

Involvement of Neuroligins and Associated Proteins in Synapse Development

by

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B.Sc., McGill University, 1999

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2010

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Abstract

Cell adhesion molecules (CAMs) have emerged as important players in synapse development; however, the precise roles of these proteins at newly formed contacts remain unknown. In this thesis, I begin by providing an overview of synaptic structure and development, as well as a review of our current understanding of how key CAMs and associated proteins fit into this framework.

In the second chapter, I demonstrate that members of the postsynaptically localized neuroligin (NL) family of CAMs, including NL1, NL2 and NL3, can trigger the formation of excitatory and inhibitory presynaptic terminals, and that while NL1 is enriched at excitatory contacts, NL2 localizes primarily to inhibitory sites. Neuroligin-mediated enhancement of inhibitory synapse density is blocked by a fusion protein containing the extracellular domain of the presynaptic neuroligin binding partner, neurexin-1 β . Furthermore, overexpression of postsynaptic density-95 (PSD-95), a postsynaptic binding partner of neuroligins, results in a shift of NL2 from inhibitory to excitatory synapses. These findings reveal that multiple neuroligins control the number of inhibitory and excitatory synapses, and that localization of NL2 can be altered by scaffolding proteins.

In the third chapter, I examine the mechanisms by which NL2 and NL3 are recruited to inhibitory and excitatory synapses, respectively. To this end, I assessed the roles of PSD-95 and gephyrin, a postsynaptic scaffolding molecule localized exclusively to inhibitory synapses, in localizing NL2 and NL3. Knockdown of gephyrin results in a shift of NL2 from inhibitory to excitatory synaptic contacts, while knockdown of PSD-95 leads to a shift of NL2 and NL3 from excitatory to inhibitory contacts. Deletion of a discrete region within the C-terminus of NL2 reveals that the intracellular tail is required for the normal synaptic clustering of this protein. Together, these data suggest that intracellular mechanisms are involved in the synaptic targeting of different neuroligin family members.

Overall, these results demonstrate an important role for neuroligins in the development of glutamatergic and GABAergic synapses, and indicate that postsynaptic scaffolding molecules modulate the targeting of neuroligins to distinct postsynaptic compartments. The final chapter of the thesis provides a general discussion relating these findings to other recent advancements in the field.

Table of Contents

Abstract.....	ii
Table of Contents	iii
List of Tables	vi
List of Figures.....	vii
List of Abbreviations	viii
Statement of Co-authorship.....	x
Acknowledgments	xi
1. Introduction.....	1
1.1 History of the Synapse.....	1
1.2 Synaptic Structure and Composition.....	2
1.2.1 Structure and Composition of the NMJ	2
1.2.2 Structure and Composition of Glutamatergic CNS Synapses.....	4
1.2.3 Structure and Composition of GABAergic CNS Synapses	11
1.3 Development of Synapses.....	15
1.3.1 General Stages of Synaptic Development.....	15
1.3.2 Development of the NMJ.....	16
1.3.3 Development of Glutamatergic Synapses.....	18
1.3.4 Development of GABAergic Synapses	23
1.4 Cell Adhesion Molecules in Synaptic Development	26
1.4.1 NCAM.....	26
1.4.2 The Cadherin Adhesion Complex.....	27
1.4.3 SynCAMs	31
1.4.4 LRR Proteins	33
1.4.5 Non-neuronal Regulators of Synaptogenesis.....	36
1.5 Neuroligins and Neurexins.....	39
1.5.1 Structure of Neuroligins and Neurexins	39
1.5.2 Spatiotemporal Distribution of Neuroligins and Neurexins.....	42
1.5.3 Functional Significance of Neuroligin-Neurexin Interaction at Synapses.....	44
1.6 Scaffolding Molecules in Synapse Development	47

1.6.1 The PSD-95 Family	47
1.6.2 The Role of PSD-95 in Synapse Development and Function	49
1.6.3 Other Synaptic Scaffolding Molecules and their Roles in Synaptic Organization	52
1.7 Synapse Balance and Excitatory/Inhibitory Ratio.....	57
1.7.1 The Ratio of Excitatory to Inhibitory Synapses and Neuronal Activity	57
1.7.2 Synaptic Imbalance and Neuroligins in Neuropsychiatric Disorders	57
1.8 Rationale and Hypothesis.....	60
1.9 References	62
2. Neuroligins Mediate Excitatory and Inhibitory Synapse Formation: Involvement of PSD-95 and Neurexin-1β in Neuroligin Induced Synaptic Specificity	86
2.1 Introduction	86
2.2 Materials and Methods.....	88
2.2.1 cDNA Cloning and Mutagenesis	88
2.2.2 Neuronal cell Culture and Transfections	88
2.2.3 Immunocytochemistry	88
2.2.4 Western Blotting and Application of Neurexin Fusion Protein	89
2.2.5 Imaging and Analysis	89
2.2.6 Electrophysiology	90
2.3 Results.....	92
2.3.1 Neuroligins Drive Excitatory and Inhibitory Presynaptic Contact Formation.....	92
2.3.2 NL-Induced Inhibitory Synapse Formation is Mediated by Neurexin-1 β	94
2.3.3 Functional Effects of Neurexin-1 β on Excitatory and Inhibitory Synapses	99
2.3.4 Localization of Endogenous NLs to Excitatory and Inhibitory Synapses.....	100
2.3.5 Manipulation of PSD-95 Levels Regulates Clustering of NLs and Shifts the Distribution of NL2 from Inhibitory to Excitatory Synapses	103
2.4 Discussion	107
2.5 References	110
3. Postsynaptic Scaffolding Molecules Modulate the Localization of Neuroligins.....	113
3.1 Introduction	113
3.2 Methods and Materials.....	117
3.2.1 cDNA Cloning and Mutagenesis	117
3.2.2 Cell Culture and Transfections	118
3.2.3 Antibody Production and Purification	119
3.2.4 Immunocytochemistry	119
3.2.5 Western Blot Analysis	120
3.2.6 Imaging and Analysis	120

3.3 Results.....	122
3.3.1 Distribution of Endogenous NL2 in Cultured Hippocampal Neurons.....	122
3.4.2 Knockdown of Gephyrin or PSD-95 Alters the Proportion of NL2 at Inhibitory and Excitatory Synapses.....	124
3.3.3 The C-terminal Domain of NL2 Modulates its Recruitment to Inhibitory Synapses.....	127
3.3.4 NL3 is Expressed in the Adult Brain and is Localized Mainly at Excitatory Synapses in Cultured Hippocampal Neurons.....	130
3.3.5 Knockdown of PSD-95 or Gephyrin Alters the Proportion of NL3 at Excitatory and Inhibitory Synapses.....	132
3.4 Discussion	134
3.5 References	138
4. General Discussion.....	141
4.1 Summary of Findings	141
4.2 Role of Neuroligin-Neurexin Interaction in Synapse Development and Specificity.....	143
4.2.1 Initiation of Synapse Formation vs. Synapse Maturation vs. Synaptic Plasticity. Which One is it?	143
4.2.2 Which Came First: The Chicken or the Egg? Presynaptic vs. Postsynaptic Mechanisms of Neuroligin-based Synapse Development.....	146
4.2.3 Involvement of Other Cell Adhesion and Scaffolding Molecules	151
4.3 Implications in Disease	154
4.4 Conclusions and Future Directions	156
4.5 References	158
Appendix A. Additional Publications and Presentations	162
A.1 List of Additional Published Works.....	162
A.2 List of Presentations	162
Appendix B. Supplemental Data	164
B.1 Localization of Neuroligin 2 in Various Neuronal Cell Types.....	164
B.1.1 Materials and Methods.....	164
B.1.2 Results.....	165
B.2 Role of ErbB4 in Synapse Formation and Dendritic Branching.....	166
B.2.1 Methods and Materials.....	166
B.2.2. Results.....	167
B.3 References.....	170

List of Tables

Table 1.1. Neuroligin-related genetic mutations and chromosomal abnormalities associated with autism spectrum disorders.	59
Table 4.1. Neuroligin and neurexin splice isoforms and their synaptic binding partners and clustering effects.	148

List of Figures

Fig. 1.1. Organization of synaptic specializations at the NMJ.....	2
Fig. 1.2. General structure and composition of CNS glutamatergic synapses.....	5
Fig. 1.3. Organization of proteins and protein-protein interactions in the PSD.....	6
Fig. 1.4. Morphology of dendritic protrusions, filopodia and spines.....	7
Fig. 1.5. Schematic diagrams illustrating structure of AMPA receptor subunits.....	8
Fig. 1.6. Schematic diagram depicting pertinent features of NMDA neurotransmitter receptors.....	9
Fig. 1.7. Schematic diagram of interactions of CAZ proteins and the resulting network at the active zone.....	10
Fig. 1.8. Structure and diversity of inhibitory ligand-gated ion channels.....	12
Fig. 1.9. Proteins that associate with GABAARs.....	13
Fig. 1.10. Synaptic adhesion molecules function throughout the life of a synapse.....	15
Fig. 1.11. Three models for spinogenesis.....	20
Fig. 1.12. Schematic diagram of the molecular structure of the cadherin superfamily and their cytoplasmic interactors.....	28
Fig. 1.13. Schematic diagram of the postsynaptic organization by LRR proteins.....	34
Fig. 1.14. Structure of neuroligins.....	40
Fig. 1.15. Neuroligin Expression in HEK293 Cells Causes Accumulation of Synapsin in Axons.....	45
Fig. 1.16. Schematic diagram of PDZ proteins.....	48
Fig. 1.17. Diagram of various GRIP1 splice variants.....	56
Fig. 2.1. Members of the NL family drive excitatory and inhibitory synapse formation.....	93
Fig. 2.2. Purification of a soluble fragment of neurexin-1 β	94
Fig. 2.3. NL induced inhibitory synapse formation involves neurexin-1 β	95
Fig. 2.4. A soluble form of neurexin blocks NL-induced inhibitory contacts.....	96
Fig. 2.5. Changes in number of VGAT-positive terminals contacting cells transfected with GFP.....	97
Fig. 2.6. Soluble neurexin-1 β induces clustering of both HA-NL1 and HA-NL2 at sites positive for PSD-95.....	98
Fig. 2.7. Expression of HA-NL1 or HA-NL2 does not alter clustering of PSD-95.....	98
Fig. 2.8. Blocking neurexin-1 β function diminishes NL1 mediated excitatory and inhibitory synaptic function.....	100
Fig. 2.9. Specificity of NL2 antibody.....	101
Fig. 2.10. Endogenous localization of NLs to excitatory and inhibitory synapses.....	102
Fig. 2.11. Localization of endogenous NL1 with excitatory postsynaptic proteins in developing neurons.....	103
Fig. 2.12. Regulation of NL2 and NL3 clustering by PSD-95.....	104
Fig. 2.13. Enhanced PSD-95 expression alters the distribution of endogenous NL2.....	105
Fig. 2.14. Model illustrating the effects of altered balance between NLs and PSD-95 on E/I ratio.....	106
Fig. 3.1. Localization of endogenous NL2 at excitatory and inhibitory synapses.....	123
Fig. 3.2. Knockdown of gephyrin and PSD-95 in COS-7 cells.....	125
Fig. 3.3. Knockdown of gephyrin or PSD-95 in cultured hippocampal neurons.....	125
Fig. 3.4. Knockdown of gephyrin or PSD-95 alters the distribution of NL2 at inhibitory and excitatory synapses.....	126
Fig. 3.5. Schematic representation of C-terminal NL2 deletion constructs.....	128
Fig. 3.6. The C-terminal domain of NL2 modulates its localization at inhibitory synapses.....	129
Fig. 3.7. NL3 is expressed in rat brain.....	131
Fig. 3.8. NL3 is found mainly at excitatory synapses in cultured hippocampal neurons.....	131
Fig. 3.9. Knockdown of PSD-95 or gephyrin alters the synaptic localization of NL3 in cultured neurons.....	133
Fig. 4.1. PSD-95 and gephyrin regulate the targeting of neuroligins.....	153
Fig. B.1. Neuroligin 2 localization in rat hippocampal and cortical cultures.....	165
Fig. B.2. shRNA-mediated knockdown of ErbB4 in GABAergic neurons reduces presynaptic maturation in contacting axonal terminals.....	168
Fig. B.3. Decreased number of primary neurites in inhibitory GABAergic neurons expressing ErbB4 shRNA.....	169

List of Abbreviations

AChE	acetylcholinesterase
AChR	acetylcholine receptor
AKAP150	A-kinase anchoring protein 150 kDa
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionate
AMPAR	AMPA receptor
ASD	autism spectrum disorder
AZ	active zone
BL	basal lamina
bp	base pair
CAM	cell adhesion molecule
CaMKII	calcium/calmodulin-dependent kinase II
CAZ	cytomatrix of the active zone
CC	clathrin-coated vesicle
CNR	cadherin-related neuronal receptor
CNS	central nervous system
DIV	days <i>in vitro</i>
Dlg	discs large
DNA	deoxyribonucleic acid
DV	dense core vesicles
E/I	excitatory/inhibitory
GABA	γ -amino butyric acid
GABAR	GABA receptor
GABARAP	GABAR-associated protein
GAP	GTPase activating proteins
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GKAP	guanylate kinase domain-associated protein
GluR	AMPA receptor subunit
GRIP	glutamate receptor-interacting protein
GST	glutathione S-transferase
HA	hemagglutinin
IRSp53	insulin receptor substrate 53 kDa
KCh	K ⁺ channel
LTD	long-term depression
LTP	long-term potentiation
MAGUIN	membrane-associated guanylate kinase-interacting protein
MAGUK	membrane-associated guanylate kinase
MC	mitochondria
mEPSC	miniature excitatory postsynaptic current
mGluR	metabotropic glutamate receptor
mIPSC	miniature inhibitory postsynaptic current
MT	microtubule
NL1-4	neuroligin 1-4
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
NMJ	neuromuscular junction
nNOS	neuronal nitric oxide synthase
NR	NMDA receptor subunit

PCR	polymerase chain reaction
PDZ	PSD-95, Discs large, Zona occludens 1
PDZb	PDZ-binding domain
PFA	paraformaldehyde
PKA	protein kinase A
PKC	protein kinase C
PLIC1	protein that links IAP to the cytoskeleton
PMSF	phenylmethylsulfonyl fluoride
PP2B	protein phosphatase 2B
PRR	proline-rich region
PSD	postsynaptic density
PSD-93	postsynaptic density protein 93 kDa
PSD-95	postsynaptic density-95
PTV	Piccolo-bassoon Transport Vesicles
RACK1	receptor for activated C-kinase
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SAP102	synapse-associated protein 102 kDa
Shank	SH3 and ankyrin repeat-containing protein
SPAR	spine-associated RapGAP
SSCAM	synaptic scaffolding molecule
SV	synaptic vesicle
SYN	synaptophysin
TARP	transmembrane AMPA receptor regulatory protein
TBST	tris-buffered saline tween-20
TM	transmembrane
TSP	thrombospondin
VGAT	vesicular GABA transporter
VGluT1	vesicular glutamate transporter 1
ZO-1	zona occludens protein 1

Statement of Co-authorship

Chapter 2: I designed, performed and analyzed all immunocytochemical experiments in primary cultured neurons, with the exception of Figure 2.13, for which Kun Huang assisted with data analysis, and Figure 2.11, for which Kimberly Gerrow performed the experiments. I also generated and purified NXN-FC fusion protein used in Figures 2.2-2.6 and 2.8. In addition, I performed experiments to test the specificity of the anti-NL2 antibody used in Figures 2.9, 2.10 and 2.13. I designed and generated the following constructs: GFP-NL2, HA-NL2, GFP-NL3, HA-NL3. Untagged NL2 and NL3 sequences used for generating the tagged versions of these proteins, as well as NXN-FC and FC-IgG constructs, were obtained from the laboratory of Thomas Sudhof. HA-NL1 was generously provided by Peter Scheiffele. GFP-NL1 and PSD-95-GFP constructs were previously generated in our laboratory. Electrophysiological experiments were performed by Nadège Chéry and Tak Pan Wong in the laboratory of Yu Tian Wang. Laboratory technician, Esther Yu, prepared the dissociated primary cultured neurons used in this study. I performed approximately 50% of the manuscript preparation, including writing and figure preparation. The remaining 50% was prepared by Alaa El-Husseini.

Chapter 3: I designed, performed and analyzed immunocytochemical experiments for Figures 3.1 and 3.6. With the assistance of Rongwen Li, I designed, performed and analyzed immunocytochemical experiments for Figures 3.3, 3.4, 3.8 and 3.9. I performed the assessment of siRNA knockdown efficiency in heterologous cells (Figure 3.2). With the assistance of Rongwen Li, I performed experiments to test the specificity of anti-NL3 antibody (Figure 3.7). I designed and generated NL2 deletion constructs used in Figure 3.6, as well as the gephyrin and PSD siRNA constructs (and corresponding control constructs) used in this study, and the construct used for generation of anti-NL3 antibody. CFP-gephyrin was generously provided by the laboratory of Ann-Marie Craig. PSD-GFP was previously designed and generated in our laboratory. Technician Cindy Jiang generated and purified GST-NL3 fusion protein for generation of anti-NL3 antibody. This antibody was purified by Rongwen Li, Rujun Kang and Hakima Moukhles. Laboratory technician, Esther Yu, assisted in the preparation of dissociated primary cultured neurons used in this study. I performed approximately 75% of the manuscript preparation, including writing and figure preparation. The remaining 25% was prepared by Shernaz Bamji.

Acknowledgments

I would like to thank, first and foremost, the late Dr. Alaa El-Husseini for his guidance and support during the majority of my Ph.D. studies. He was a wonderful teacher, mentor and friend, and his passion for science and love for life are greatly missed. The work described in this thesis is dedicated to his memory.

I can never fully express my appreciation for the support and assistance provided by my “foster” supervisor, Dr. Shernaz Bamji. I deeply thank her for her generosity in taking me into her lab during a tough time, making a potentially difficult transition go as smoothly as humanly possible and truly treating me as one of her own. I hope she realizes how important her dedication and positive spirit were in guiding me through the last leg of my Ph.D. I would also like to thank my Ph.D. supervisory committee, consisting of Drs. Vanessa Auld, Yu Tian Wang and Anthony Phillips, for their guidance and support throughout my degree.

I also send thanks to a myriad of fellow students, colleagues and lab mates for their indispensable presence throughout my Ph.D. I would like to give special mention to Drs. Rujun Kang, Kun Huang, Rochelle Hines, Catherine Campbell, Dove Keith, Kimberly Gerrow, Oliver Prange as well as Mytyl Aiga, Fergil Mills, Sun Yu, Stefano Brigidi and Eileen Yoshida. As well, I thank my dear friends, Pamela Arstikaitis, Dr. Marie-France Lisé, Dr. Austen Milnerwood, Soroush Moghaddam, Dr. Lucia Tapia and Ainsley Coquinco for keeping me well entertained. I also want to thank Drs. Andy Shih and Herman Fernandes for SAT and the procrastination train.

A big thanks, as well, to my “outside world” friends. In particular, Moris Markarian, Vijay Viswanathan and Calvin Penfound are the very best friends a guy could ever hope for.

Although far away, I could constantly feel the love of my family, especially my parents and sister. Thank you so much, Mom, Dad and Julie for all your support and advice over the years. And thanks, to my father in particular, for putting up with all the stress caused by my last-minute nature.

Last, but certainly not least, I want to thank my beautiful wife, Seanna, for her love and support, especially during some of my more cranky moments while writing this thesis. Her sweet and kind nature, as well as her extreme patience, has helped me more than she knows.

During my dissertation, I was supported by the Michael Smith Foundation for Health Research (MSFHR) and the Canadian Institutes for Health Research (CIHR). I thank these agencies for their generous financial support.

1. Introduction

1.1 History of the Synapse

At the beginning of the twentieth century, using light microscopy, Santiago Ramon y Cajal and his student, Fernando Tello, began to examine the intricacies of the neuromuscular junction (NMJ), the interface between motor neuron and muscle fiber (described in (Ramon y Cajal, 1928)). Over the pursuing century, scientists continued to exploit the large size, relative simplicity and accessibility of the NMJ (reviewed in (Sanes and Lichtman, 1999)), further building upon the initial foundation erected by Ramon y Cajal and his students. Indeed, in the 1930s, Dale and colleagues, using the vertebrate NMJ, conducted the first sets of experiments to demonstrate chemically-based synaptic transmission. Shortly after this, it was discovered that the chemical responsible for neurotransmission at these synapses was acetylcholine, and that this neurotransmitter was released from membranous vesicular structures in a quantal manner (described in (Katz, 1966)). In the 1950s, with the advent of electron microscopy and electrophysiological techniques, the detailed structure and function of the NMJ began to unfold. As molecular cloning tools became available in later decades, some of the protein constituents of the NMJ were identified in a number of species, beginning with the receptor for acetylcholine (AChR), the first neurotransmitter receptor to be cloned (reviewed in (Duclert and Changeux, 1995)). In recent years, advanced imaging techniques, *in vitro* and *in vivo*, have permitted a great deal of insight into the various steps of NMJ development.

As we have begun to probe the central nervous system (CNS) in recent decades, it has become clear that some of the fundamental discoveries made regarding the NMJ hold true for the structure, function and development of synapses in general. Thus, the work done over the last hundred years on the NMJ has paved the way for elucidating the inner function of CNS synapses.

