 1015-1023.
- Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chishti, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Cantley, L. C.

- (1997). Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* 275, 73-77.
- Srinivasan, Y., Elmer, L., Davis, J., Bennett, V., and Angelides, K. (1988). Ankyrin and spectrin associate with voltage-dependent sodium channels in brain. *Nature* 333, 177-80.
- Stevenson, B. R., Siliciano, J. D., Mooseker, M. S., and Goodenough, D. A. (1986). Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol* 103, 755-66.
- Stricker, N. L., Christopherson, K. S., Yi, B. A., Schatz, P. J., Raab, R. W., Dawes, G., Bassett, J., D. E., Bredt, D. S., and Li, M. (1997). PDZ Domain of Neuronal Nitric Oxide Synthase Recognizes Novel C-Terminal Peptide Sequences as Determined by *In Vitro* Selection. *Nature Biotechnology* 15, 336-342.
- Sunada, Y., Bernier, S. M., Kozak, C. A., Yamada, Y., and Campbell, K. P. (1994). Deficiency of merosin in dystrophic dy mice and genetic linkage of laminin M chain gene to dy locus. *J Biol Chem* 269, 13729-32.
- Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y., and Ozawa, E. (1994). Molecular organization at the glycoprotein-complex-binding site of dystrophin. Three dystrophin-associated proteins bind directly to the carboxy-terminal portion of dystrophin. *Eur J Biochem* 220, 283-92.
- Suzuki, A., Yoshida, M., and Ozawa, E. (1995). Mammalian alpha 1- and beta 1-syntrophin bind to the alternative splice-prone region of the dystrophin COOH terminus. *J Cell Biol* 128, 373-81.
- Taglialetela, M., Wible, B. A., Caporaso, R., and Brown, A. M. (1994). Specification of pore properties by the carboxyl terminus of inwardly rectifying K⁺ channels. *Science* 264, 844-7.
- Takao, K., Yoshii, M., Kanda, A., Kokubun, S., and Nukada, T. (1994). A region of the muscarinic-gated atrial K⁺ channel critical for activation by G protein beta gamma subunits. *Neuron* 13, 747-55.

- Tinsley, J., Blake, D., Zuellig., and Davies, K. (1994). Increasing complexity of the dystrophin-associated protein complex. *Proc. Natl. Acad. Sci.* *91*, 8307-8313.
- Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M., and Zuker, C. S. (1997). A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. *Nature* *388*, 243-249.
- Vincent, S. R., and Hope, B. T. (1992). Neurons that say NO. *Trends Neurosci* *15*, 108-13.
- Wagner, K. R., Cohen, J. B., and Huganir, R. L. (1993). The 87K postsynaptic membrane protein from Torpedo is a protein-tyrosine kinase substrate homologous to dystrophin. *Neuron* *10*, 511-22.
- Wallace, B. G. (1992). Mechanism of agrin-induced acetylcholine receptor aggregation. *J Neurobiol* *23*, 592-604.
- Wang, T., Xie, Z., and Lu, B. (1995). Nitric oxide mediates activity-dependent synaptic suppression at developing neuromuscular synapses. *Nature* *374*, 262-6.
- Wang, W. H., and Giebisch, G. (1991). Dual modulation of renal ATP-sensitive K⁺ channel by protein kinases A and C. *Proc Natl Acad Sci U S A* *88*, 9722-5.
- Webster, C., Silberstein, L., Hays, A. P., and Blau, H. M. (1988). Fast muscle fibers are preferentially affected in Duchenne muscular dystrophy. *Cell* *52*, 503-13.
- Wible, B. A., Taglialatela, M., Ficker, E., and Brown, A. M. (1994). Gating of inwardly rectifying K⁺ channels localized to a single negatively charged residue. *Nature* *371*, 246-9.
- Willott, E., Balda, M. S., Fanning, A. S., Jameson, B., Van Itallie, C., and Anderson, J. M. (1993). The tight junction protein ZO-1 is homologous to the *Drosophila* discs-large tumor suppressor protein of septate junctions. *Proc Natl Acad Sci U S A* *90*, 7834-8.

- Wischmeyer, E., and Karschin, A. (1996). Receptor stimulation causes slow inhibition of IRK1 inwardly rectifying K⁺ channels by direct protein kinase A-mediated phosphorylation. *Proc. Natl. Acad. Sci. USA* 93, 5819-5823.
- Woods, D. F., and Bryant, P. J. (1991). The discs-large tumor suppressor gene of *Drosophila* encodes a guanylate kinase homolog localized at septate junctions. *Cell* 66, 451-64.
- Wu, H. H., Williams, C. V., and McLoon, S. C. (1994). Involvement of nitric oxide in the elimination of a transient retinotectal projection in development. *Science* 265, 1593-6.
- Xie, J., Roddy, P., Rife, T. K., Murad, F., and Young, A. P. (1995). Two closely linked but separable promoters for human neuronal nitric oxide synthase gene transcription. *Proc Natl Acad Sci U S A* 92, 1242-6.
- Yang, B., Ibraghimov-Beskrovnaya, O., Moomaw, C. R., Slaughter, C. A., and Campbell, K. P. (1994). Heterogeneity of the 59-kDa dystrophin-associated protein revealed by cDNA cloning and expression. *J Biol Chem* 269, 6040-4.
- Yang, B., Jung, D., Rafael, J. A., Chamberlain, J. S., and Campbell, K. P. (1995). Identification of alpha-syntrophin binding to syntrophin triplet, dystrophin, and utrophin. *J Biol Chem* 270, 4975-8.
- Yang, J., Jan, Y. N., and Jan, L. Y. (1995). Control of rectification and permeation by residues in two distinct domains in an inward rectifier K⁺ channel. *Neuron* 14, 1047-54.
- Zagotta, W. N., Hoshi, T., and Aldrich, R. W. (1990). Restoration of inactivation in mutants of Shaker potassium channels by a peptide derived from ShB. *Science* 250, 568-71.
- Zhang, Z. G., Reif, D., Macdonald, J., Tang, W. X., Kamp, D. K., Gentile, R. J., Shakespeare, W. C., Murray, R. J., and Chopp, M. (1996). ARL 17477, a potent and selective neuronal NOS inhibitor decreases infarct volume after transient middle cerebral artery occlusion in rats. *J Cereb Blood Flow Metab* 16, 599-604.



For reference

Not to be taken
from the room.