1.2 Synaptic Structure and Composition

1.2.1 Structure and Composition of the NMJ

The NMJ consists of highly specialized structures from three different cell types which together form a unit that can rapidly and reliably respond to incoming neuronal stimuli (Fig. 1.1; reviewed in (Ogata, 1988; Sanes and Lichtman, 1999)). On the postsynaptic side, once mononucleated myoblasts have migrated to appropriate target sites, they align in rows and fuse to form multinucleated structures called myotubes. Upon maturation of myotubes, myonuclei migrate from central positions to the periphery of the cylinder, whereby the structure becomes known as a muscle fibre.

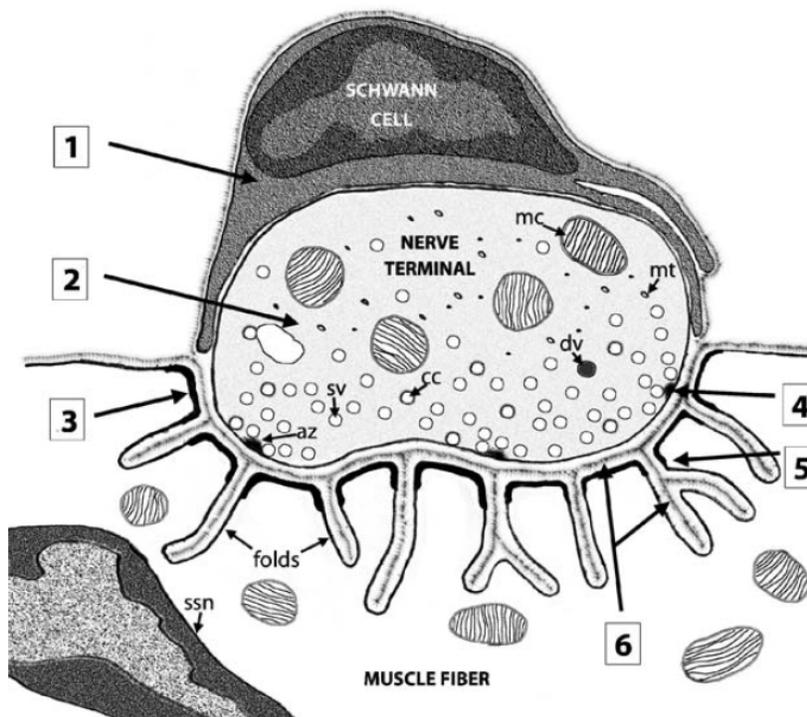


Fig. 1.1. Organization of synaptic specializations at the NMJ. Neuromuscular synapses comprise primary and secondary specializations in three cells. (1-3) Primary specializations include a terminal Schwann cell that caps rather than wraps the motor nerve; varicose terminal branches in the motor axon, which accumulate mitochondria (MC) and synaptic vesicles (SV); and an AChR-rich postsynaptic endplate, shown as thickened portions of the muscle membrane. (4-6) Secondary specializations which appear as NMJs mature postnatally enhance neurotransmission and signal transduction. In the nerve, neurosecretory “active zones” (AZ) form along the junctional

surface (4), and the distribution of intra-terminal organelles becomes asymmetric with respect to the synaptic cleft. In the muscle, the formation of secondary synaptic clefts creates folds in the postsynaptic membrane (5); endplate associated membrane proteins are distributed asymmetrically in postsynaptic membranes, with AChRs concentrated in the primary (thickened) postsynaptic membrane, and voltage-gated sodium channels (not shown) concentrated in secondary postsynaptic membranes (6). Importantly, these specializations are accurately co-localized across a biochemically-specialized portion of the muscle fiber basal lamina (BL). Synaptic BLs contain specialized components, which organize synaptic specializations in all three cells. MT, microtubules; DV, dense core vesicle; CC, clathrin-coated vesicle; sub-synaptic myonuclei. Reproduced with permission from Patton, 2003.

Presynaptically, motor neuron axons reach their target muscle tissue as individual myoblasts are fusing to form myotubes, where synaptic transmission between motor terminal and myotube begins shortly thereafter. The motor terminal is highly enriched for proteins and organelles that enable it to efficiently release the neurotransmitter, acetylcholine, into the synaptic cleft. Among these specialized constituents are acetylcholine-filled synaptic vesicles, which concentrate at very high densities at spatially restricted, morphologically defined sites, known as active zones (AZs). These juxtamembrane regions contain a vast array of scaffolding and other molecules, and are themselves present in multiple numbers within each axonal terminal. In addition, each synaptic vesicle contains hundreds of proteins required for the synthesis and packaging of neurotransmitter into vesicles, as well as the fusion of vesicles with the plasma membrane of the motor terminal (reviewed in (Sudhof, 1995; Calakos and Scheller, 1996)). Additional organelles present in large numbers in motor axon terminals are mitochondria, which sustain the energy-demanding processes of neurotransmitter synthesis and release.

Directly beneath the motor terminal, shallow depressions in the postsynaptic membrane are formed, in which can be found deep, evenly spaced invaginations (Fig. 1.1). The openings of these folds are directly apposed to presynaptic active zones, suitably positioned to receive neurotransmitter released from the nerve terminal. The molecular composition of these folds in the postsynaptic membrane varies in different regions. At the crests of the folds and running partway down into them, high concentrations of AChRs can be found (Salpeter and Loring, 1985; Flucher and Daniels, 1989), while molecules such as sodium channels and cell adhesion molecules are found in high concentrations at the base of the folds (Covault and Sanes, 1986; Flucher and Daniels, 1989). It has been proposed that the polarized nature of these folds contributes to the efficiency of synaptic transmission (Wood and Slater, 1997). Cytoskeletal proteins that are likely involved in both formation and polarization of the folds are also heterogeneously distributed within them (reviewed in (Sanes and Lichtman, 1999)).

The third type of cell present at the NMJ is the Schwann cell. At the pre-terminal portion of the motor axon, Schwann cells generate a myelin sheath, which aids in the propagation of the action potential down the axon. The motor axon terminal is physically insulated by a structurally and functionally distinct subtype of Schwann cell, which may also provide factors required for normal NMJ development (reviewed in (Mirsky and Jessen, 1996)).

Ensheathing the cells of the NMJ and extending into muscle fiber folds is a well-defined basal lamina. This complex, highly-organized network of proteins is composed of extracellular matrix proteins such as collagen, laminin and heparan sulfate proteoglycans, and serves as a docking point for agrin, a protein required for postsynaptic clustering of AChRs (Sanes and Lichtman, 1999). The basal lamina of the NMJ is thought to be important for the normal formation of NMJ synapses during development, as well as for synaptic transmission in adult animals (Patton, 2003).

1.2.2 Structure and Composition of Glutamatergic CNS Synapses

Glutamatergic synapses constitute the major class of excitatory synaptic contact in the adult mammalian brain. As is the case with NMJs, glutamatergic synapses consist of an axonal terminal closely associated with a postsynaptic membrane that is densely packed with neurotransmitter receptors and other molecules required for synaptic organization and transduction of neuronal signals. However, in the case of central glutamatergic synapses, the postsynaptic membrane is found on the dendritic process of a neighbouring neuron, and is characterized by the presence of a postsynaptic density (PSD), a specialized membrane microdomain apposed specifically to the active zone of glutamate-releasing presynaptic terminals (Fig. 1.2). The PSD consists of a large protein matrix containing a plethora of proteins, among which are neurotransmitter receptors, scaffolding proteins and molecules important for signal transduction and cytoskeletal organization. These proteins are arranged into an intricate meshwork associated with the cytoskeleton, and ensure that the postsynaptic compartment is competent to receive input from its associated axonal terminal and transduce these signals to other subcellular regions (Fig. 1.3) (Kennedy, 1997; Kornau et al., 1997; Rao et al., 1998; Kim and Huganir, 1999; Lee and Sheng, 2000).

The majority of excitatory synapses in the CNS are made onto discrete morphological structures which house PSDs, referred to as spines. These structures, first described by Ramon y Cajal in 1888 (Ramon y Cajal, 1888), are small, actin-rich protrusions located along the length of dendrites. Dendritic spines exist in a variety of morphologies, ranging from short stubs emerging from the dendritic shaft (stubby), to thin, more elongated structures with a bulbous head (thin), to prominent protrusions with a long, thin neck, and a large, mushroom-shaped head (mushroom;

Fig. 1.4) (Peters and Kaiserman-Abramof, 1970). In the adult brain, the most commonly found type of spines are of the mushroom-shaped class (Miller and Peters, 1981). A great deal of evidence indicates that spines play an important role in synapse development and function. For instance, synapse formation has been shown to occur on spines (Passafaro et al., 2003), and the size of spines correlates with postsynaptic AMPA receptor (AMPA) clustering (Nusser et al., 1998; Takumi et al., 1999; Nusser, 2000; Matsuzaki et al., 2001), which in turn is linked to postsynaptic current strength (Malenka and Nicoll, 1999; Malinow et al., 2000). In addition, long-term potentiation (LTP), a form of synaptic plasticity believed to be correlated with learning and memory, is associated with the appearance of dendritic spines, as well as the accumulation of AMPARs at these sites (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Shi et al., 1999; Toni et al., 1999). Other than providing a spatially isolated subcellular location for the formation of synapses, spines also allow for biochemical compartmentalization. It has been found, for example, that the geometry of spine necks affects the flow of calcium from spines into the dendritic shaft, and is therefore an important factor for calcium signaling through NMDA receptors (NMDARs) in spines (Noguchi et al., 2005).

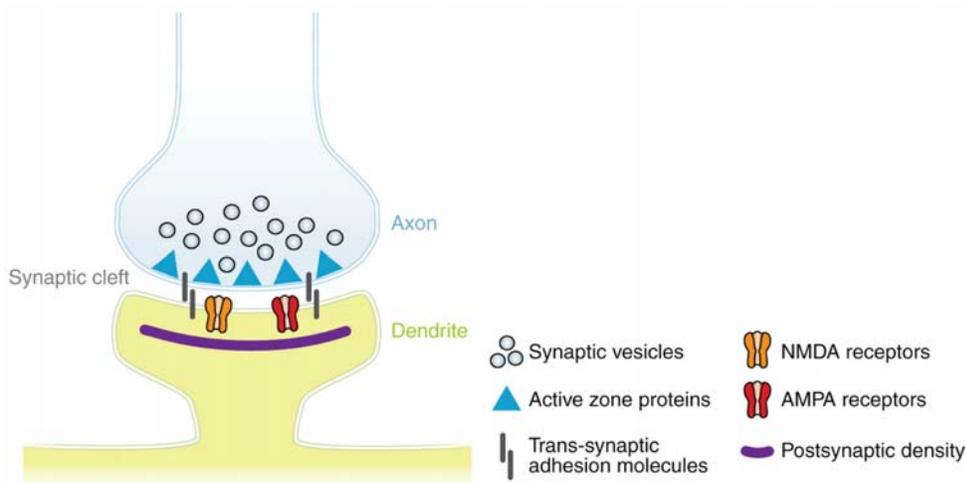


Fig. 1.2. General structure and composition of CNS glutamatergic synapses. Central glutamatergic synapses are comprised of several major protein classes. Synaptic vesicles containing the neurotransmitter glutamate cycle at the active zone, which is composed

of many kinds of proteins including presynaptic scaffolding proteins. The presynaptic terminal is separated from the postsynaptic dendrite by the synaptic cleft; a number of trans-synaptic adhesion molecules span this cleft, providing a molecular connection between the pre- and postsynaptic membranes. Glutamate receptors, including AMPA and NMDA receptors, are found in the postsynaptic membrane, where they are associated with a large number of scaffolding and signaling proteins that together comprise the postsynaptic density. Although glutamatergic synapses are usually located on dendritic spines in the adult, these synapses are more often found on dendritic shafts and filopodia in the CNS during the initial stages of synaptogenesis. Adapted with permission from McAllister, 2007.

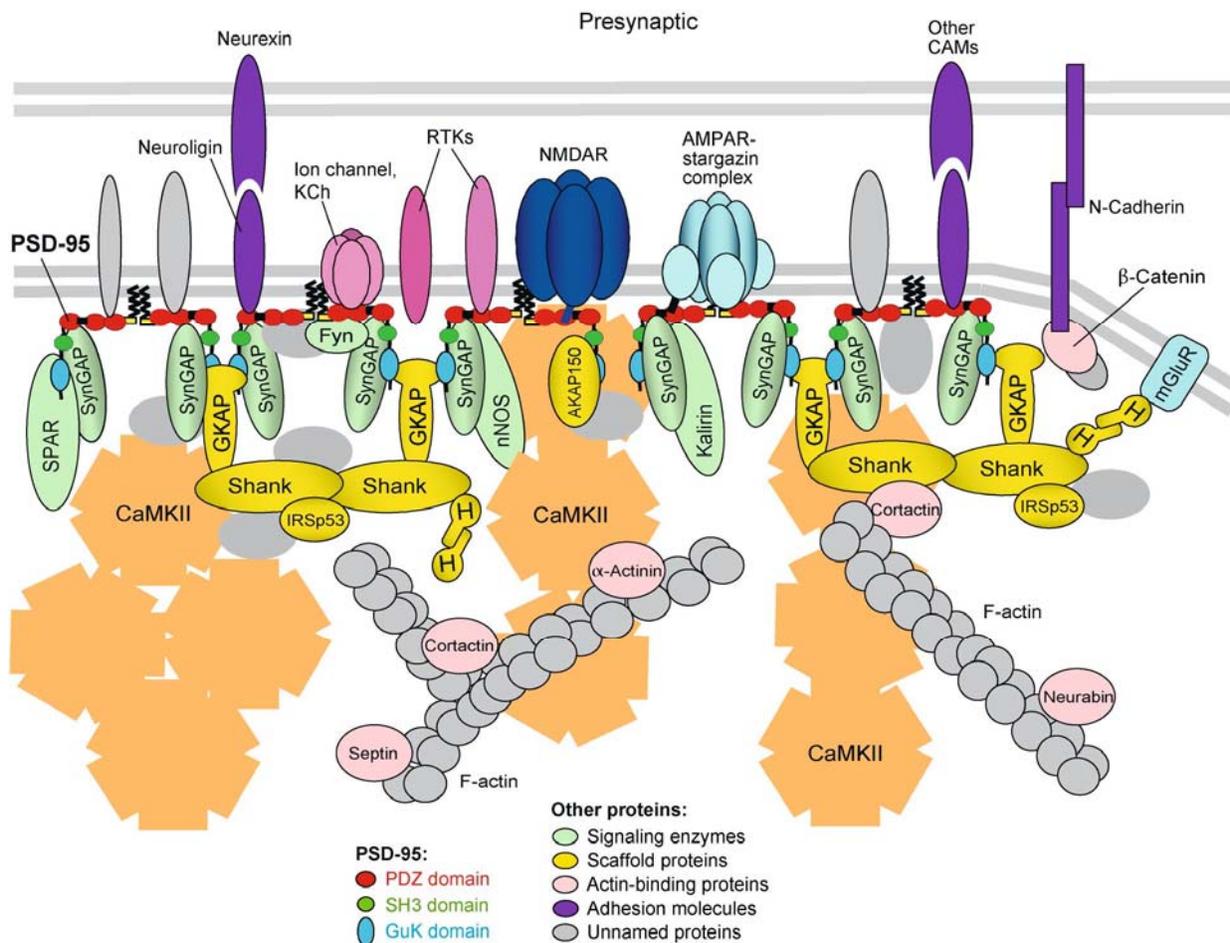


Fig. 1.3. Organization of proteins and protein-protein interactions in the PSD. Schematic diagram of the network of proteins in the PSD, with edge of PSD depicted at right. Only major families and certain classes of PSD proteins are shown [in approximate stoichiometric ratio and scaled to molecular size, if known]. Contacts between proteins indicate an established interaction between them. Domain structure is shown only for PSD-95 (PDZ domain, SH3 domain, GuK domain). Other scaffold proteins are colored yellow; signaling enzymes, green; actin binding proteins, pink. CaMKII (calcium/calmodulin-dependent protein kinase II) is depicted as a dodecamer. Unnamed proteins signify the many other PSD proteins that are not illustrated in this diagram. Abbreviations: AKAP150, A-kinase anchoring protein 150 kDa; CAM, cell adhesion molecule; Fyn, a Src family tyrosine kinase; GKAP, guanylate kinase-associated protein; H, Homer; IRSp53, insulin receptor substrate 53 kDa; KCh, K⁻ channel; mGluR, metabotropic glutamate receptor; nNOS, neuronal nitric oxide synthase; RTK, receptor tyrosine kinases (e.g., ErbB4, TrkB); SPAR, spine-associated RapGAP. Reproduced with permission from Sheng and Hoogenraad, 2007.

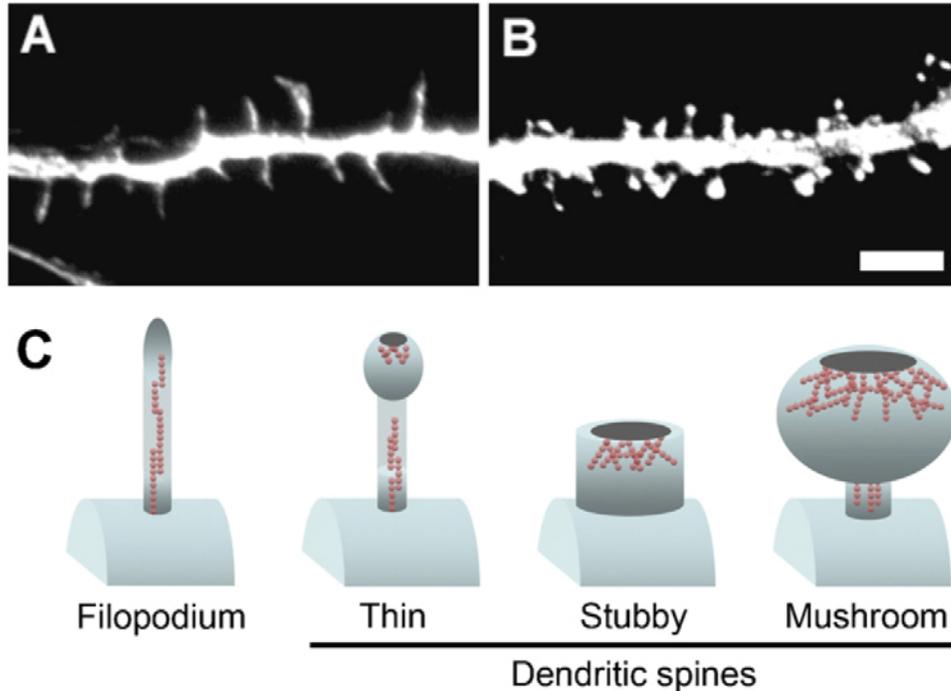
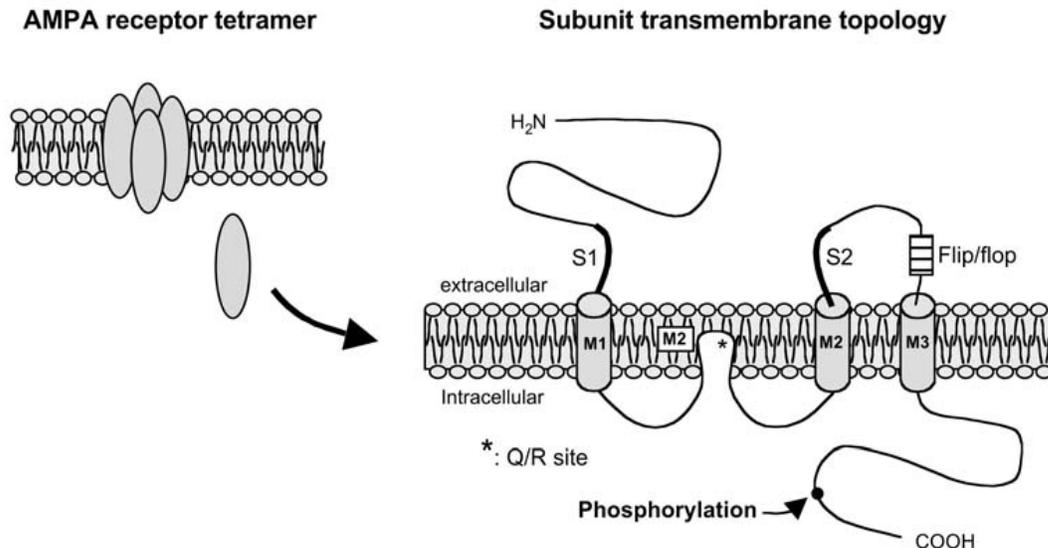


Fig. 1.4. Morphology of dendritic protrusions, filopodia and spines. Dendrites of GFP-transfected hippocampal neurons cultured for 7 days (A) and 21 days (B). At the immature stage (7 days), dendritic protrusions are very thin and long; these protrusions are called dendritic filopodia. In contrast, at the mature stage (21 days), dendrites are covered by dendritic spines, which commonly have an expanded head and a narrow neck. Scale bar, 5 μm . (C) Schematic representation of morphologies of a filopodium and three types of dendritic spine: thin type, stubby type and mushroom type. Gray disks represent the PSD structure and chains of red circles represent F-actin. Reproduced with permission from Sekino et al., 2007.

Activation of AMPARs by release of glutamate provides the majority of excitatory depolarization in the brain (reviewed in (Bredt and Nicoll, 2003)). AMPARs assemble in various combinations of four subunits, GluR1-4 (Fig. 1.5). The particular subunit stoichiometry of each receptor is a key factor in determining its synaptic function. Alternative splicing in the ligand-binding domains of AMPAR subunits, for example, accelerates desensitization and resensitization of AMPARs in a developmentally regulated manner (Jonas, 2000; Palmer et al., 2005; Valente and Nishikura, 2005). In addition, RNA editing within the pore loop of GluR2 confers the ability of this subunit to render AMPARs calcium-impermeable (Seeburg et al., 1998; Palmer et al., 2005). NMDARs, on the other hand, consist of seven distinct subunits, NR1, NR2A-D and NR3A-B (Fig. 1.6) (Anne Stephenson et al., 2008). For each subunit, alternative splicing results in the generation of several subunit subtypes (reviewed in (Dingledine et al., 1999)). NMDARs are generally thought to be tetrameric in nature, including two NR1 and two

NR2 subunits. Indeed, the incorporation of at least one NR1 and one NR2 subunit is required for the formation of functional receptors in experiments involving expression of recombinant receptors (reviewed in (Dingledine et al., 1999)). In some cases, however, NR3 has been observed to assemble into tetrameric complexes along with NR1 and NR2 subunits (Sasaki et al., 2002).



Amino acid sequences in the C terminus:

GluR1: EFCYKSRS**ES**KRMKG**F**CLIPQ**Q**SINEAIR**T**STLPR**NS**GAGASGGGGSGENGRVVSQDFPKSMQ**S**IPCM**SH**SSGMPLGATGL
 Ser831 Ser845

GluR2: EFCYKSRA**E**AKRMKVAKNPQ**NI**NPSS**Q**NSQN**F**ATYKEGY**N**VY**G**IES**V**KI
 Ser863 Tyr876 Ser880

GluR3: EFCYKSRA**E**SKRMKLT**K**NTQ**N**FKP**A**PATNTQ**NY**ATYREGY**N**VY**G**T**E**SVKI

GluR4: EFCYKSRA**E**AKRMK**L**TFSEAIR**N**KAR**L**SITGS**V**GENGRVLYPDCPKAVHTGT**A**IRQSSGLAVIASD**L**P
 Thr830 Ser842

Fig. 1.5. Schematic diagrams illustrating structure of AMPA receptor subunits. AMPA receptors exist as heteromeric tetramers through the cell membrane (top left panel). The transmembrane topology of a single subunit (top right panel) includes the flip/flop alternatively spliced exon, the two ligand-binding domains (S1 and S2) in the extracellular domain, and a Q/R site (glutamine: specific for GluR1, GluR3, and GluR4; arginine: specific for GluR2). The cytoplasmic C-terminus provides amino acid sites for phosphorylation and interactions with submembraneous proteins. Phosphorylation of GluR1 subunits occurs at serine residues 831 and 845, GluR2 subunits at serines 863 and 880 and tyrosine 876, and GluR4 at threonine 830 and serine 842. Bold and underlined amino acids represent phosphorylation sites. Reproduced with permission from (Wang et al., 2005).

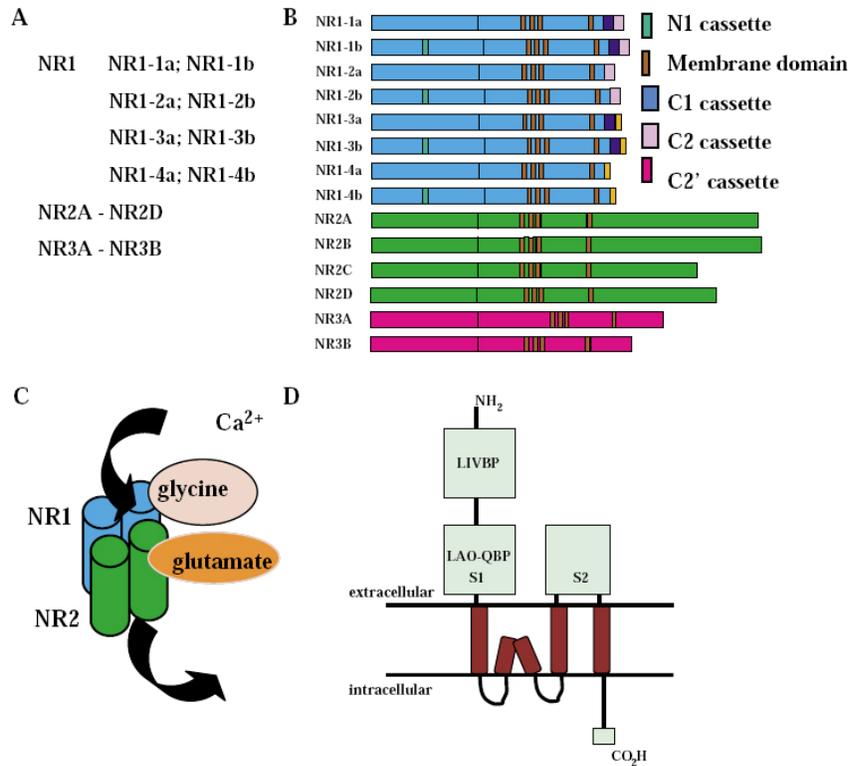


Fig. 1.6. Schematic diagram depicting pertinent features of NMDA neurotransmitter receptors. (A) Summarizes NMDA receptor subunits; (B) schematic of the NMDA receptor NR1, NR2 and NR3 subunits, including all the NR1 splice variants; (C) shows a model of a tetrameric NMDA receptor comprised of two NR1 and two NR2 subunits; (D) shows the domain structure that is shared between both NR1 and NR2 subunits where the S1 and S2 domains form the glycine (NR1) or L-glutamate (NR2) binding sites, the Ca^{2+} -permeable pore is formed by the re-entrant membrane domain M2. Reproduced with permission from (Anne Stephenson et al., 2008).

The part of the presynaptic terminal directly apposed to the PSD is composed of an intricate matrix of electron-dense, proteinaceous material, the cytomatrix of the active zone (CAZ). This web-like grid, consisting of a regular array of cone-shaped particles extending approximately 50 nm into the cytoplasm, is tightly associated with the actin cytoskeleton and provides a docking platform for synaptic vesicle fusion required for release of neurotransmitter from the presynaptic cell (Phillips et al., 2001). Although the low solubility of the CAZ has made identification of its components difficult, a good deal of progress has been made on this front. A myriad of proteins from a number of classes have been identified as key components of the CAZ (Fig. 1.7). These include CAMs, scaffolding proteins, synaptic vesicle fusion machinery, as well as voltage-gated calcium channels which act as calcium sensors for triggering the release of neurotransmitter. Another important class of molecules found in excitatory presynaptic terminals comprises proteins required for the synthesis and vesicular packaging of glutamate, key processes in glutamatergic neurotransmission. The synthesis of glutamate from glutamine occurs directly in the cytoplasm of presynaptic terminals and is thought to be catalyzed by the enzyme, phosphate-activated glutaminase (Takamori, 2006). Vesicular uptake of glutamate then occurs, driven by a

proton-dependent electrochemical gradient across the vesicular membrane, mediated by members of the vesicular glutamate transporter (VGluT) protein family (Bellocchio et al., 2000; Takamori et al., 2000). To date, three VGluT isoforms have been identified in mammals. The proteins are members of the type I phosphatase transporter family, and are evolutionarily conserved among a wide range of organisms, including *C. elegans*, *Drosophila*, zebrafish and *Xenopus laevis* (reviewed in (Takamori, 2006)). Despite efforts to identify biochemical differences between the three mammalian VGluT isoforms, no such differences have yet been established. These proteins show highly distinct distributions in the adult rodent brain. VGluT1 and VGluT2 are found at excitatory synapses in broad, yet mostly exclusive sets of brain regions. Surprisingly, however, VGluT3, while sparsely found throughout the brain, often colocalizes with markers for non-glutamate neurotransmitters, including GABA and serotonin (Fremeau et al., 2002; Schafer et al., 2002; Herzog et al., 2004).

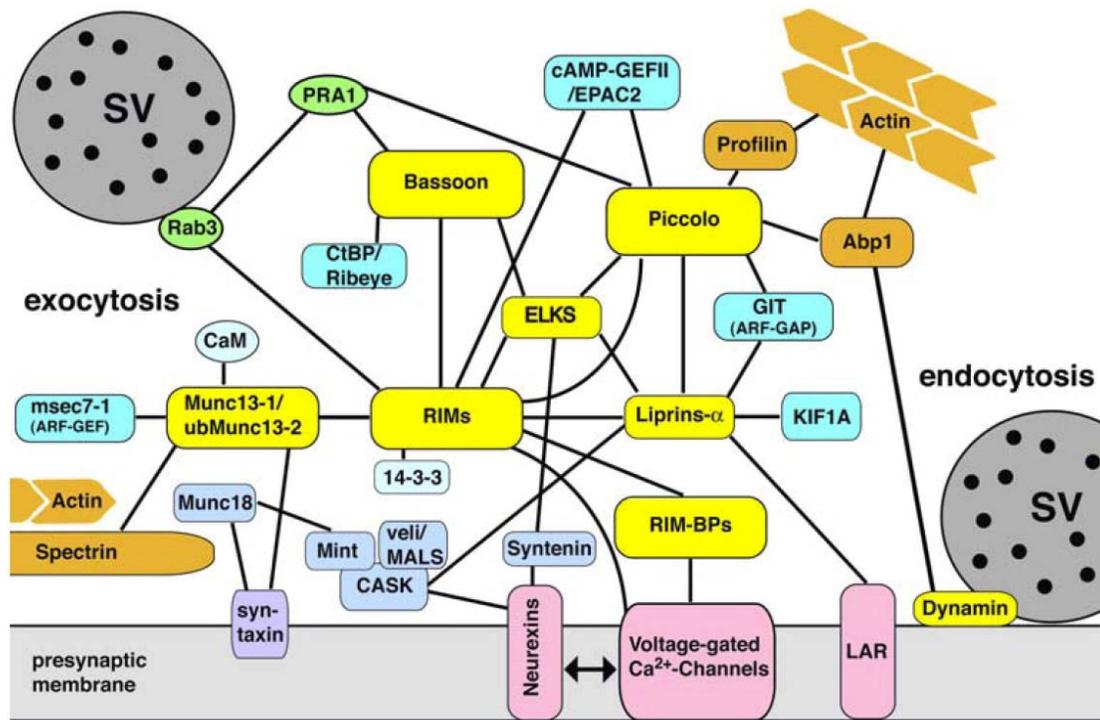


Fig. 1.7. Schematic diagram of interactions of CAZ proteins and the resulting network at the active zone. The CAZ contains a myriad of proteins from diverse groups, all of which form a complex network critical for a properly functioning presynaptic terminal. Adapted with permission from (Schoch and Gundelfinger, 2006).

1.2.3 Structure and Composition of GABAergic CNS Synapses

The majority of inhibitory synapses in the mature mammalian CNS consist of γ -aminobutyric acid (GABA)-releasing axonal terminals apposed to dendritic shafts containing GABA receptors (GABARs) and other specialized proteins required for GABAergic transmission. Unlike glutamatergic synapses, the majority of which are formed on dendritic spines, GABAergic contacts generally develop directly on the shafts of target dendrites. GABA-releasing terminals also commonly contact the soma and axon initial segments, placing them close to the sites of action potential initiation, where they can exert a proportionately stronger influence on neuronal firing.

GABARs fall into three distinct classes: GABA_A, GABA_B and GABA_C receptors. The ionotropic GABARs, GABA_A and GABA_C receptors, are members of the ligand-gated superfamily of ion channels, which also includes glycine receptors, nicotinic acetylcholine receptors, serotonin type 3 receptors, and the 5-hydroxytryptamine type 3 (5-HT₃) receptor (Connolly and Wafford, 2004). The receptor subunits within this superfamily assemble into hetero-pentameric channels and are thought to share common structural features that differ substantially in their C-terminal topology from glutamate receptor subunits (Unwin, 1998). GABA_A and GABA_C receptors can be differentiated on a structural and pharmacological basis. GABA_ARs consist of 19 different subunits, α 1–6, β 1–3, γ 1–3, δ , ρ 1–3, ϵ , π and θ , each encoded by a distinct gene (Fig. 1.8). Alternative splicing is known to generate additional subunit complexity (Whiting et al., 1999). The wide variety of GABA_AR subunits expressed in the CNS produces a great diversity of receptor subtypes, allowing the fine-tuning of GABAergic transmission within different brain regions and neuronal populations. GABA_CRs, which are expressed only in the retina within the CNS, have a simpler subunit makeup, being composed of only three different ρ subunits that are thought to assemble into homomeric or heteromeric pentamers (Fig. 1.8) (Cutting et al., 1991; Lukasiewicz, 1996; Moss and Smart, 2001).

An increasing body of research has begun to focus on the postsynaptic constituents of GABAergic synapses; however, yeast-two hybrid has proven relatively ineffective for teasing out molecular interactions involving components of this synapse type, and, due to the lack of a PSD-like structure, a well-established biochemical fractionation method for selective isolation of

inhibitory postsynaptic compartments has not been achieved to date. As such, the molecular composition of GABAergic synapses is not as well defined as that of glutamatergic synaptic contacts. While no distinct morphological structures exist to delineate postsynaptic sites of inhibitory contact, extensive protein networks have been implicated in the development and function of this synapse type (Fig. 1.9). As is the case for glutamatergic synapses, the proteins found at GABAergic synapses encompass a number of classes, including CAMs, scaffolding proteins and signaling molecules. Some of the specific proteins found in this network are common to both synapse types, including cadherins, GRIP1 and neuroligin family members, and are involved in cell-cell contact, subcellular targeting of neurotransmitter receptors and other components and modulation of inhibitory synaptic transmission (Yamagata et al., 2003; Hoogenraad et al., 2005; Levinson et al., 2005; Li et al., 2005). However, molecules specific to inhibitory synapses, such as the cytoplasmic multidomain protein, gephyrin, have also been identified (Kneussel and Loeblich, 2007). Molecules important for inhibitory synaptic development and function are described in detail below.

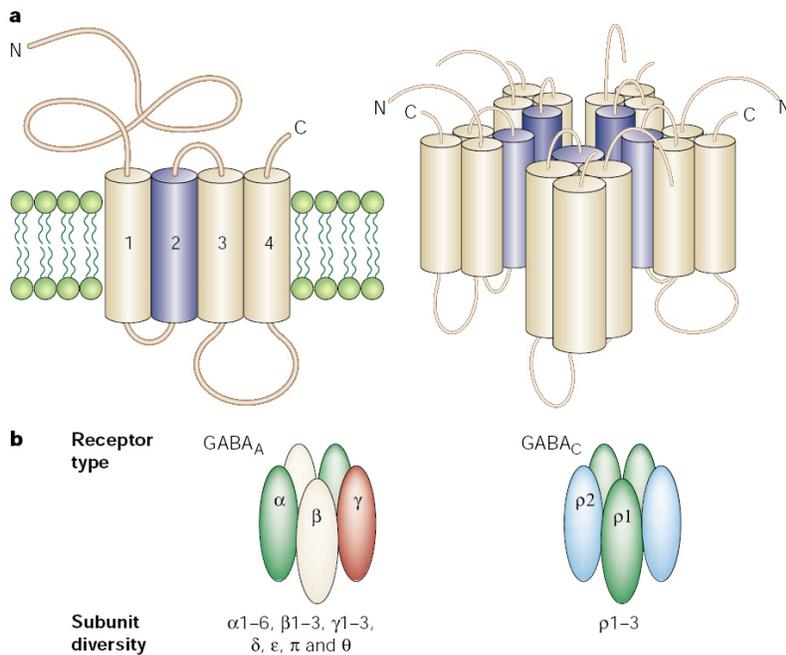


Fig. 1.8. Structure and diversity of inhibitory ligand-gated ion channels. (A) Proposed structure of a ligand-gated ion channel. Receptor subunits contain four hydrophobic transmembrane (TM) domains. TM2 is believed to form the lining of the ion channel. The large amino-terminal domain is located extracellularly and is believed to incorporate neurotransmitter and some modulator binding sites. The intracellular domain between TM3 and TM4 comprises ~10% of the mass of each subunit. This domain is the most divergent part of individual receptor subunits and contains numerous consensus sites for the action of both

serine/threonine and tyrosine protein kinases. (B) Proposed pentameric structures of GABA_A and GABA_C receptor subtypes. Most GABA_A receptors are believed to be composed of α , β and γ subunits in the ratio of 2:2:1. The δ , ϵ and θ subunits can replace the γ subunit in some receptor subtypes. GABA_C receptors are constructed from $\rho 1$ – $\rho 3$ subunits either as heteromeric or homomeric pentameric assemblies. Their stoichiometry is unknown. Adapted with permission from Moss and Smart, 2001.

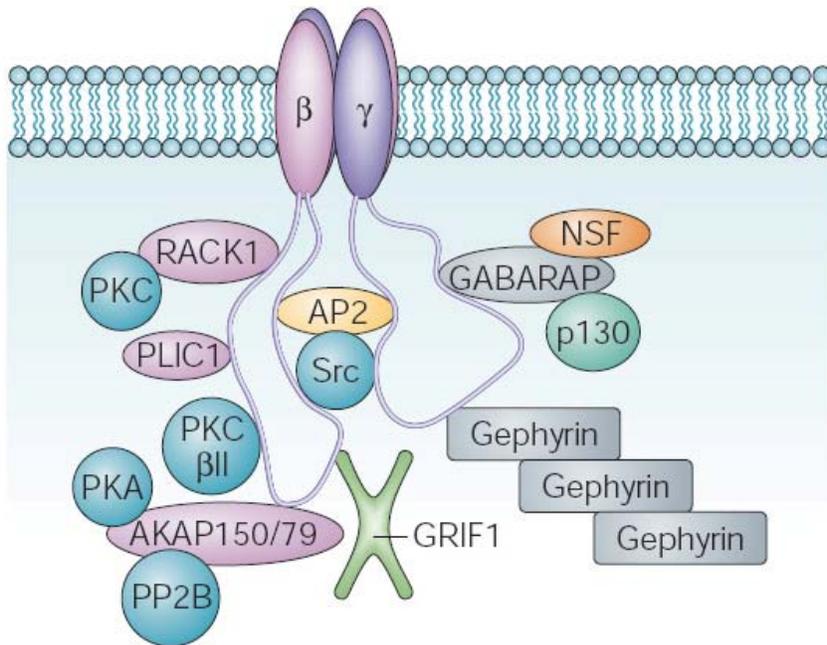


Fig. 1.9. Proteins that associate with GABA_ARs. Multi-domain scaffolding proteins: the trimeric scaffolding protein gephyrin for γ -subunits of GABA_ARs. NSF (*N*-ethylmaleimide-sensitive factor) associates with γ -subunits through its interaction with GABARAP (GABAR associated protein). AP2 (adaptor complex): interaction with the β - and γ -subunits. AKAPs (A-kinase-anchoring proteins): AKAP150/79 directly interacts with the β -subunit. Proteins that interact with PKC (protein kinase C) (PKC chaperones): RACK1 (receptor for activated C-kinase) binds to β -subunits.

Cytoskeleton-interacting proteins: GABARAP interacts with γ -subunits, and PLIC1 (protein that links IAP to the cytoskeleton) interacts with β -subunits. Motor proteins: GRIF1, a hypothesized motor protein, interacts directly with β -subunits. Ubiquitination pathway proteins: PLIC1, which regulates ubiquitination, interacts directly with β -subunits. PKA, protein kinase A; PP2B, protein phosphatase 2B. Adapted with permission from Collingridge et al., 2004.

Presynaptically, GABAergic terminals are morphologically indistinguishable from glutamatergic boutons. These terminal types also share much of the same fundamental molecular machinery, including many of the molecules used to regulate calcium-dependent synaptic vesicle fusion and recycling. Some differences, however, do exist in this system. The SNARE complex is involved in vesicle exocytosis at presynaptic terminals; while the protein, SNAP-25, functions as an important member of this complex at glutamatergic synapses, GABAergic hippocampal neurons lack SNAP-25 and are resistant to botulinum toxins A and E, which are known to cleave this protein and inhibit neurotransmitter release (Ashton and Dolly, 1988; Verderio et al., 2004).

GABAergic terminals also differ from terminals of other synapse types in that they contain the biosynthetic and transport molecules specific for the synthesis and packaging of GABA into synaptic vesicles. Biosynthesis of GABA is achieved through the decarboxylation of glutamate via L-glutamic acid decarboxylase (GAD). Unlike other neurotransmitter systems, GABA synthesis is mediated by two different enzymes, GAD65 and GAD67 (Bu et al., 1992). Each

enzyme, however, shows distinct localization patterns, regulatory properties and functions. GAD65 is concentrated in presynaptic terminals and produces GABA for use in neurotransmission; GAD67, on the other hand, is found diffusely throughout the cytoplasm and is thought to synthesize GABA that participates in a number of neurotransmission-independent processes, including synaptogenesis and protection after neuronal injury (Pinal and Tobin, 1998).

Once GABA synthesis occurs, it must be packaged into synaptic vesicles via active transport from the cytosol. The transporter responsible for translocation of GABA into synaptic vesicles was first identified in *C. elegans shrinker* mutants deficient in the *unc-47* gene (McIntire et al., 1993). Based on this finding, two independent studies using either positional cloning of *unc-47* or prediction of a putative *unc-47* sequence based on sequencing of the *C. elegans* genome, later identified a mammalian homolog, designated VGAT (vesicular GABA transporter) or VIAAT (vesicular inhibitory amino acid transporter) (McIntire et al., 1997; Sagne et al., 1997). Several distant homologs have subsequently been identified in a number of other animals, fungi and plants (reviewed in (Gasnier, 2000)). VGAT is believed to enable vesicular packaging of GABA by catalyzing the exchange of one proton for one neutral amino acid (Hell et al., 1990; McIntire et al., 1997).

1.3 Development of Synapses

1.3.1 General Stages of Synaptic Development

Synapse development is a highly orchestrated process requiring bidirectional communication between pre- and postsynaptic membranes (Fig. 1.10) (Bourne and Harris, 2007). During the initial stages of this process, synaptic partners must seek each other out among near-overwhelming populations of surrounding neurites, initiate contact with one another, and begin the intricate process of building a synapse with properly matched and fully functional pre- and postsynaptic elements. As CAMs are transmembrane proteins that can mediate either homo- or heterophilic binding across the synaptic cleft, they are thought to play important roles in the initial contact between axon and dendrite (Sperry, 1963; Lardi-Studler and Fritschy, 2007). Cadherins, for instance, which constitute a large family of homophilic CAMs that cluster early at synaptic sites and are involved primarily in early stages of synapse development (Benson and Tanaka, 1998; Togashi et al., 2002; Bozdagi et al., 2004; Jontes et al., 2004), are ideally suited for this role.

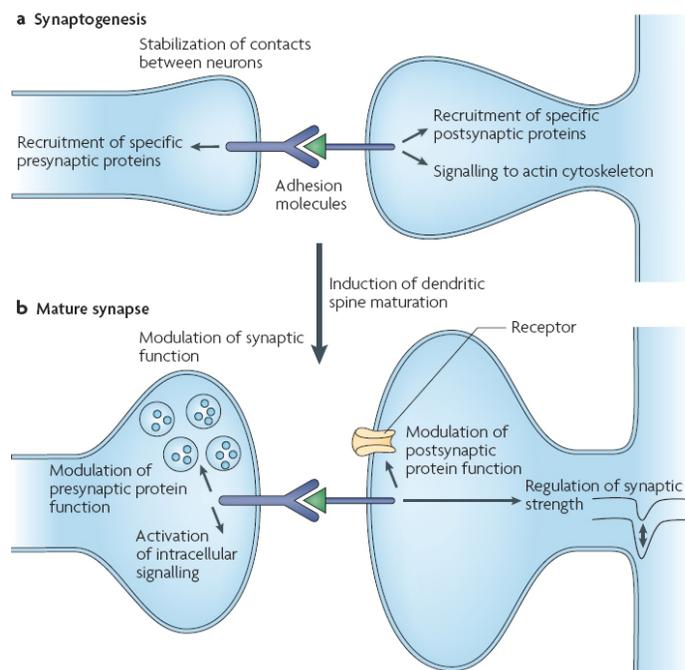


Fig. 1.10. Synaptic adhesion molecules function throughout the life of a synapse.

(A) At the nascent synaptic site, synaptic adhesion molecules stabilize the initial contact between axons and dendrites. Clustering and binding of adhesion proteins can lead to the recruitment of synaptic proteins via specific cytoplasmic or extracellular domains on these molecules, including PDZ-binding domains. Interactions between adhesion molecules can also lead to the activation of intracellular signaling events that can drive synapse maturation. In particular, signaling to the actin cytoskeleton can lead to the induction of dendritic spine formation. (B) In the mature or maturing synapse, synaptic adhesion molecules can interact with channels and other synaptic proteins to modulate their function, either by direct interaction with these proteins or through

the activation of intracellular signaling events. In addition, synaptic adhesion proteins can regulate synaptic plasticity. Reproduced with permission from (Dalva et al., 2007).

Following the establishment of points of contact between axon and dendrite, cells must recruit the appropriate pre- and postsynaptic proteins required for further synapse development, and ultimately, those needed for synaptic activity. This includes a wide array of proteins, ranging from presynaptic vesicle release machinery in the presynaptic terminal, to neurotransmitter receptors on the postsynaptic side, to additional cell adhesion and scaffolding molecules on both sides of the synapse.

As synapse development progresses, further steps are required to obtain fully functional contacts capable of undergoing normal synaptic activity. This maturation process involves further recruitment of synaptic proteins, including additional CAMs and scaffolding proteins, which serve to stabilize the contact site. Other molecules that accumulate at this time include those that constitute the functional machinery of a fully developed synapse, such as neurotransmitter receptors and intracellular signaling proteins.

During later stages of brain development, refining of neural networks occurs in part from removal of pre-existing synaptic contacts (reviewed in (Goda and Davis, 2003; Waites et al., 2005)). This elimination process can even occur in adult brain. In a study by Trachtenberg et al., *in vivo* imaging of mouse barrel cortex in sensory-deprived animals revealed the presence of synapses on retracting spines that were eventually eliminated (Trachtenberg et al., 2002). Another important role for synapse elimination is in the case of synapse mismatching. Although association of a particular presynaptic terminal with a postsynaptic partner containing an inappropriate complement of neurotransmitter receptors occurs rarely, mismatched synaptic pairs that remain stable for several days can be observed (Lardi-Studler and Fritschy, 2007).

1.3.2 Development of the NMJ

The current model for describing NMJ development centers around “reciprocal induction” (reviewed in (Goda and Davis, 2003)). The earliest event that can be observed at the NMJ occurs at a time that coincides with the arrival of the motor neuron, and involves patterned reorganization of AChRs within the muscle fiber, from a diffuse distribution throughout the postsynaptic membrane, to concentrating in small clusters in the central portion of the muscle. This process is referred to as pre patterning, stemming from evidence that formation of these

initial AChR “hot spots” does not require either the presence of motor neurons or motor neuron-derived signals. For instance, a study examining mice deficient for DNA topoisomerase IIb, in which motor axons fail to contact skeletal muscles, found that AChRs still formed clusters in the central region of muscle fibers (Yang et al., 2000). Also, in embryonic skeletal muscle lacking motor neuron innervation, AChR clusters are observed in the central region. However, the lack of motor axons results in an expanded zone of AChR expression, suggesting that neurally-derived signals refine the pre patterning process (Yang et al., 2001).

During the next stage of mammalian NMJ development, arrival of the motor axon growth cone induces further differentiation of the postsynaptic specialization, including further clustering and stabilization of AChRs, as well as preferential AChR expression from nuclei most proximal to the site of nascent contact formation (McMahan, 1990; Hall and Sanes, 1993; Bowe and Fallon, 1995). The heparan sulfate protein, agrin, initially isolated from Torpedo electric organ, was shown to be sufficient for the aggregation of AChRs, as well as other components of the postsynaptic membrane, the cytoskeleton and the extracellular matrix (Nitkin et al., 1987; Wallace, 1989; McMahan, 1990; Tsen et al., 1995). Several other presynaptically released factors were identified as being potentially responsible for the clustering of AChRs in the postsynaptic membrane, however only agrin appears to play an important role *in vivo* (Sanes and Lichtman, 2001). Indeed, AChR cluster size and density are significantly reduced in agrin-deficient mice (Gautam et al., 1996). Furthermore, transfection of denervated muscle or ectopic regions of innervated muscle with recombinant neuronal agrin results in the formation of protein clusters and morphological features characteristic of normal NMJ postsynaptic sites (Cohen et al., 1997; Meier et al., 1997). In addition, while agrin is expressed in both motor neurons and muscle, the alternatively spliced version of agrin, z⁺ agrin, which is expressed exclusively in neurons, is 1000-fold more potent in AChR clustering assays than its isoforms expressed in muscle (Hoch et al., 1994; Gesemann et al., 1995). In an elegant set of experiments, Burgess and colleagues showed that chimeric mice that had agrin-deficient muscle fibers innervated by neurons expressing z⁺ agrin had apparently normal NMJ synapses (Burgess et al., 1999). Thus, many lines of evidence exist to support a role for agrin as a key motor neuron-derived organizer of the postsynaptic apparatus.

The next steps following clustering of AChRs and other related proteins in the postsynaptic membrane involve both morphological rearrangement of the muscle fiber, as well as progression of the motor axon growth cone into a more mature endplate structure. On the postsynaptic side, formation of the membrane folds occurs at this time, and proteins within the muscle fiber membrane become polarized within the folds. Presynaptically, active zones begin to form, and accumulation of neurotransmitter-filled synaptic vesicles occurs in the side of the motor terminal facing the muscle fiber. Differentiation of the presynaptic terminal at NMJs appears to require retrograde signaling from muscle. In mice mutant for agrin, intramuscular nerve branching is reduced, and motor axon terminals are poorly differentiated (Gautam et al., 1996). Moreover, the finding that muscle-derived z- agrin is dispensable for normal NMJ development, while z+ agrin secreted from motor terminals is necessary for this process, further boosts the idea that agrin acts indirectly on presynaptic maturation through its direct effects on muscle differentiation (Burgess et al., 1999). Also, in MuSK knockout mice, presynaptic maturation occurs abnormally, with motor nerve endings acquiring a pre-terminal region-like appearance (DeChiara et al., 1996). As MuSK expression is absent in motor neurons (Valenzuela et al., 1995), these defects in presynaptic maturation are most likely a result of lack of retrograde signaling due to defective muscle development. Further evidence comes from studies of chimeric mice, in which wild-type mice were transplanted with muscle from MuSK- or rapsyn-deficient animals (Nguyen et al., 2000). In these animals, some presynaptic differentiation did occur, but terminals contacting implanted muscle ultimately matured to a much lesser extent. Together, these data suggest that normal maturation of motor axon terminals depends on signals derived from the muscle that they innervate.

1.3.3 Development of Glutamatergic Synapses

Historically, the majority of research on synapse development in the brain has focused on excitatory synaptic contacts; as such, a great deal is known about the molecular mechanisms responsible for their proper development and function. The bulk of excitatory synapse formation in the developing brain occurs during the second and third weeks of postnatal development (Eroglu, 2009). As described above, in the case of NMJ development, axons generally play a dominant role, sending out foraging growth cones that actively seek out relatively stationary postsynaptic muscle fiber targets (although it is now known that filopodia-like structures, called

myopodia, are involved in guiding synapse assembly at the NMJ; (Ritzenthaler et al., 2000; Misgeld et al., 2002)). It has become apparent, however, that dendrites play a more active role in the initial process of synapse formation in the CNS. For instance, dendrites are known to extend motile structures resembling axonal growth cones, on which synapse formation occurs (Saito et al., 1997; Fiala et al., 1998; Cline, 2001). In addition, numerous elongated, motile protrusions, ranging from 2-20 μm in length, decorate the dendritic shaft during early stages of neuronal development in a variety of organisms, including *Xenopus laevis*, zebrafish and mammalian species (Dailey and Smith, 1996; Ziv and Smith, 1996; Wu et al., 1999; Jontes et al., 2000; Jontes and Smith, 2000; Yuste and Bonhoeffer, 2004). These tapered membraneous extensions, called filopodia, show high levels of motility during periods of active synaptogenesis (Dailey and Smith, 1996; Ziv and Smith, 1996; Marrs et al., 2001). Combined with various experiments showing that synapses often form at the tips of these structures (Vaughn et al., 1974; Saito et al., 1997; Fiala et al., 1998; Niell et al., 2004), these observations have led to the idea that dendritic filopodia function in synaptogenesis in an exploratory capacity, probing the environment for nearby axons, and guiding them to the dendrite (Fig. 1.11). Thus, it appears that dendrites are highly dynamic structures that may actively contribute to synapse formation. In addition to their roles as active synaptic foragers, it has been proposed that dendritic filopodia also serve as direct structural precursors for spines (Dailey and Smith, 1996; Ziv and Smith, 1996). In support of this, as animals mature, a progressive loss of filopodia is accompanied by a concomitant increase in spine formation (Zuo et al., 2005a; Zuo et al., 2005b). In addition, dendritic protrusions present at early developmental stages become shorter and less motile as maturation progresses (Portera-Cailliau et al., 2003). However, this concept is a controversial one, with no direct evidence that spines emerge from the morphological transformation of filopodia. Indeed, in at least some circumstances, spine growth can precede synapse formation (Knott et al., 2006). One alternative mechanism appears to involve the complete retraction of filopodia to leave shaft synapses, which subsequently develop into spines through a process of outgrowth (Fiala et al., 1998). Conversely, spines and filopodia could represent two completely independent processes. It is unclear which model best explains the nature of spine formation. However, it is likely that multiple mechanisms of spinogenesis co-exist, with spine development proceeding differently under various conditions.

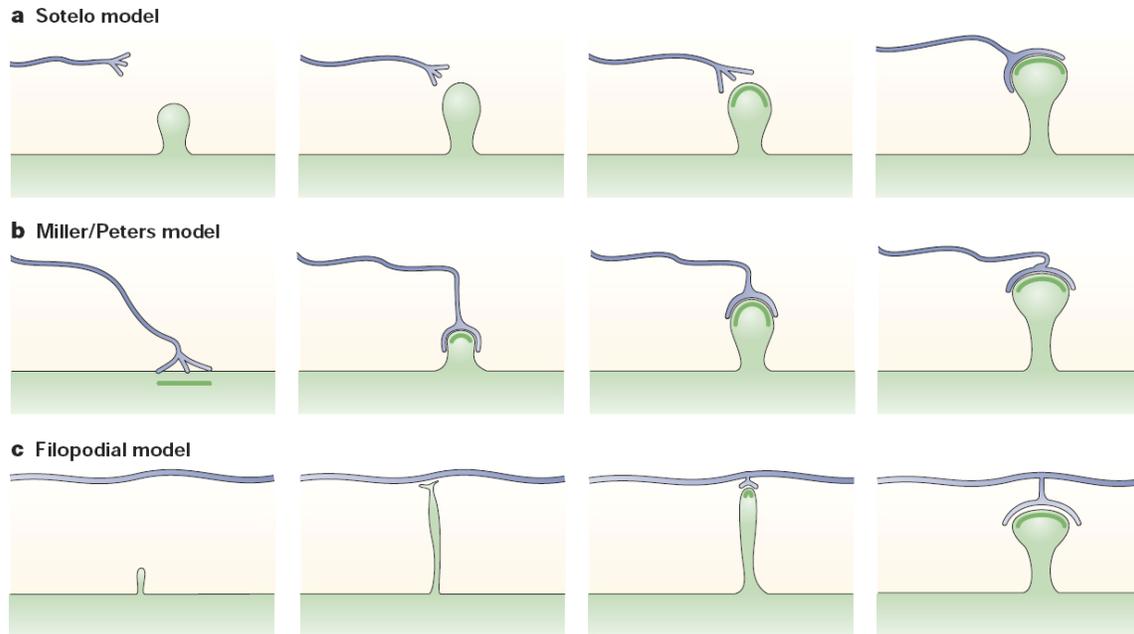


Fig. 1.11. Three models for spinogenesis. This diagram illustrates the essential features of the three models of spinogenesis. In the Sotelo model (a), spines emerge independently of the axonal terminal. In the Miller/Peters model (b), the terminal actually induces the formation of the spine. Finally, in the filopodial model (c), a dendritic filopodium captures an axonal terminal and becomes a spine. Reproduced with permission from (Yuste and Bonhoeffer, 2004).

A broad set of proteins from many different classes have been implicated in spine morphology and dynamics. Cell surface proteins have been shown to exert modulatory effects on spine morphology, including EphB receptors and their ephrin-B ligands, neuroligins and neuroligins, cadherins and members of the immunoglobulin superfamily of CAMs (Ethell and Pasquale, 2005). In addition, scaffolding molecules such as PSD-95 and Shank mediate the formation of spines (Migaud et al., 1998; El-Husseini et al., 2000a; Sala et al., 2001). Intracellular signaling pathways mediated by several members of the Rho GTPase family, as well as actin-binding proteins such as Arp1/3, cortactin and drebrin, also alter spine morphology and dynamics (Hering and Sheng, 2001; Takahashi et al., 2003; Carlisle and Kennedy, 2005), tying these processes to the actin cytoskeleton.

On the presynaptic side, AZ assembly begins early in synapse development, shortly after initial contact between pre- and postsynaptic membranes. Evidence suggests that the active zone assembly can occur as early as 1-2 hours after initial contact (Friedman et al., 2000; Antonova et al., 2001; Colicos et al., 2001; Okabe et al., 2001), generating a need for rapid recruitment of

synaptic proteins. One possible mechanism which may be able to support such a demand could involve the transport of packets containing a number of proteins to sites of nascent contact, allowing simultaneously delivery of multiple synaptic components. This is indeed the case for active zone and synaptic vesicle proteins (reviewed in (McAllister, 2007)). Piccolo and bassoon, two of the earliest mammalian active zone proteins to arrive at nascent presynaptic sites, are transported to synapses, along with N-cadherin and proteins involved in synaptic vesicle exocytosis, in so-called piccolo transport vesicles (PTVs), dense-core vesicles that rapidly shuttle along axons (Zhai et al., 2001; Shapira et al., 2003). In addition, various synaptic vesicle-associated proteins are transported to presynaptic terminals in highly mobile, morphologically heterogeneous organelles, referred to as synaptic transport vesicles (STVs; (Kraszewski et al., 1995; Dai and Peng, 1996; Nakata et al., 1998; Ahmari et al., 2000; Kaether et al., 2000; Sabo et al., 2006)). Transport of protein packets to the synapse is consistent with evidence suggesting the CAZ may be constructed in a gradual manner through incorporation of unitary building blocks, as indicated by observations of polyhedral cages associated with synaptic vesicles at active zone sites (Zampighi et al., 2008).

A number of CAMs have come to the forefront as key players in the development of excitatory synapses. An important role for CAMs has been identified at every step of excitatory synapse development, ranging from initial formation and stabilization of these contacts, to long-term stability, maturation, and synaptic activity (Washbourne et al., 2004; Gerrow and El-Husseini, 2006; Ko and Kim, 2007). Neurofascin186, for instance, has been implicated in the initiation of contact formation. In the cerebellum, a gradient of neurofascin186 along the axon initial segment of Purkinje cells has been shown to provide a directional guide for innervation by basket cell axons, thus participating in the proper matching of pre- and postsynaptic processes (Ango et al., 2004). In *C. elegans*, UNC-40 and SYG-1 have been associated with active zone assembly. These CAMs act as guidepost proteins, specifying distinct domains of plasma membrane where active zone components will eventually accumulate (Shen et al., 2004; Colon-Ramos et al., 2007). Postsynaptically, CAMs such as NCAM, neuroligins, SynCAMs, cadherins, LRR family proteins and secreted non-neuronal factors have been implicated in excitatory synapse formation. These proteins will be discussed in detail in later sections.

Scaffolding proteins are thought to form the architectural framework on both sides of the synapse. Scaffolding proteins found early at developing presynaptic sites include the vertebrate-specific proteins, Piccolo and Bassoon. Piccolo, while not required for formation of glutamatergic synapses, is important for the regulation of synaptic vesicle exocytosis, and therefore, synaptic activity (Leal-Ortiz et al., 2008). Bassoon, on the other hand, is required for normal AZ formation and synaptic function (reviewed in (Schoch and Gundelfinger, 2006)). Among the postsynaptic scaffolding molecules which have been shown to be critical for normal excitatory synapse development and function are PSD-95, GKAP, Shank and GRIP1, all of which are multidomain proteins thought to crosslink various components of the PSD, including neurotransmitter receptors, signal transduction components and CAMs (Srivastava and Ziff, 1999; Okabe, 2007). The roles of these proteins in synapse development are also discussed in detail below.

Following the recruitment of scaffolding proteins and other molecules required for establishing the initial framework of the synapse, stepwise accumulation of excitatory neurotransmitter receptors occurs. NMDARs are the first to be recruited to nascent synapses. At this stage, synapses are functionally silent at resting membrane potential, due to the lack of AMPARs as well as Mg^{2+} block of NMDARs. As synapse maturation progresses, this silent stage is followed by the appearance of AMPARs, which are the main source of excitatory current in mature neuronal circuits (Bredt and Nicoll, 2003). Synaptic recruitment of NMDARs during synaptogenesis has been reported as occurring in both a modular fashion and by gradual accumulation of protein (Washbourne et al., 2002; Bresler et al., 2004). PSD-95 has been suggested to play a role in targeting of NMDARs to synapses, based on detection of direct PDZ-mediated interactions between these proteins (Niethammer et al., 1996). However, recent reports suggest that binding to PSD-95 may not be a key mechanism for synaptic recruitment of NMDARs *in vivo*. For instance, clustering of NMDARs is unaffected by mutations in the cytoplasmic tails of NMDAR subunits, by interfering peptides that disrupt PSD-95 clustering, or by mutation of PSD-95 in mice (Migaud et al., 1998; Sprengel et al., 1998; Passafaro et al., 1999). Recruitment of AMPARs to the synapse appears to occur by two distinct mechanisms. One mechanism involves bidirectional shuttling of receptors between the cell surface and internal endosomal pools, and relies on proteins involved in exo- and endocytosis (Malinow and

Malenka, 2002; Song and Huganir, 2002; Brecht and Nicoll, 2003; Collingridge et al., 2004). AMPARs are also known to be inserted first into extrasynaptic regions of the dendritic plasma membrane, and subsequently clustered at synaptic sites through lateral diffusion, representing a second mechanism for synaptic insertion of these neurotransmitter receptors (Groc et al., 2004; Adesnik et al., 2005; Ashby et al., 2006). In addition to these mechanisms of AMPAR recruitment being important for the transition of synapses from silent to active during synaptic development, insertion of AMPARs into the synaptic membrane is also thought to underlie changes in synaptic plasticity, such as those that occur during long-term potentiation (LTP) and long-term depression (LTD). A number of PDZ domain-containing postsynaptic scaffolding proteins have been implicated in the synaptic delivery of AMPARs, including protein kinase C-interacting protein (PICK), glutamate receptor binding protein (GRIP/ABP) and synapse-associated protein 97 (SAP-97) (Kim and Sheng, 2004). Furthermore, through interactions with the adaptor protein, stargazin, PSD-95 indirectly regulates the synaptic targeting of AMPARs (Chen et al., 2000; Schnell et al., 2002; Bats et al., 2007). Thus, the recruitment of neurotransmitter receptors to excitatory synaptic sites is a dynamic and highly regulated process.

1.3.4 Development of GABAergic Synapses

GABAergic synapse development shares the same basic steps as excitatory synapses, however a number of key differences exist. In the hippocampus, for example, GABAergic contacts on pyramidal neurons form early on in development, before the appearance of glutamatergic synapses (Hennou et al., 2002). However, at this time, GABAergic synapses mediate excitatory postsynaptic responses, due to differences in the Cl⁻ gradient compared to later stages of development (Ben-Ari, 2002).

At the molecular level, less is understood about the development of inhibitory as compared to excitatory synapses; despite this, several molecules have been well established as involved in the formation and maturation of inhibitory synaptic contacts. As is the case for the development of excitatory synapses, CAMs have been implicated in various stages of inhibitory synapse development. The dystrophin glycoprotein complex (DGC) was among the first adhesive macromolecules identified at GABAergic synapses. This multimolecular complex consists of an extracellular α -dystroglycan (DG) subunit that binds to ECM protein, as well as a

transmembrane β -DG component that anchors α -DG to the membrane (Sunada et al., 1994; Yoshida et al., 1994). Intracellular components of the DGC include dystrophin and utrophin, and bind F-actin, linking the extracellular matrix to the actin cytoskeleton (de Arruda et al., 1990; Suzuki et al., 1994; Jung et al., 1995). The DGC has been well studied with respect to its role at the skeletal neuromuscular junction, where it is required for the maturation and maintenance of this structure (Higginson and Winder, 2005; Barresi and Campbell, 2006). However, recent studies have shown members of the DGC to be expressed at CNS synapses as well (Moore et al., 2002; Montanaro and Carbonetto, 2003). A study by Levi et al. (Levi et al., 2002) shows that although the DGC is not essential for GABAergic synaptogenesis, it is likely to function in modulating inhibitory synapses or conferring specialized properties to a subset of them. DGC components localize to inhibitory but not excitatory synapses, where they are coupled to presynaptic neuroligins (Levi et al., 2002; Li et al., 2005). Moreover, S-SCAM, β -DG and NL2 are partially co-localized at inhibitory synaptic sites in rat hippocampal neurons. Indeed, these proteins have been shown to form a tripartite complex *in vitro* (Sumita et al., 2006), suggesting that dystroglycans may act as modulators of the neuroligin-neurexin complex at inhibitory sites. Other CAMs implicated in inhibitory synapse development include L1-CAMs, protocadherins and NL2 (Bliss et al., 2000; Saghatelian et al., 2004; Chih et al., 2005; Weiner et al., 2005). Disruption of the activities of these proteins results in decreased numbers of inhibitory synapses, as well as deficient postsynaptic inhibitory currents.

Proteins involved in the recruitment of GABA_ARs have, for the most part, eluded detection. However, some advances in this area have been made in recent years. The inhibitory synaptic multidomain protein, gephyrin, for example, associates with a number of proteins at inhibitory contacts, including GABA and glycine receptors, tubulin, microfilaments and key regulators of microfilament dynamics (Kirsch et al., 1991; Kneussel and Betz, 2000; Kneussel, 2002; Giesemann et al., 2003; Harvey et al., 2004). As such, gephyrin is believed to link inhibitory neurotransmitter receptors with microtubules and the actin cytoskeleton, and evidence indeed shows that gephyrin is involved in the synaptic localization of both GABA and glycine receptors (Kneussel et al., 1999b; Kneussel et al., 2001; Levi et al., 2004). GRIP1, a multi-PDZ scaffolding protein well-known to bind multiple AMPAR subunits at glutamatergic synapses, has recently been identified at GABAergic contacts (Dong et al., 1997; Dong et al., 1999; Charych et

al., 2004; Li et al., 2005). In intact brain, the GRIP1 splice isoforms, GRIP1a/b, GRIP1c 4-7, GRIP1d and GRIP1e 4-7, have all been found to localize at both excitatory and inhibitory synaptic sites (Charych et al., 2004; Li et al., 2005; Charych et al., 2006). However, it remains unclear how these GRIP1 isoforms may participate in GABAergic synapse development or function. These molecules will be discussed in more detail in later sections.

GABA itself also appears to be involved in the development of inhibitory synapses. This can be seen, for example, in the mouse visual cortex, where knockdown of GAD67, the rate-limiting GABA synthetic enzyme (Asada et al., 1997; Soghomonian and Martin, 1998; Ji et al., 1999), results in deficient maturation of perisomatic synapses from basket interneurons (Chattopadhyaya et al., 2007). This effect was rescued by suppression of GABA reuptake and by application of GABAR agonists. In addition, overexpression of GAD67 was found to enhance the maturation of perisomatic innervation. Together, these results suggest that presynaptically released GABA is required for activity-dependent regulation of inhibitory innervation.

Although our understanding of the development of GABAergic synapses has advanced at a slower pace than has been seen in the case of glutamatergic synapse development, recent research has yielded a wealth of information in this regard, significantly narrowing the gap in our knowledge. As described later in this thesis, the identification of a growing number of molecules and mechanisms involved in GABAergic synapse development, as well as uncovering how these mechanisms relate to the formation and maturation of glutamatergic synapses, has opened up new doors for the exploration of synapse development in general.

1.4 Cell Adhesion Molecules in Synaptic Development

1.4.1 NCAM

NCAM is a member of the immunoglobulin (Ig) superfamily of CAMs, and was one of the first membrane proteins to be linked to neuronal adhesion (Thiery et al., 1977). Alternative splicing of NCAM produces three membrane-associated isoforms, NCAM-120, -140 and -180 (Garrow and El-Husseini, 2006). NCAM-180 and -140 both contain an extracellular region with five Ig-like domains and two fibronectin III-like repeats, followed by a single transmembrane domain (Dityatev et al., 2008), but differ in their intracellular tails (Murray et al., 1986). NCAM-120, however, is tethered to the extracellular surface of the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (Murray et al., 1986).

NCAM interacts with several ligands, but homophilic NCAM interactions have also been observed (Scheiffele, 2003). Although the presence of NCAM in PSDs was discovered in the late 1980s (Persohn et al., 1989), its role in synaptic function was not assessed until several years later, when it was determined that disruption of the interaction between NCAM and another CAM of the Ig family, L1, results in reduced LTP in the hippocampus (Luthl et al., 1994). Supporting the involvement of NCAM in synaptic plasticity *in vivo*, NCAM knockout mice show impaired LTP in the CA1 of the hippocampus (Muller et al., 1996; Cremer et al., 1998; Bukalo et al., 2004).

NCAM is an unusual CAM, in that it is the principal carrier of polysialic acid (PSA) in the mammalian brain (Dityatev et al., 2008). PSA is a long-chain polyanionic carbohydrate that, through its posttranslational modification of NCAM, has been implicated in various aspects of synaptic function. For example, removal of PSA from NCAM inhibits LTD and LTP, as well as spatial learning and morphological changes to synapses associated with LTP (Becker et al., 1996; Muller et al., 1996; Dityatev et al., 2004). Moreover, mice with genetically manipulated ST8SiaIV, one of the two polysialyltransferases (PSTs) required for transfer of PSA to NCAM, showed deficits in LTP and LTD in the CA1 region of the hippocampus, suggesting an essential role for PSA in hippocampal synaptic plasticity (Eckhardt et al., 2000). Conversely, mice deficient for ST8SiaII, the other PST involved in the polysialylation of NCAM, showed no deficits in synaptic plasticity in the hippocampus. Instead, these animals showed abnormal

axonal targeting and formation of ectopic synapses in this region, as well as higher exploratory drive and reduced hippocampus- and amygdala-dependent fear conditioning (Angata et al., 2004). Studies looking at the effects of PSA on neurotransmitter receptors have shown that PSA directly potentiates the opening of AMPARs and inhibits the activity of NR2B-containing extrasynaptic NMDARs (Vaithianathan et al., 2004; Hammond et al., 2006), suggesting possible mechanisms for the effects of PSA on synaptic plasticity. In addition, the presence of PSA on NCAM has been shown to sterically hinder intercellular homophilic NCAM and cadherin adhesion, suggesting additional mechanisms through which PSA-NCAM exerts its effects (Johnson et al., 2005).

Additional functions have been attributed to NCAM, independent of the presence of PSA. For example, the non-receptor tyrosine kinase, p59^{fyn}, is required for intracellular NCAM signaling related to axonal outgrowth (Beggs et al., 1994; Beggs et al., 1997). In addition, NCAM-dependent neurite outgrowth also seems to depend on signaling of NCAM through FGF receptors-phospholipase C-related pathway (Saffell et al., 1997; Doherty et al., 2000).

1.4.2 The Cadherin Adhesion Complex

Cadherins comprise a superfamily of homophilic CAMs that contains more than 80 members, and are found in a wide variety of organisms across the animal kingdom (Yagi and Takeichi, 2000). Members of this family are characterized by distinctive, tandemly repeated cadherin domains present in their extracellular regions (Overduin et al., 1995; Shapiro et al., 1995b; Shapiro et al., 1995a) and are divided into subfamilies, including classical cadherins, protocadherins, Fat cadherins, cadherin-related neuronal receptors (CNRs) and seven-pass transmembrane cadherins (Fig. 1.12) (Yagi and Takeichi, 2000). The majority of cadherin family members share a similar overall molecular architecture, with identical C-terminal domains, and extracellular regions differing in the number of cadherin repeats. Classical cadherins are further classified as type I or II cadherins (Nollet et al., 2000), distinguished by differences in distribution and adhesive interface properties (Nishimura et al., 1999; Van Aken et al., 2000; Patel et al., 2006). Although some binding between different cadherins has been observed (Inuzuka et al., 1991; Matsunami et al., 1993; Murphy-Erdosh et al., 1995; Nakagawa and Takeichi, 1995), homophilic binding is thought to underlie the majority of cadherin interactions

(reviewed in (Redies and Takeichi, 1996)). This binding specificity conferred by the cadherin adhesion system may be a critical feature relating to the involvement of cadherins in synapse development.

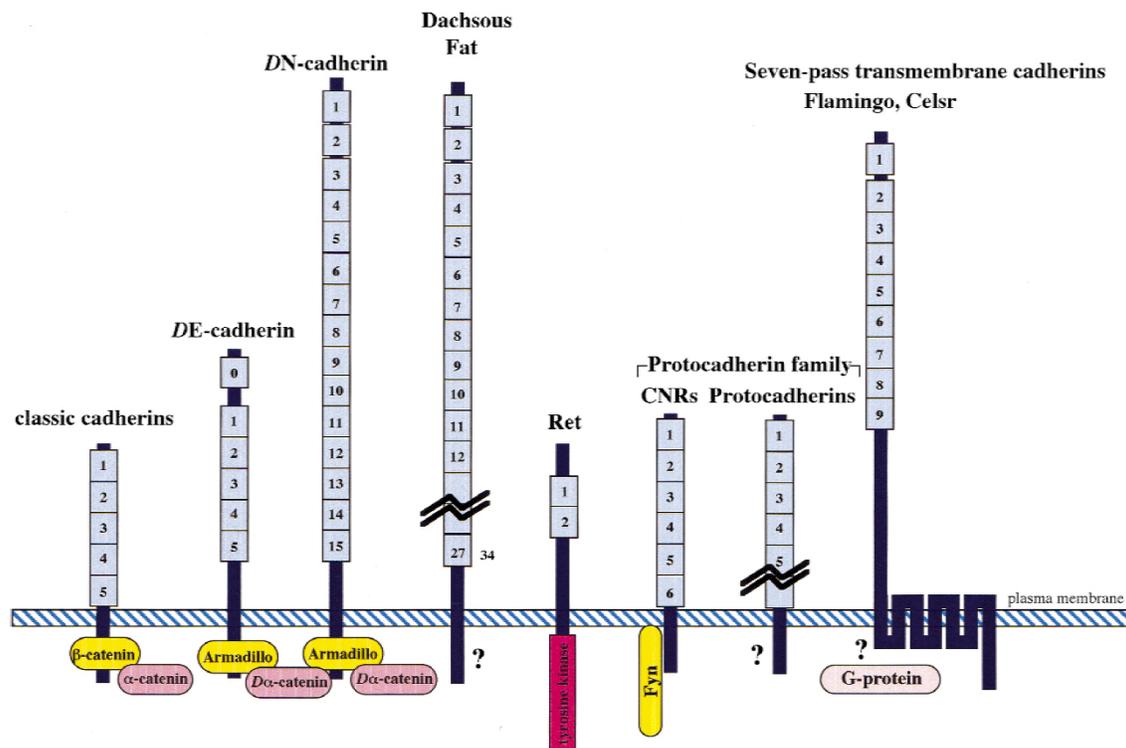


Fig. 1.12. Schematic diagram of the molecular structure of the cadherin superfamily members and their cytoplasmic interactors. Classic cadherins have been primarily isolated as Ca^{2+} -dependent cell adhesion molecules. Activity of cell adhesion is regulated by catenins bound to their cytoplasmic regions. Approximately 80 members of the cadherin superfamily have been isolated. Most members are expressed in the CNS. Cadherin-related neuronal receptors (CNRs) bind to Fyn-tyrosine kinase in their cytoplasmic region. Interestingly, *Drosophila* cadherins have many extracellular tandem repeats; the number of these repeats varies. Cadherin superfamily proteins are shown in blue, while their cytoplasmic interactors are shown in yellow and pink. Reproduced with permission from (Yagi and Takeichi, 2000).

Through interaction with a diverse set of molecules, cadherins and their interacting proteins are thought to participate in a broad range of cellular pathways and processes. For instance, the cytoplasmic domains of cadherins contain known binding sites for several intracellular proteins, including β -catenin, which in turn binds the soluble actin-binding protein, α -catenin (reviewed in (Arikkath and Reichardt, 2008)), linking adhesion at the cell surface to the actin cytoskeleton. In addition to its ability to bind α -catenin, the C-terminal region of β -catenin has been shown to contain a transcriptional activation motif, as well as a PDZ-binding motif that allows interaction

with various PDZ-containing proteins present at pre- and postsynaptic sites. Interactions between β -catenin and a number of proteins involved in intracellular signaling have also been reported.

Members of a distinct family of catenins, p120 catenin (p120ctn) and δ -catenin, are known to bind the juxtamembrane region of classical cadherins (Peifer et al., 1994; Lu et al., 1999). p120ctn, the founding member of this family of catenins, has been shown to be important for cytoskeletal organization, through its role in regulating the adhesive activity and stability of cadherins (Thoreson et al., 2000; Ireton et al., 2002). Unlike p120ctn, δ -catenin contains a C-terminal PDZ-binding motif, allowing it to interact with a number of postsynaptic proteins, including Densin-180, Erbin, SSCAM and PSD-95 (reviewed in (Kosik et al., 2005)). Finally, cadherins play roles in various intracellular signaling processes, through interactions with a wide array of proteins, including kinases, phosphatases, GTPases and members of the Arp2/3 complex.

A number of studies provide functional evidence for involvement of cadherin-catenin interactions in synapse formation. Indeed, interfering with cadherin-based adhesion, either through the use of anti-cadherin antibodies or through expression of a dominant-negative form of N-cadherin, disrupts several aspects of synapse formation, including reduced contact between pre- and postsynaptic elements, altered PSD formation and compromised synaptic function (Sanes and Yamagata, 1999; Honjo et al., 2000; Bozdagi et al., 2004). Furthermore, expression of dominant-negative N-cadherin leads to altered distribution of pre- and postsynaptic proteins, as well as the appearance of elongated membrane protrusions reminiscent of immature spines (Togashi et al., 2002), or inhibition of spine expansion (Okamura et al., 2004). Similarly, deletion of β -catenin in cultured mouse neurons results in the increased appearance of filopodia-like structures, accompanied by a decrease in the number of mushroom-shaped spines (Okuda et al., 2007). In intact mice, deletion of β -catenin results in smaller synaptic vesicle reserve pools, as indicated by EM and paired-pulse facilitation experiments *in vivo* (Bamji et al., 2003). These effects were found to be dependent on PDZ interactions mediated by the C-terminal of β -catenin, but were not influenced by deletion of the β -catenin N-terminal, suggesting an adhesion-independent role for cadherin-catenin interaction in synapse development. A later study by Sun

et al. showed that vesicle clustering induced by cadherin- β -catenin is mediated by the leucine-rich repeats and PDZ domains (LAP) protein family member, scribble (Sun et al., 2009).

The type I cadherin, N-cadherin, is the most widely distributed family member in neurons. During development of the nervous system, N-cadherin shifts from being homogeneously distributed at nascent synapses at early stages, to being localized in a more discrete, punctate pattern at regions surrounding the synaptic clefts of more mature contacts (Elste and Benson, 2006). Also, in cultured hippocampal neurons, N-cadherin is initially found at both excitatory and inhibitory synapses early in synaptic development, but is selectively lost from inhibitory synapses in more mature neurons (Benson and Tanaka, 1998), suggesting a potential role in synaptic specificity. Experiments examining the effects of N-cadherin disruption provide support for a role for this CAM in synapse formation. For example, knockdown of N-cadherin in young neurons results in decreased spine density (Saglietti et al., 2007), and defects in synapse assembly caused by expression of dominant-negative N-cadherin occur early in synapse development (Togashi et al., 2002). Interestingly, disruption of N-cadherin does not appear to affect later stages of synapse development (Togashi et al., 2002; Saglietti et al., 2007), suggesting that N-cadherin plays a specific role in the initial stages of synapse formation.

The role of the cadherin-catenin complex at inhibitory synapses has not been well defined. However, despite the selective loss of N-cadherin from inhibitory synapses during development, β -catenin is retained at this synaptic subtype. Thus, interactions between β -catenin and other cadherin family members may occur at inhibitory contacts and contribute to their formation. Indeed, cadherins 11 and 13 have been shown to play a role in inhibitory synapse development in cultured hippocampal neurons (Paradis et al., 2007).

In addition to their apparent roles in synapse formation, cadherins and catenins have also been implicated in control of synaptic activity and plasticity at mature synapses. For instance, both N-cadherin and β -catenin localization are altered by synaptic activity (Bozdagi et al., 2000; Tanaka et al., 2000; Murase et al., 2002; Shan et al., 2002). Conversely, LTP is modulated by disruption of cadherin function (Tang et al., 1998; Manabe et al., 2000).

1.4.3 SynCAMs

Synaptic Cell Adhesion Molecules (SynCAMs) 1-4, encoded by four distinct genes, comprise another subfamily of Ig superfamily proteins with both homophilic and heterophilic, calcium-independent binding properties (Biederer, 2006; Fogel et al., 2007). SynCAMs possess an extracellular region with three Ig-like domains, a transmembrane domain, and a short cytoplasmic tail with a protein 4.1-ezrin-radixin-moesin (FERM)- and a PDZ-binding motif that mediate binding to intracellular proteins such as CASK and syntenin (Biederer, 2006; Gerrow and El-Husseini, 2006). All SynCAM proteins are widely expressed throughout the developing and adult nervous systems (Maurel et al., 2007; Spiegel et al., 2007; Thomas et al., 2008). In the brain, SynCAMs are found primarily in neurons, both excitatory and inhibitory, each with a distinct spatiotemporal expression pattern (Thomas et al., 2008). In the peripheral nervous system, SynCAMs are found both in myelinated axons and in Schwann cells, and are thought to mediate adhesion at neuron-Schwann cell contact points (Maurel et al., 2007; Spiegel et al., 2007). In addition, SynCAM 1 expressed in mast cells has been shown to mediate interaction of these cells with neurites of superior cervical ganglion neurons (Furuno et al., 2005). In PC12 cells, SynCAM 1 has been proposed to be involved in the neuron-like differentiation of these cells, based on the finding that the gene for SynCAM 1 is induced by retinoic acid (Urase et al., 2001). SynCAM 1 has also been implicated in various roles outside of the nervous system. In testis, for instance, SynCAM 1 (known as SgIGSF in this context) is required for normal spermatid maturation during spermatogenesis (Fujita et al., 2006), and SynCAM 1 has been identified as a tumour-suppressor gene in a small-cell lung cancer cell line (Kuramochi et al., 2001).

Using an antibody that recognizes SynCAM 1-3, fractionation and immunofluorescence labelling, as well as immunoelectron microscopy experiments, have shown that SynCAMs are present in both pre- and postsynaptic membranes (Biederer et al., 2002). The first functional evidence demonstrating a role for SynCAMs in the brain emerged from a study searching for vertebrate Ig domain and PDZ-binding domain-containing CAMs involved in the alignment and differentiation of pre- and postsynaptic elements during synaptogenesis (Biederer et al., 2002). In this study, expression of SynCAM 1 in non-neuronal cells co-cultured with hippocampal neurons was found to induce the formation of presynaptic protein clusters in contacting axons, in a

manner dependent on the extracellular Ig domains of SynCAM 1. Furthermore, the use of FM5-95 dye showed that these clusters of presynaptic proteins corresponded to sites of active synaptic vesicle exo- and endocytosis, and spontaneous and evoked excitatory postsynaptic responses were reconstituted in SynCAM-expressing non-neuronal cells upon co-expression of GluR2 (Biederer et al., 2002; Sara et al., 2005). Thus, SynCAM 1 appeared to have synaptogenic properties, in that its expression in an artificially generated “postsynaptic” setting was sufficient for inducing the assembly of a fully functional secretory apparatus in the presynaptic terminals of contacting axons. Interestingly, expression of SynCAM 1 in hippocampal neurons results in increased spontaneous and evoked EPSC frequency; however, unlike expression in heterologous cells, neuronal expression of SynCAM 1 does not increase the number of morphologically identifiable synaptic contacts (Biederer et al., 2002; Sara et al., 2005). This may be a reflection that, in neurons, SynCAM specifically increases synaptic vesicle pool size, leading to enhanced synaptic efficacy, without a corresponding increase in the number of synapses.

These results are consistent with the idea that SynCAMs mediate trans-synaptic adhesion to exert their effects on synapse development. While SynCAM 1 was initially identified as a homophilic adhesion molecule (Biederer et al., 2002; Sara et al., 2005), recent studies have shown that SynCAMs participate preferentially in strong, specific heterophilic interactions. In particular, SynCAM 1/2, SynCAM 2/4 and SynCAM 3/4 binding pairs are most prominently observed in a series of affinity chromatography, co-immunoprecipitation and cell-binding experiments (Fogel et al., 2007; Thomas et al., 2008). Furthermore, in addition to expression of SynCAM 1 or 2 in non-neuronal cells inducing the clustering of synaptic vesicle proteins in contacting axons (Biederer et al., 2002; Sara et al., 2005; Fogel et al., 2007), non-neuronal expression of either protein leads to clustering of the other in contacting presynaptic terminals, indicating mutual recruitment of SynCAMs 1 and 2 into neuronal adhesion complexes (Fogel et al., 2007). Thus, heterophilic, trans-synaptic adhesion between different SynCAM family members is likely to play a significant role in the development of synaptic contacts. This concept is supported by the observation that different regions in the developing and mature mouse brain exhibit distinct spatiotemporal SynCAM expression patterns (Thomas et al., 2008). Although the extracellular regions of SynCAM 1 and 3 are also known to heterophilically interact with proteins such as nectins 1 and 3 (Shingai et al., 2003; Kakunaga et al., 2005), as well as the T-cell receptor,

CRTAM (Arase et al., 2005; Boles et al., 2005), the physiological relevance of these interactions remains unknown.

1.4.4 LRR Proteins

Synaptic members of the leucine-rich repeat (LRR) family of proteins form another group of CAMs and CAM-like molecules that have been implicated in the regulation of synapses (reviewed in (Ko and Kim, 2007)). The LRR family is composed of a large number of proteins, the majority of which possess tandemly repeated protein-binding motifs of 20-29 amino acids containing a conserved 11-amino acid, leucine-rich sequence (Kobe and Kajava, 2001). Neuronal LRR proteins are known to play roles in the regulation of neurite outgrowth and migration (Wong et al., 2002; Filbin, 2003; Chen et al., 2006); however, a number of synaptically localized LRR proteins have recently been implicated in synapse development. These include members of the LAP (LRR and PDZ), the netrin-G-ligand (NGL) and the synaptic adhesion-like molecule (SALM) families (Fig. 1.13).

The best studied members of the LAP family are densin-180 and Erbin, both of which are concentrated in the PSD in neurons (Apperson et al., 1996; Huang et al., 2001). Although densin-180 possesses a predicted transmembrane domain and is thus thought to mediate cell adhesion, densin-180 expressed in cultured hippocampal neurons does not bind extracellular biotin, and may therefore not be an integral membrane protein (Izawa et al., 2002). Binding of densin-180 to a number of synaptic proteins physically links it to the PSD, and implicates it in various cellular processes. For instance, densin-180 forms a ternary complex with the α subunit of calcium/calmodulin-dependent kinase II (CaMKII α) and α -actinin (Walikonis et al., 2001; Robison et al., 2005), proteins involved in intracellular signaling and regulation of actin dynamics, respectively. These interactions have been proposed to play a role in the synaptic localization of CaMKII α (Walikonis et al., 2001). Densin-180 has also been shown to bind the postsynaptic adaptor proteins, MAGUIN and Shank (Ohtakara et al., 2002; Quitsch et al., 2005). While binding of MAGUIN multimers to PSD-95 forms a link between these proteins and densin-180, the interaction between Shank and densin-180 appears to be involved in regulating the effects of densin-180 on dendritic branching (Ohtakara et al., 2002; Quitsch et al., 2005).

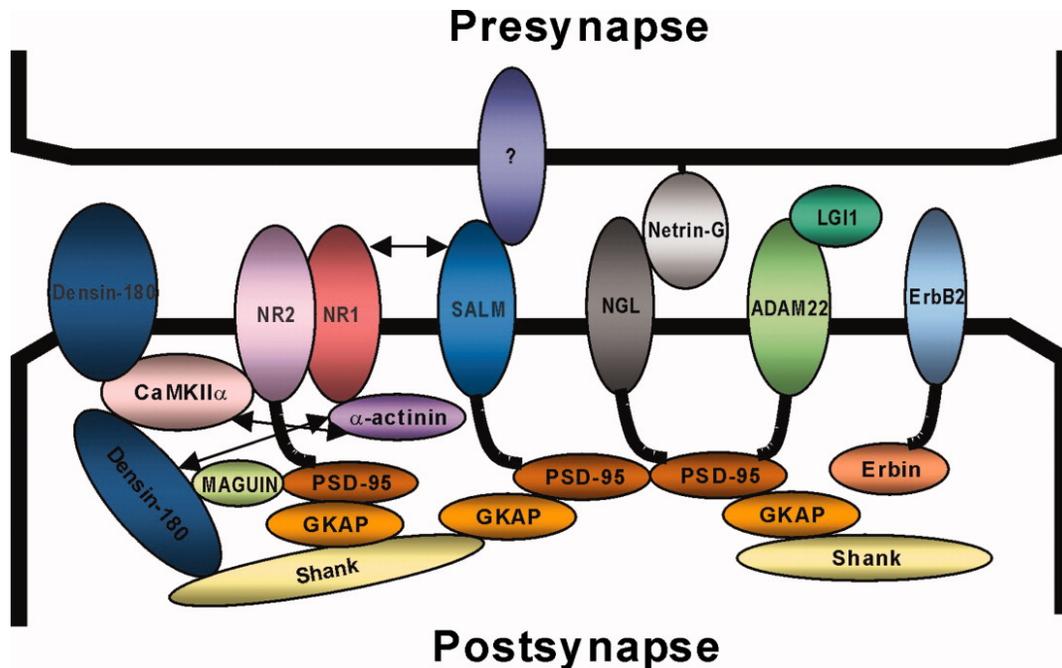


Fig. 1.13. Schematic diagram of postsynaptic organization by LRR proteins. The C-terminal cytoplasmic tails of membrane proteins are indicated by black lines. Specific protein–protein interactions are indicated by direct contacts of the proteins or arrows. Netrin-G is tethered to the plasma membrane through a GPI anchor. Densin-180 may be either a membrane or cytoplasmic protein. Proteins: CaMKII α , the α subunit of calcium/calmodulin-dependent kinase II; ADAM22, a disintegrin and metalloprotease 22; PSD-95, postsynaptic density 95; GKAP, guanylate kinase-associated protein; Shank, SH3 and ankyrin repeat-containing protein; MAGUIN, membrane-associated guanylate kinase-interacting protein; NR1, NMDA receptor subunit 1; NR2, NMDA receptor subunit 2. Reproduced with permission from Ko and Kim, 2007.

Three members of the NGL family, NGL-1, NGL-2 and NGL-3, have so far been identified (Lin et al., 2003; Zhang et al., 2005; Kim et al., 2006). NGL-1, originally identified as a receptor for netrin-G1, has been shown to promote the outgrowth of thalamocortical axons (Lin et al., 2003). NGL-2, on the other hand, was initially identified as LRRC4, a protein involved in maintaining normal function and suppressing tumorigenesis in the CNS (Zhang et al., 2005). NGLs are transmembrane proteins, composed of an extracellular domain consisting of nine LRRs followed by an Ig domain, and an intracellular region containing a C-terminal PDZ-binding motif. NGLs are found specifically at excitatory synaptic sites, where they bind to PSD-95 intracellularly, and interact trans-synaptically with netrin-G1 and netrin-G2 (Lin et al., 2003; Kim et al., 2006), netrin-like adhesion molecules found mainly in the brain (Nakashiba et al., 2000; Nakashiba et al., 2002; Yin et al., 2002). Genetic analysis has linked netrin-G and NGL proteins to brain disorders such as schizophrenia and Rett Syndrome (Aoki-Suzuki et al., 2005; Borg et al., 2005),

further supporting the notion that these proteins play an important role in development of the nervous system. Interestingly, NGL-2 expressed in non-neuronal cells or presented on the surface of beads induces presynaptic differentiation in contacting axons, indicating a synaptogenic role for this protein (Kim et al., 2006). In addition, clustering of PSD-95 and other excitatory postsynaptic proteins is induced by direct aggregation of NGL-2 on the surface membrane of dendrites (Kim et al., 2006). Furthermore, while overexpression of NGL-2 in cultured neurons enhances the formation of dendritic protrusions containing PSD-95, knockdown of NGL-2 reduced excitatory synapse density and currents, having no effect on inhibitory synapses (Kim et al., 2006). Moreover, treatment with a soluble form of NGL-2 inhibits excitatory synapse formation (Kim et al., 2006). Together, these data suggest that NGL is specifically involved in excitatory synapse development.

SALMs were initially identified in two independently conducted yeast two-hybrid screens, one looking for SAP97-binding molecules, the other for molecules that bind PSD-95 (Ko et al., 2006; Wang et al., 2006). Proteins of the SALM family, which consists of five members (SALM1-5), are expressed primarily in brain (Ko et al., 2006; Morimura et al., 2006; Wang et al., 2006). The domain structure of SALMs is similar to that of NGLs, with the exceptions that SALMs have an additional fibronectin III adhesion domain, and SALM4 and SALM5 do not contain PDZ-binding motifs (Ko et al., 2006; Morimura et al., 2006). Also similar to NGLs, SALMs appear to be involved in synaptic development. For instance, subcellular fractionation reveals that SALM1 is found in the synaptic membrane and in postsynaptic densities (Wang et al., 2006), and immunolocalization experiments have revealed that SALM2 clusters at excitatory, but not inhibitory synapses (Ko et al., 2006). Furthermore, overexpressing SALM1 in cultured neurons enhances the recruitment of PSD-95 and NR2A to synapses in a PDZ-mediated fashion. Together with the finding that SALM1 forms a complex with NMDARs in brain (Wang et al., 2006), these data suggest that SALMs may play a role in the synaptic recruitment of NMDARs *in vivo*. Similar to SALM1, low-level overexpression of SALM2 leads to increased numbers of excitatory synapses and dendritic protrusions (Ko et al., 2006). In addition, bead-induced aggregation of SALM2 causes clustering of excitatory postsynaptic scaffolding proteins and neurotransmitter receptors. Conversely, either RNAi knockdown or high levels of SALM2 overexpression, which leads to dispersal of this protein in dendrites, decreases excitatory synapse

and spine formation (Wang et al., 2006). Interestingly, SALM2 overexpression did not have synapse-inducing effects when expressed in young neurons, suggesting that this protein may influence later stages of synaptic development (Ko et al., 2006).

Recently, a screen for synaptogenic factors present in the rat brain revealed a new class of LRR proteins involved in presynaptic differentiation (Linhoff et al., 2009). All four members of this family, originally identified as leucine-rich repeat transmembrane (LRRTM) proteins in the brain (Lauren et al., 2003), are localized to excitatory synapses, have the ability to induce clustering of presynaptic proteins in contacting axons when expressed in non-neuronal cells, and, when artificially clustered in dendrites, lead to postsynaptic differentiation (Linhoff et al., 2009). Moreover, disruption of LRRTM1 in mice disrupts the normal distribution of VGluT1, indicating an important role for this protein *in vivo* (Linhoff et al., 2009).

1.4.5 Non-neuronal Regulators of Synaptogenesis

Astrocytes are the most abundant cell type in the mammalian brain (Eroglu, 2009). Each astrocyte has a highly complex network of processes that can intimately associate with thousands of synapses in its surrounding area, allowing these cells to actively participate in synaptic activity (Bushong et al., 2002; Eroglu, 2009). Indeed, the three-way synaptic structure consisting of presynaptic terminal, postsynaptic membrane and astrocytic process has been termed a “tripartite synapse,” highlighting the apparent importance of astrocytes in normal synaptic function (Araque et al., 1999). For many years, it has been known that astrocytes are crucial for neuronal survival and proper neurite development, both *in vitro* and *in vivo* (Banker, 1980; Craig and Banker, 1994; Shaham, 2006). Retinal ganglion cells (RGCs) cultured in the absence of astrocytes, for example, fail to make synapses, suggesting that factors secreted from this cell type are important for the development of synaptic contacts (Meyer-Franke et al., 1995; Pfrieger and Barres, 1997; Ullian et al., 2001). Despite this, it remains unclear exactly how these “support” cells contribute to synapse formation and function.

Recent findings have identified the astrocyte-secreted proteins of the thrombospondin (TSP) family as being important factors in synaptogenesis. TSP protein is detected in the brains of embryonic and early postnatal animals, but decreases dramatically in the adult CNS, becoming

restricted to areas of neurogenesis and plasticity (O'Shea and Dixit, 1988; O'Shea et al., 1990b; O'Shea et al., 1990a; Hoffman et al., 1994; Christopherson et al., 2005). Moreover, TSP1 and 2 transcripts can be found in astrocytes isolated from early postnatal mouse cortex, but are downregulated in astrocytes from mature brain (Cahoy et al., 2008). In a screen for synaptogenic molecules present in astrocyte-conditioned medium (ACM), recombinant TSP1 and 2 were found to be sufficient for mimicking the ACM-induced increase in synapse number in cultured RGCs, as assessed by staining for overlapping pre- and postsynaptic markers, as well as by electron microscopy (Christopherson et al., 2005). Furthermore, immunodepletion of TSP2 from ACM indicates that this family member is necessary for the synapse-inducing effects of ACM. The number of total and docked vesicles in presynaptic terminals of ACM- or TSP1-induced synapses was comparable to control cells cultured in the presence of astrocytes, and the postsynaptic densities of these synapses appeared unaltered, indicating that these synapses were ultrastructurally normal. However, examination of synapse function revealed that neither ACM- nor TSP1/2-induced synapses were synaptically active, as measured by whole-cell patch clamp recording. When the effects of pre- vs. postsynaptic function were dissected, it was determined that, while presynaptic terminals underwent active vesicular recycling to similar extents in the case of treatment with TSP1, ACM or an astrocyte feeding layer, ACM and TSP1 induced postsynaptically silent synapses, lacking a full complement of functional AMPA receptors. These data suggest that astrocytes mediate two independent steps in synapse development: (1) Recruitment of pre- and postsynaptic specializations, mediated by TSP1 and 2, and (2) insertion of glutamate receptors into the postsynaptic membrane, mediated by an as yet unidentified astrocytic signal (Christopherson et al., 2005). To confirm the effects of TSP1 and 2 on synapse formation *in vivo*, TSP1/2 double knockout mice were generated (Christopherson et al., 2005). These mice showed a 25-30% reduction in the number of cortical excitatory synapses during the first and third weeks of postnatal development. TSPs therefore appear to play an important role in synapse formation.

In recent years, other astrocyte-secreted proteins have also been implicated in CNS development. Tenascin-C, for example, is involved in neuronal migration and maturation, while SPARC participates in neurite outgrowth (Eroglu, 2009). In addition, hevin is currently being investigated for its possible role in synapse formation and function (Eroglu, 2009). Thus, despite

the classic view that astrocytes are passive bystanders in relation to synapse development, accumulating evidence suggests that they in fact significantly contribute to this process in a number of ways.

1.5 Neuroligins and Neurexins

1.5.1 Structure of Neuroligins and Neurexins

Neuroligins: Neuroligin 1 was originally identified as a synaptic cell surface protein acting as a ligand for β -neurexins, members of a family of presynaptic cell adhesion molecules known to bind the spider venom, α -latrotoxin (Ushkaryov et al., 1992; Ichtchenko et al., 1995). Since this initial finding, a number of other neuroligin family members have been identified in the genomes of several species, ranging from *C. elegans* and *Drosophila*, to rat and human (Gilbert and Auld, 2005). In mouse and rat, four individual neuroligin proteins are known to be encoded, each from separate genes, while five neuroligin genes have been described in humans (Bolliger et al., 2001; Jamain et al., 2003; Bolliger et al., 2008).

Neuroligins are single-pass transmembrane proteins, consisting of a large extracellular domain with sequence homology to acetylcholinesterase (AChE), a carbohydrate linker region and a short intracellular tail containing a C-terminal PDZ-binding motif (Fig. 1.14) (Ichtchenko et al., 1995). The presence of a cholinesterase-like domain (CLD) in the extracellular portion of neuroligins places them in a family of similarly structured proteins, referred to as cholinesterase-like adhesion molecules (CLAMs), members of which are found in a wide range of cell types and organisms (reviewed in (Gilbert and Auld, 2005)). All members of this family possess an extracellular domain with homology to cholinesterases that mediates heterophilic cell adhesion. Separating the CLAMs from the rest of the cholinesterase family, however, is the loss of enzymatic activity in the cholinesterase-like domain of CLAMs, due to the absence of at least one residue forming the catalytic triad.

Sequence similarity between the different neuroligin family members is unevenly distributed (Ichtchenko et al., 1996). The highest levels of sequence similarity can be seen in the extracellular and transmembrane domains, with 55% and 91% sequence identity, respectively. Within the intracellular C-terminal regions of neuroligins, however, despite the presence of a PDZ-binding motif in all neuroligins, only 31% identity is observed. The relatively high level of sequence similarity in the extracellular region is likely reflective of the common abilities of all

neuroligins so far examined to bind neurexins. Conversely, the higher level of sequence divergence observed in the C-terminal tails of neuroligins may allow various family members to interact with differing subsets of intracellular binding partners.

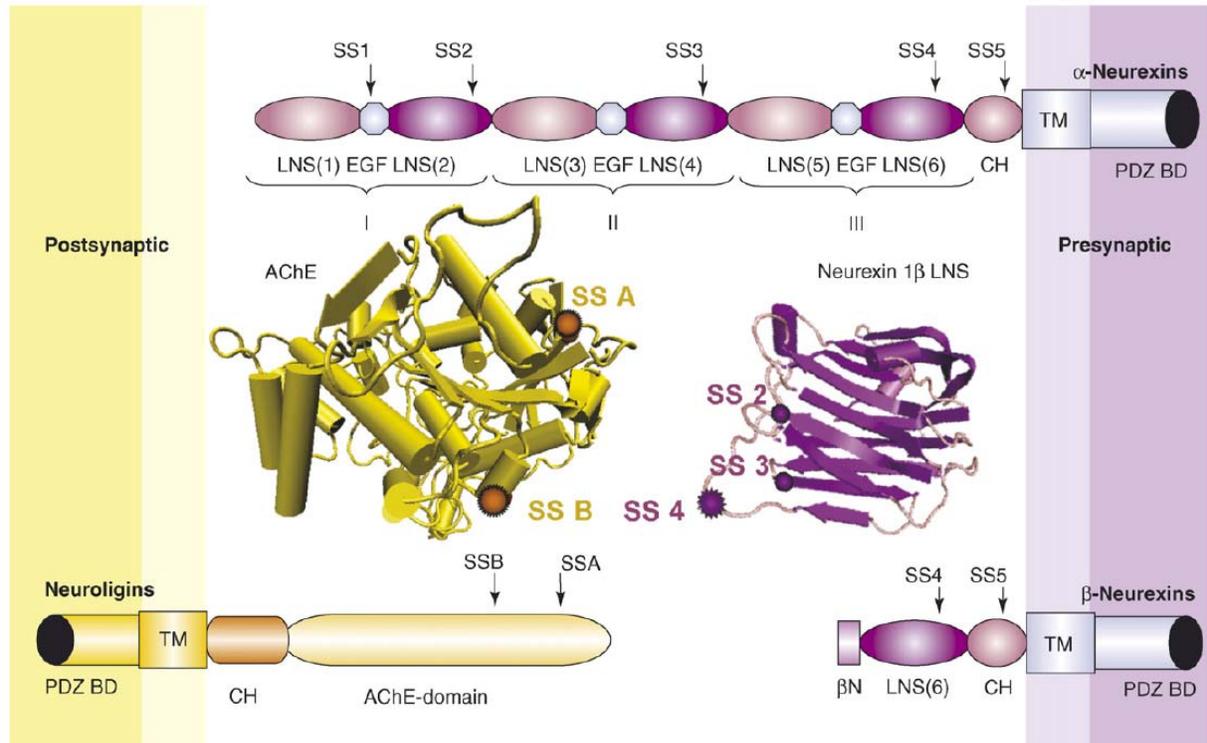


Fig. 1.14. Structure of neurexins and neuroligins. In humans, there are three neurexin genes and five neuroligin genes. Each neurexin gene uses an upstream promoter to generate the larger α -neurexins and a downstream promoter to generate the smaller β -neurexins. Thus, β -neurexins can be thought of as N-terminally truncated α -neurexins that have a short β -specific leader (β N). In α -neurexins, the LNS domains are organized with EGF-like domains into three homologous modules, I–III. The position of each of five sites of alternative splicing (SS1–SS5) is indicated. Neuroligins contain an extracellular AChE-homologous domain that contains one or two sites of alternative splicing (SSA, plus SSB in the case of neuroligin 1). Both neurexins and neuroligins contain a highly glycosylated region (CH) and a transmembrane domain (TM; not present in some splice variants of neurexin 3), and terminate in PDZ-domain-binding sites (PDZ BD). Shown between the neurexins and neuroligins are structures of AChE, a model for the AChE-homologous domain of neuroligins, and the neurexin 1 β LNS domain. The position of splice sites SS2–SS4 is shown on a single LNS domain for simplicity, although SS2 and SS3 actually occur in different LNS domains of α -neurexins. Reproduced with permission from Craig and Kang, 2007.

Alternatively spliced inserts have been identified in the CLD of neuroligins. NL1 contains two alternative splice sites, A and B, while NL2 and NL3 contain alternative inserts only at site A (Ichtchenko et al., 1995; Ichtchenko et al., 1996). Despite the presence of inserts at site A in all three neuroligins, however, the sequences of the inserts differ between NL1 and NL2. In

addition, NL3 contains two possible splice insert sequences at site A; one with homology to NL1, and one with homology to NL2 (Ichtchenko et al., 1996). Alternative splicing at these sites has been shown to be important for the binding activity and synaptic localization of different neuroligin family members (Boucard et al., 2005; Chih et al., 2006).

Neurexins: Three neurexin genes (1-3) have been identified in mammals, each one encoding both an α and β isoform, driven by different promoters within each gene (Ushkaryov et al., 1994; Tabuchi and Sudhof, 2002). Thus, six principal isoforms of neurexins are encoded in mammals; namely neurexin 1 α , 2 α and 3 α , as well as neurexin 1 β , 2 β and 3 β . However, the presence of five alternative splice sites in α -neurexins and two in β -neurexins, allow for the potential for generating a huge number of multiple splice variants of each isoform (Ullrich et al., 1995; Missler et al., 1998; Missler and Sudhof, 1998). Counterparts to the mammalian neurexins are found in a number of invertebrate species, however only one variant (a form of α -neurexin) exists in *Drosophila* and *C. elegans* (Tabuchi and Sudhof, 2002). While the extracellular domains of α -neurexins consist of six LNS domains (named after homology to repeating sequences in laminin, neurexin and sex hormone-binding protein) arranged in modules with epidermal growth-factor (EGF)-like repeats, the extracellular regions of β -neurexins are much smaller, consisting of only a 38-amino acid β -neurexin-specific sequence, the last LNS domain of α -neurexin and a highly glycosylated linker region (Figure 1.14) (Ushkaryov et al., 1992; Ullrich et al., 1995; Missler et al., 1998; Missler and Sudhof, 1998). Following the extracellular region of neurexins is a single-pass transmembrane domain, as well as a short C-terminal tail that is conserved among all neurexin isoforms. The type II PDZ-binding motif present at the C-terminus of all neurexins likely mediates intracellular protein-protein interaction. In addition, alternative splicing at splice site 4 of neurexin-1 β modulates interaction with neuroligins (Ichtchenko et al., 1995; Graf et al., 2006).

Crystal structure of the neuroligin-neurexin complex: Two recent studies have examined the high resolution crystal structures for the extracellular domains of NL1 and NL4, both in isolation and in complex with β -neurexin (Arac et al., 2007; Fabrichny et al., 2007). From these studies, it is apparent that neuroligins exist as constitutive dimers, as suggested by findings from previous functional studies (Dean and Dresbach, 2006; Comoletti et al., 2007). It also becomes clear that

the general structure of neuroligins diverges from that of AChEs, due mainly to differences in conformation and length of several surface loops, preventing substrate from entering the active site. The Cys-loop of neuroligins was shown to display flexibility relative to that of the AChEs, providing a looser connection between the molecular core and the four-helix bundle involved in dimerization of neuroligin molecules. This molecular flexibility may be important for regulating the adhesive properties of neuroligins and neurexins. Structures for NL1 and NL4 bound to neurexin-1 β demonstrate that the binding interface between these molecules is conserved throughout all neuroligins, but is absent in AChEs. The specific arrangement of the neuroligin/neurexin complex consists of two neurexin protomers bound to opposite surfaces of the neuroligin dimer. This organization is consistent with these molecules bridging the 20 nm space between the presynaptic terminal and dendritic spine or shaft. Although the structure of the binding interface is well conserved within the neuroligin family, some variations exist at the edges of this interface which could be responsible for differing affinities between various neuroligin and neurexin proteins. In addition, neuroligins lack a permanent dipole, which may facilitate fluctuating interactions, a feature that is likely important in the context of cell adhesion.

Data obtained from the crystal structures validate previous findings pointing to crucial roles for alternative splice insertions in neuroligins and neurexins in modulating interactions between these proteins. The structures reveal that neuroligins splice site B is located at the edge of the binding interface, consistent with insertion at this site hindering interaction between neuroligins and α -neurexins, as indicated by previous functional studies (Boucard et al., 2005; Chih et al., 2006; Graf et al., 2006; Arac et al., 2007; Fabrichny et al., 2007). The crystal structures of these proteins therefore support the concept that these splice sites introduce a regulatory point for controlling the strength of interaction, and ultimately the nature of action at a particular contact site.

1.5.2 Spatiotemporal Distribution of Neuroligins and Neurexins

NL1 protein is found almost exclusively in neurons throughout the brain of rats, including hippocampal pyramidal neurons and cerebellar Purkinje cells (Song et al., 1999). Although neuroligin expression is low in embryonic brain, its expression is dramatically increased following birth, and levels off during a later period of heavy synaptogenesis. NL2 protein is

distributed throughout the brain, where it is enriched in the synaptosomal membrane fraction, consistent with its targeting to the synaptic plasma membrane; it is absent, however, from non-nervous tissue (Varoqueaux et al., 2004). NL2 expression is widely distributed throughout the brain, with particularly high expression levels in the cortex and hippocampus, among various other regions. The expression of NL2 protein first becomes apparent at embryonic day 16 (E16), and increases steadily throughout early postnatal development, until it plateaus at postnatal day 15-21 (P15-21). NL3 protein is also present in the brains of embryonic mice, and increases postnatally (Varoqueaux et al., 2006; Budreck and Scheiffele, 2007). *In situ* hybridization experiments show that NL3 mRNA is found in almost all neuronal populations of the brains of both newborn and adult rats, where it is co-expressed with NL1 and NL2 (Varoqueaux et al., 2006). However, some evidence shows that NL3 expression may not be restricted to neurons. In rodents, NL3 has been observed in a variety of glial cells, including immature astrocytes, Schwann cells, satellite glia and olfactory ensheathing cells (Gilbert et al., 2001). In addition, NL3 expression has been detected in non-nervous tissue, such as muscle and pancreas (Philibert et al., 2000). Unlike the other neuroligins, NL4 is lacking in the brains of both newborn brain and more mature mice (Varoqueaux et al., 2006). In humans, however, NL4 expression occurs in a number of other tissues, such as heart and skeletal muscle, pancreas and liver (Bolliger et al., 2001).

Neurexin expression seems to begin earlier in development than that of neuroligins. Transcripts for both α - and β -isoforms of all three neurexin genes have been detected throughout the nervous system in embryonic mice, with increased levels of expression as maturation proceeds (Puschel and Betz, 1995). A study looking at neurexin expression in embryonic chick brain showed the presence of neurexin-1 in telencephalon, optic tectum and hindbrain as early as E4, which persisted at least until E17 (Paraoanu et al., 2006). In the same study, neurexin-1 transcript was detected on either side of the inner plexiform layer of the developing retina throughout embryonic development. The expression of neurexins at such early developmental stages may reflect the involvement of these proteins in processes other than synapse development. In adult rats, neurexin-1 α mRNA is seen throughout the brain, while expression of neurexin-1 β is more restricted, found only in regions such as the cortex and hippocampus (Ullrich et al., 1995). Conversely, expression of neurexin-2 α is more spatially limited than that of neurexin-2 β . High

levels of neurexin-3 β were found equally in all brain regions, while neurexin-3 α was expressed at low levels in most brain areas. Within the hippocampus, neurexins show differential regional and cell type expression patterns. For instance, pyramidal neurons of the CA3 region do not express neurexin-1 β , while CA1 pyramidal cells co-express all six principal neurexin isoforms.

1.5.3 Functional Significance of Neuroligin-Neurexin Interaction at Synapses

Numerous recent studies have examined the role of the neuroligin family of postsynaptic cell adhesion molecules in synapse development. Although a great deal of data showing biochemical interaction between neuroligins and neurexins had been accumulated in the late 1990s, the first solid evidence suggesting such a role for neuroligins came from a pivotal study by Scheiffele and colleagues showing that NL1 or NL2 expressed in non-neuronal cells is capable of inducing differentiation in contacting pontine axons of cerebellar granule cells (Fig. 1.15) (Scheiffele et al., 2000). A great deal of progress has been made since then in understanding how coupling of neuroligins to their synaptic binding partners is involved in neuronal contact formation. A later study, for instance, determined that NL1 in fact mediates its effects on presynaptic terminal maturation through trans-synaptic interaction with and clustering of β -neurexin (Dean et al., 2003). In this study, application of purified neuroligin protein to neurons was sufficient to induce presynaptic clustering of neurexin, which in turn led to the recruitment of the neurotransmitter release machinery in a manner dependant on the cytoplasmic tail of neurexin. In support of this, incubation with a soluble fragment of neurexin-1 β that interferes with NL1-neurexin-1 β binding blocks the effects of NL1 on synapse formation (Scheiffele et al., 2000). In addition, co-culturing of neurons with β -neurexin-expressing non-neuronal cells induces the clustering of PSD-95 and NMDARs in contacting dendrites (Nam and Chen, 2005). Providing evidence for a connection to synapse “unsilencing,” AMPARs were recruited to PSD-95/NMDAR scaffolds upon stimulation with glutamate, or through neuronal expression of a constitutively active form of CaMKII. Furthermore, expression of a dominant-negative form of NL1 lacking its PDZ-binding domain (rendering it unable to bind PSD-95) resulted in reduced clustering of both PSD-95 and AMPARs. Confirming a specific role of this system in the assembly of glutamatergic contacts, expression of dominant-negative NL1 impaired excitatory, but not inhibitory, synaptic function.

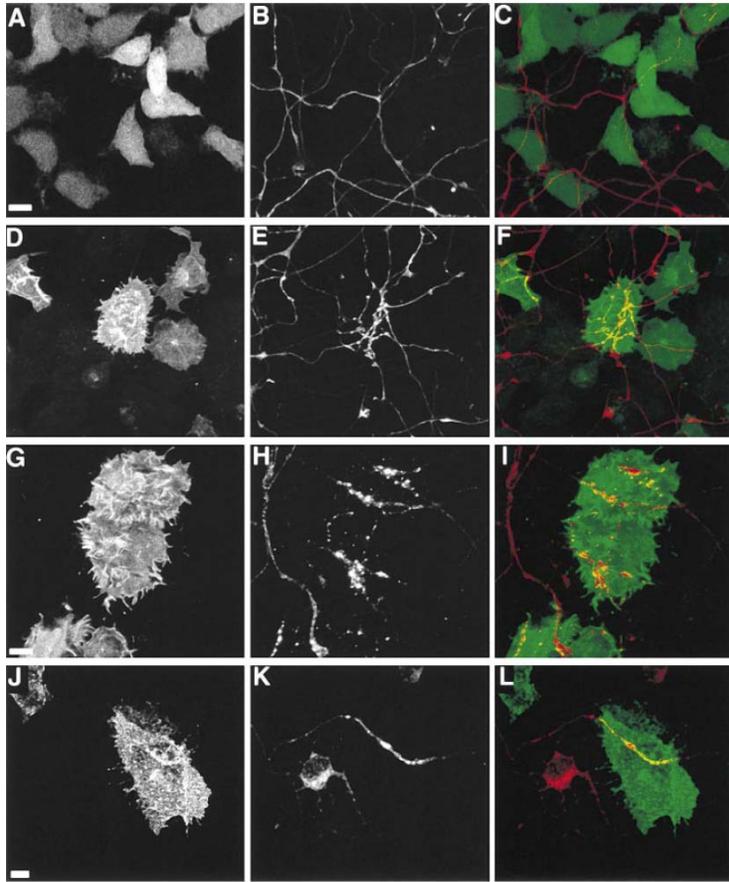


Fig. 1.15. Neuroigin Expression in HEK293 Cells Causes Accumulation of Synapsin in Axons. HEK293 cells were transiently transfected with GFP (A–C), neuroigin-1*ab* (D–F and J–L), or neuroigin-2 (G–I) and were co-cultured with pontine explants (A–I) or purified cerebellar granule cells (J–L). Fixed, immunostained cultures were analyzed by confocal microscopy. Fluorescence of GFP (A) or immunostaining with anti-HA antibodies to detect neuroigins (D, G, and J) is displayed in green in the overlay (C, F, I, and L). Immunostaining for synapsin in the pontine axons (B, E, H, and K) is displayed in red in the overlay (C, F, I, and L). The scale bar in (A) represents 20 μ m, 10 μ m in (G), and 12 μ m in (J). Reproduced with permission from Scheiffele et al., 2000.

Despite these major advances in uncovering factors involved in maturation of excitatory synapses, molecules that induce inhibitory synaptic contact formation were lacking, until a more recent study by Prange et al. provided new insights into a novel role for neuroigins in inhibitory synapse development (Prange et al., 2004). Unexpectedly, overexpression of NL1 in hippocampal neurons was found to induce not only excitatory synapses, but also robustly enhance the number and activity of GABAergic contacts. These results provided the first evidence that neuroigins may be directly involved in inhibitory synapse formation. More recent studies have shown that other members of the neuroigin family, namely NL2, NL3 and NL4, exert similar effects on both types of synapses (Graf et al., 2004; Chih et al., 2005; Levinson et al., 2005). This emerging role in inhibitory synapse formation seems to be characteristic of neuroigins, but has not been observed for other known synaptogenic molecules such as SynCAM1 (Sara et al., 2005), pointing to a novel and specific role for neuroigins in the development of inhibitory synapses.

A study by Boucard et al. further underlines the importance of neuroligin-neurexin interaction in synapse development (Boucard et al., 2005). In the initial neuroligin-neurexin binding studies, NL1 was found to biochemically associate only with β -neurexins lacking an insert at splice site 4, a 30-amino acid sequence under developmental and spatial regulation (Ichtchenko et al., 1995; Ullrich et al., 1995; Ichtchenko et al., 1996). However, an unexpected result came when it was discovered that the previously untested form of NL1 lacking splice site B was able to robustly bind α -neurexins in addition to β -neurexins, regardless of the presence of splice site 4 (Boucard et al., 2005). The basis for a lack of binding between NL1 with splice site B and α -neurexin was determined to relate to the presence of N-glycosylated residues within splice site B that sterically hinder interaction between these molecules. Moreover, while NL1 that binds only β -neurexins induces the formation of synapses, NL1 that binds both α -neurexin and β -neurexins promotes the expansion of individual synaptic sites. Excitingly, it was later found that the presence of splice site B in NL1 prevents it from inducing inhibitory presynaptic differentiation, and neurexin isoforms that specifically bind NL1 lacking splice site B exclusively enhance the assembly of inhibitory postsynaptic components (Nam and Chen, 2005; Chih et al., 2006; Graf et al., 2006). These results bolster the idea that different neuroligin family members play a significant role in synaptic specificity, and may be required for normal E/I balance.

1.6 Scaffolding Molecules in Synapse Development

1.6.1 The PSD-95 Family

Studies examining the overall protein makeup of the PSD have shown that one of its most abundant constituent proteins is the synaptic scaffolding protein, PSD-95 (also known as SAP90), a member of the membrane-associated guanylate kinase (MAGUK) protein superfamily (Chen et al., 2005; Sugiyama et al., 2005; Cheng et al., 2006; Sheng and Hoogenraad, 2007). One of the best studied PSD proteins, much is known about the structure, function and regulation of PSD-95.

PSD-95 is organized in a highly modular fashion (Fig. 1.16). At its N-terminus, PSD-95 contains three type I PDZ domains, named after the proteins in which these sequences were first discovered (PSD-95, discs large, zona occludens 1; (Kim and Sheng, 2004)). PDZ domains are 90 amino acid sequences that bind to short motifs at the C-termini of many proteins, thereby mediating protein-protein interactions. C-terminal to the PDZ domains of PSD-95 is a src homology 3 (SH3) protein interaction module, known to bind proline-rich tracts in other proteins, followed by a guanylate kinase (GK) domain, which retains the GMP-binding capability of yeast guanylate kinase, but lacks its kinase activity (Kistner et al., 1995).

PSD-95 exists in a multimeric state. Palmitoylation of two cysteine residues at the N-terminus of PSD-95, as well as intermolecular interaction between its SH3 and GK domains, mediate head-to-head and tail-to-tail multimerization, respectively, of PSD-95 (Hsueh and Sheng, 1999; McGee et al., 2001; Tavares et al., 2001; Christopherson et al., 2003). Moreover, the minor splice variant of PSD-95 (referred to as PSD-95 β) contains an alternatively spliced sequence at its N-terminus, within which is an L27 domain that mediates protein multimerization in SAP97, and is thought to do so as well in PSD-95 (Chetkovich et al., 2002; Nakagawa et al., 2004). Such multimerization leads to self-assembly, resulting in the formation of PSD-95 clusters, a process that may be important for the nucleation of large, multivalent, PSD-95-interacting scaffolds required for assembly of the PSD.

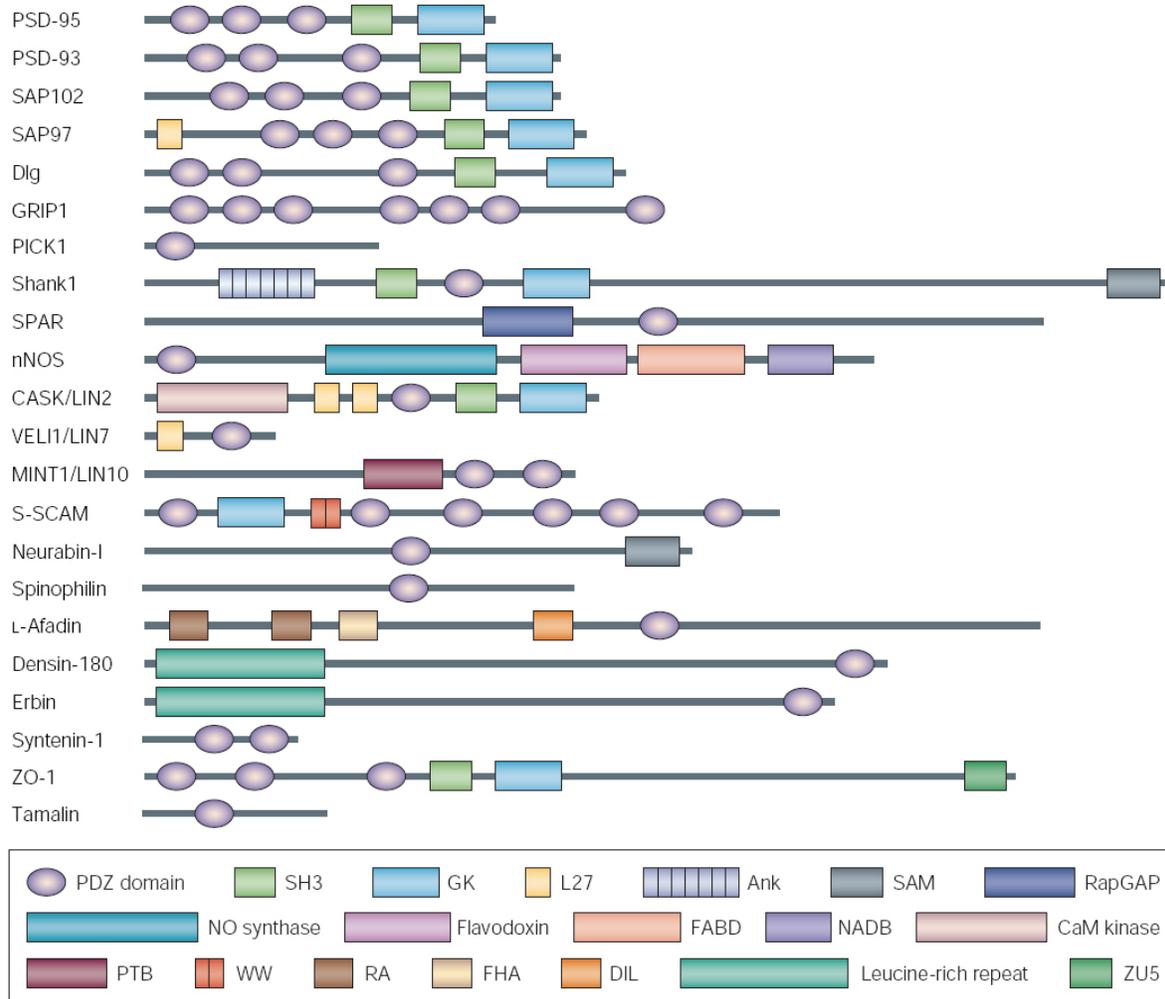


Fig. 1.16. Schematic diagram of PDZ proteins. PDZ domains are often found in scaffold proteins as multiple tandem arrays and/or linked to other kinds of modular protein-interaction domain. Domains are as indicated: Ank, ankyrin repeats; CaM kinase, calmodulin-dependent kinase (CaMK)-like domain; DIL, dilute domain; FABD, FAD binding domain; FHA, forkhead-associated domain; GK, guanylate kinase-like domain; L27, domain initially found in LIN2 and LIN7; NADB, NAD-binding domain; NO, nitric oxide; PTB, phosphotyrosine-binding domain; RA, RAS association domain; RapGAP, Rap GTPase-activating protein; SAM, sterile α motif; SH3, Src homology 3 domain; WW, domain with two conserved Trp (W) residues; ZU5, domain present in ZO-1 and UNC5-like netrin receptors. Adapted with permission from Kim and Sheng, 2004.

Although PSD-95 does not contain any transmembrane regions, immunoelectron microscopy analysis shows that this protein lies within close proximity to the postsynaptic plasma membrane, at a mean distance of 12 nm from the extracellular face (Valtschanoff and Weinberg, 2001; Petersen et al., 2003). This close association of the major PSD-95 isoform (PSD-95 α) with the postsynaptic surface is thought to be achieved via the palmitoylated cysteine residues at its N-terminus (Topinka and Brecht, 1998). Indeed, in addition to the first two PDZ domains and a

targeting motif within the C-terminal of PSD-95, palmitoylation at these sites is required for the proper synaptic accumulation of PSD-95 (Craven et al., 1999).

In addition to it being a member of the MAGUK superfamily, PSD-95 belongs to a smaller family of vertebrate synaptic scaffolding proteins with similar domain organization, comprised of PSD-95, PSD-93/chapsyn-110, SAP97 and SAP102. The various PSD-95 family proteins are differentially distributed within individual neurons. While PSD-95 and PSD-93 are highly concentrated at postsynaptic sites, SAP97 and SAP102 are expressed in both dendrites and axons, and can be found in more diffuse, cytoplasmic expression patterns (El-Husseini et al., 2000b; Valtschanoff et al., 2000; Sans et al., 2001; Rumbaugh et al., 2003). Furthermore, these proteins show distinct temporal expression patterns. For instance, SAP102, which is expressed at high levels shortly after birth, tapers off as development progresses. Conversely, PSD-95 and PSD-93 levels are initially low following birth, but subsequently increase along with the formation of new synapses (Sans et al., 2000). PSD-95 family members also show differences in protein interaction and function *in vivo*. For example, PSD-95 and PSD-93 are associated primarily with NR2A in mature synapses, while SAP102 preferentially interacts with NR2B in immature synapses (Sans et al., 2000; Townsend et al., 2003). Moreover, a study comparing the effects of different PSD-95 family members on presynaptic terminals showed that, compared to PSD-95 and SAP102, overexpression of SAP97 results in the most dramatic increase in the accumulation of active zone and synaptic vesicle proteins (Regalado et al., 2006). This study also showed that the trans-synaptic effect of SAP97 is dependent on a number of CAMs, including cadherins, integrins and EphB/ephrin.

1.6.2 The Role of PSD-95 in Synapse Development and Function

Through its modular, multi-domain structure, PSD-95 regulates several aspects of synapse development and function. Many of the functions of PSD-95 are mediated by its ability to bind to and cluster a wide variety of proteins within the synapse. For instance, overexpression of PSD-95 induces clustering of AMPARs at excitatory postsynaptic sites in both cultured neurons and brain slices (El-Husseini et al., 2000a; Schnell et al., 2002), while knockdown of PSD-95 in brain slices reduces the number of surface GluR2 puncta in spines (Elias et al., 2006). Synaptic clustering of AMPARs by PSD-95 occurs indirectly, however, via the AMPAR-interacting

protein, stargazin, a member of the transmembrane AMPA receptor regulatory protein (TARP) family (Tomita et al., 2003). Mutation of stargazin in mice leads to the Stargazer phenotype, characterized by an absence of epilepsy and cerebellar ataxia, due to reduced surface levels of AMPARs (Chen et al., 2000). Through interaction between the first two PDZ domains of PSD-95 and the C-terminal of stargazin, synaptically localized PSD-95 reduces diffusion of AMPARs along the cell surface, thereby trapping them in the PSD (Schnell et al., 2002; Bats et al., 2007).

PSD-95 has also been implicated in the clustering of NMDARs. In this case, however, although PSD-95 directly interacts with NR2 subunits, its role in the clustering of these receptors is less apparent (El-Husseini et al., 2000a; Nakagawa et al., 2004; Elias et al., 2006). Instead, evidence suggests that PSD-95 may control NMDAR internalization, making it important for modulating synaptic plasticity (Lin et al., 2004). In addition, PSD-95 has been shown to regulate the functional properties of NMDARs, such as receptor gating and desensitization, providing another means through which PSD-95 can regulate synaptic plasticity (Li et al., 2003; Lin et al., 2004). PSD-95 is also known to regulate the clustering and functional aspects of potassium channels, as seen in the case of Kv1.4 and Kir2.3 channels, respectively (Kim et al., 1995; Nehring et al., 2000).

In addition to regulating the clustering and functional properties of multiple surface proteins, PSD-95 interacts with a number of intracellular signaling cascades. For example, PSD-95 forms a tertiary complex with NMDARs and calcium-calmodulin-activated nNOS (Christopherson et al., 1999; Sattler et al., 1999). Formation of this complex may serve to functionally couple influx of calcium through activated NMDARs with NO production. PSD-95 also associates with members of the tyrosine kinase family, which regulate NMDAR activity and NMDAR-dependent synaptic plasticity. It may therefore be the case that PSD-95 mediates access of tyrosine phosphorylation cascades to NMDARs, facilitating phosphorylation-based regulation of these neurotransmitter receptors. Relating to AMPARs, PSD-95 binds to SynGAP, a suppressor of the Ras-extracellular signal-regulated kinase (ERK) that has been implicated in synaptic delivery of AMPARs and regulation of synaptic plasticity (Kim et al., 1998; Komiyama et al., 2002; Zhu et al., 2002). In addition, further implicating PSD-95 and PSD-93 in the regulation of AMPAR-mediated activity, these proteins are known to interact with AKAP79/150, a scaffold protein for PKA, PKC and PP2B. Through PSD-95/93-mediated coupling of GluR1 to the

AKAP79/150 scaffold, PKA, PKC and PP2B are brought in close proximity to AMPARs, where they can exert their regulatory effects on AMPAR activity. Indeed, normal communication between dopaminergic and glutamatergic synapses, for instance, requires GluR1-AKAP79/150-PSD-95 complex formation (Swayze et al., 2004).

PSD-95 is also involved in regulation of spine formation, morphology and dynamics, as evidenced from increases in spine size and density resulting from overexpression of PSD-95 in cultured neurons (El-Husseini et al., 2000a). Conversely, knockdown of PSD-95 in neurons attenuates the increase in spine density and size, as well as the shift from stubby to mushroom spines, that normally occurs between DIV7 and DIV10 *in vitro* (Ehrlich et al., 2007). These effects correlate with a lack of increased synaptic strength typically observed during this stage of development (Ehrlich et al., 2007). Furthermore, knockdown of PSD-95 reduces the increase in spine size induced during LTP in more mature neurons, and spines were less stable in these neurons, resulting in a more immature phenotype (Ehrlich et al., 2007). Providing a possible explanation for the effects of PSD-95 on spine development, PSD-95 has been shown to bind directly to the GEF, kalirin-7, an activator of Rac1 known to promote spine formation. It is unknown, however, how Rac1 is linked to the actin cytoskeleton and how its interaction with PSD-95 ultimately modulates spine growth. Spine-associated RapGAP (SPAR), an inhibitory GAP for the Rap GTPase, is another spine-associated signaling protein that may play a role in PSD-95-mediated spine growth. Although SPAR binds to PSD-95, and degradation of SPAR by the ubiquitin-proteasome pathway leads to reduced PSD-95 clustering and loss of synapses, it is unclear if these proteins cooperate in the regulation of spine development (Pak and Sheng, 2003).

PSD-95 also associates, either directly or indirectly, with other scaffolding proteins within the PSD. Three groups independently identified a small family of proteins that bind directly to the GK domain of PSD-95, and named them guanylate kinase-associated protein (GKAP), SAP90/PSD-95-associated protein (SAPAP) and DLG-associated protein (DAP) (Kim et al., 1997; Naisbitt et al., 1997; Satoh et al., 1997; Takeuchi et al., 1997). GKAP/SAPAP/DAP in turn binds to the Shank/ProSAP family of proteins, via PDZ interaction, providing a link between Shank and PSD-95 (Boeckers et al., 1999; Naisbitt et al., 1999; Tu et al., 1999). Interestingly, the Shank1b isoform has also been implicated in the transformation of filopodia to spine-like

structures (Sala et al., 2001; Arstikaitis et al., 2008). In addition, binding of Shank to Homer, a scaffolding molecule that binds group 1 metabotropic glutamate receptors, may link PSD-95 and ionotropic glutamate receptors to metabotropic glutamate receptor pathways (Naisbitt et al., 1999; Tu et al., 1999; Xiao et al., 2000).

Another new paradigm which has come to light indicates that differential association of neuroligins with scaffolding proteins, including PSD-95, modulates synaptic specificity, and thus, the balance between excitatory and inhibitory synapses (Levinson and El-Husseini, 2005). When PSD-95 and NL1 are coexpressed in hippocampal neurons, the effects of NL1 on inhibitory synapses is abolished, with a corresponding increase in NL1 accumulation at excitatory contacts (Prange et al., 2004; Levinson et al., 2005). Consistent with this, overexpression of PSD-95 alone enhances presynaptic maturation, and electrophysiological recordings of PSD-95 transfected cells show an overall increase in the ratio of excitatory to inhibitory (E/I) synaptic currents (El-Husseini et al., 2000a; Prange et al., 2004). Conversely, RNA knockdown of PSD-95 reduces the E/I synapse ratio (Prange et al., 2004; Gerrow et al., 2006). These observations indicate that relative levels of scaffolding proteins that regulate excitatory synapse maturation may modulate the E/I ratio by sequestering members of the neuroligin family at excitatory contacts at the expense of inhibitory contact formation. Together, the changes observed upon manipulation of the levels of PSD-95 provide new clues to a potential mechanism involved in controlling the E/I ratio.

1.6.3 Other Synaptic Scaffolding Molecules and their Roles in Synaptic Organization

S-SCAM is a synaptic, multidomain PDZ protein with an overall molecular organization similar to that of PSD-95 (Fig. 1.16) (Hirao et al., 1998). Like PSD-95, S-SCAM is a member of the MAGUK family; however, its domain organization is inverted with respect to that of PSD-95, with a GK domain at the N-terminus, followed by two WW protein-protein interaction modules and five C-terminal PDZ domains (Hirao et al., 1998; Ilsley et al., 2002). S-SCAM was initially identified in a yeast two-hybrid screen due to the ability of its GK domain to bind the PSD protein, GKAP/SAPAP/DAP (Hirao et al., 1998). S-SCAM was also found to interact with NMDA receptor subunits through its fifth PDZ domain, and with NL1 via its first PDZ domain, as well as its WW domains (Hirao et al., 1998; Iida et al., 2004). As the binding of NL1 to the

WW domain of S-SCAM is mediated by a region in the middle of the NL1 C-terminal tail, the possibility exists that one NL1 molecule could bind S-SCAM and PSD-95 simultaneously (Iida et al., 2004). It was determined that, in cultured neurons, highly overexpressing a fragment of S-SCAM that contains one of the NL1-binding regions (PDZ1), disrupts the synaptic localization of NL1 and PSD-95, while overexpression of the wild-type form of S-SCAM has the opposite effect, inducing clustering of NL1 (Iida et al., 2004). Overexpression of the cytoplasmic tail of NL1, however, has no effect on the clustering of S-SCAM at synapses. Thus, these data suggest that S-SCAM is involved in the synaptic targeting of NL1, but not vice versa. Other work has demonstrated that β -catenin is as an additional S-SCAM-interacting partner, and is involved in the synaptic localization of S-SCAM (Nishimura et al., 2002). In addition, S-SCAM has recently been implicated in inhibitory synapse development, colocalizing with β -DG and NL2 at GABAergic synaptic sites, suggesting a role for it at both excitatory and inhibitory synapses (Sumita et al., 2006).

Gephyrin is a cytoplasmic multidomain scaffolding protein found almost exclusively at sites apposed to GABAergic and glycinergic presynaptic terminals, and is thought to link inhibitory neurotransmitter receptors to the cytoskeleton, as well as to certain signal transduction pathways involving Cdc42 (Kneussel and Loeblich, 2007). Gephyrin was originally identified in a glycine receptor co-purification assay (Pfeiffer et al., 1982), and has since been shown to be important for clustering of both glycine and GABA_ARs (Kirsch et al., 1993; Essrich et al., 1998; Feng et al., 1998; Kneussel et al., 1999b; Levi et al., 2004; Tretter et al., 2008). RNA depletion of gephyrin, as well as gephyrin knockout in mice, causes severe reductions in glycine receptor accumulation at synaptic and extrasynaptic sites in spinal cord neurons (Kirsch et al., 1993; Feng et al., 1998). This effect correlates with direct binding of gephyrin to glycine receptors via the intracellular loop of the glycine receptor β -subunit (Meyer et al., 1995; Kneussel et al., 1999a). Although no such direct biochemical interaction has been demonstrated in the case of GABA_ARs, various studies indicate that gephyrin is required for normal clustering of these receptors at specific subsets of GABAergic synapses (Essrich et al., 1998; Kneussel et al., 1999b; Kneussel et al., 2001; Levi et al., 2004). Additional proteins such as collybistin and members of the dystrophin glycoprotein complex (DGC) are thought to affect clustering of gephyrin, surface expression and function of GABA_ARs, and form complexes with NL2 and scaffolding molecules

implicated in targeting to inhibitory synapses (Kins et al., 2000; Harvey et al., 2004; Sumita et al., 2006). Gephyrin consists of three major domains, namely an N-terminal domain with sequence similarity to the *E. coli* MogA protein, a C-terminal domain sharing homology with *E. coli* MoeA and a linker region. The majority of proteins shown to interact with gephyrin do so via the MoeA domain, however protein interactions also occur via the linker region (Paarmann et al., 2006). Another inhibitory synaptic protein, collybistin, shows striking homology to Dbl-like GEFs, which regulate small G proteins of the Rho/Rac family. Previous studies have shown that co-expression of gephyrin with collybistin in heterologous cells results in redistribution of gephyrin (Kins et al., 2000; Harvey et al., 2004). In addition, deletion of the RhoGEF domain in collybistin abolished gephyrin-collybistin colocalization and GFP-gephyrin submembrane targeting upon co-expression in HEK cells (Harvey et al., 2004). Furthermore, heterologous expression of collybistin lacking the pleckstrin homology (PH) domain resulted in the production of cytoplasmic collybistin-gephyrin aggregates, indicating the PH domain is critical for normal targeting of this protein (Harvey et al., 2004). Deletion of the PH domain also interfered with clustering of gephyrin in neurons, almost completely eliminating dendritic clusters of endogenous gephyrin (Harvey et al., 2004). Another study showed that collybistin II expression can induce the formation of submembrane gephyrin microaggregates (Kins et al., 2000). These microaggregates resemble those seen in the case of glycine receptor clustering at differentiating postsynaptic sites (Kirsch and Betz, 1995; Bechade et al., 1996). Indeed, the same study showed that peripheral, submembrane collybistin II/gephyrin microaggregates were capable of recruiting glycine receptor α/β subunit heteromers in HEK cells. Thus, collybistin II can cause the formation of submembrane gephyrin clusters that can recruit glycine receptors to the plasma membrane. However, several isoforms of collybistin I and II exist, each lacking or containing an SH3 domain (Harvey et al., 2004); only collybistin II without the SH3 domain-containing cassette is capable of redistributing gephyrin in heterologous cells and neurons (Harvey et al., 2004).

A PDZ domain protein known to be present at both excitatory and inhibitory synapses is GRIP1. GRIP1 was originally identified through its ability to specifically interact with the C-termini of the AMPAR subunits, GluR2 and GluR3 (Dong et al., 1997). GRIP1, which contains 7 PDZ domains, is widely expressed in brain, and is enriched near the PSD in dendritic spines, where it

colocalizes with AMPARs (Dong et al., 1999; Wyszynski et al., 1999; Burette et al., 2001). As such, GRIP1 is thought to be an adaptor protein linking AMPARs to other synaptic proteins, and is required for synaptic clustering of AMPARs and for the activity-dependent synaptic reorganization of AMPARs during LTD (reviewed in (Song and Huganir, 2002)). GRIP1 exists in a number of alternatively spliced isoforms (Fig. 1.17), including cytoplasmically localized, non-palmitoylated forms, and a palmitoylated form that associates with membranes (Yamazaki et al., 2001; Charych et al., 2006). Recently, several of these isoforms, including GRIP1a/b, GRIP1c 4-7, GRIP1d and GRIP1e 4-7 have been identified at GABAergic contacts, some postsynaptically, and some in presynaptic terminals (Yamazaki et al., 2001; Charych et al., 2004; Li et al., 2005; Charych et al., 2006). Although the role of GRIP1 in the recruitment of AMPARs to excitatory synapses has been reasonably well-established, the functions that the various GRIP1 isoforms serve at inhibitory synapses remain unknown.

A growing number of scaffolding proteins are being implicated in the development of both excitatory and inhibitory synapses. As we broaden our knowledge of these molecules and their synaptic binding partners, it is becoming clear that they play important roles as key synapse organizers, both on the pre- and postsynaptic sides. Furthermore, recent evidence indicating that various scaffolding proteins may modulate the balance between excitatory and inhibitory synapse formation suggests that these molecules could mediate functional interactions between different synapse types during their development.

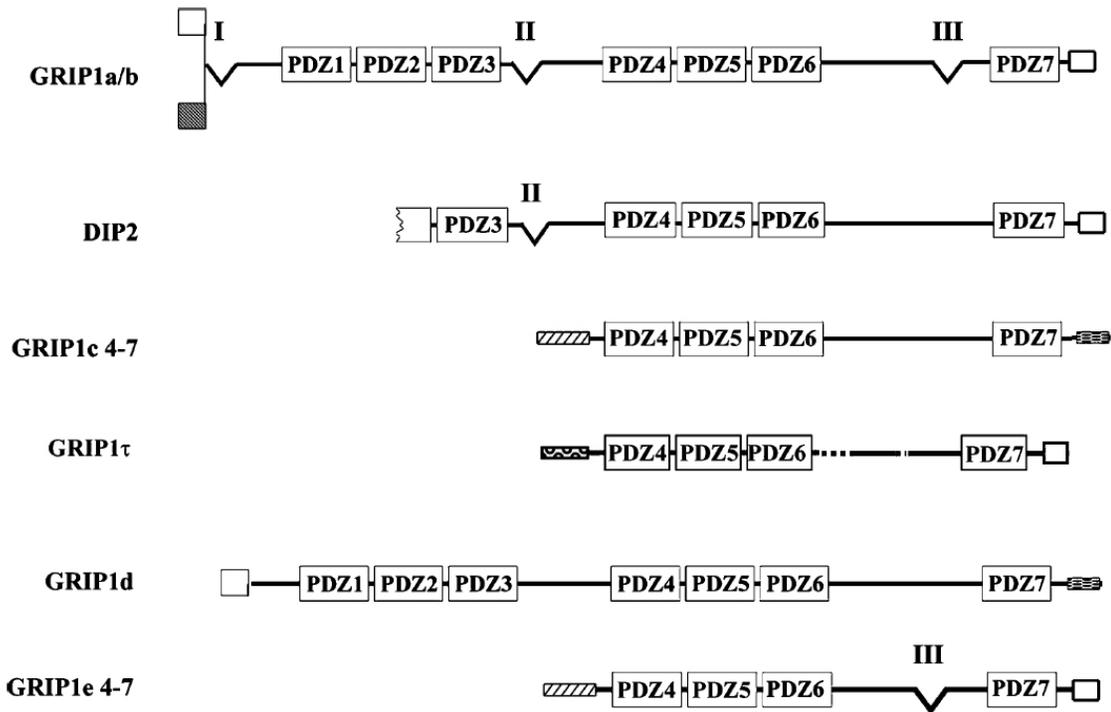


Fig. 1.17. Diagram of various GRIP1 splice variants. All are expressed in brain except GRIP1 τ . GRIP1a and GRIP1b differ in alternate N-terminal 18 and 19 amino acid peptides, as shown by empty and shaded boxes, respectively. However, they share the 12 amino acid C-terminus C1 peptide (empty box). DIP2 is a 90 kDa short form of GRIP1 which is missing the N-terminal leader peptide, PDZ domain 1, part of PDZ domain 2, and a 41-amino acid peptide between the third and fourth PDZ domains of GRIP1a/b. DIP2 is expressed in embryonic forebrain. GRIP1c 4–7 is a short splice variant of GRIP1 with alternate N- (35 amino acids) and C-terminal (12 amino acids) peptide regions (filled boxes). GRIP1c 4–7 has the 12 amino acid C2 peptide at the C-terminus. GRIP1 τ contains four PDZ domains that are identical to those of GRIP1c 4–7, and an N-terminal 35 amino acid region that is 64% identical to the corresponding region of GRIP1c 4–7. The 12-amino acid C-terminus of GRIP1 τ , however, bears no similarity to GRIP1c 4–7, but is identical to the corresponding region of GRIP1a/b. Moreover, it also contains two large deletions between PDZ6 and PDZ7. GRIP1 τ contains the 12-amino acid C1 peptide at the C-terminus (empty box) identical to that of GRIP1a/b. GRIP1d is a 7-PDZ domain-containing splice form of GRIP1a that contains the C-terminal 12-amino acid C2 peptide of GRIP1c 4–7. The GRIP1e 4–7 form is a 4-PDZ domain-containing splice form similar to GRIP1c 4–7, except that it contains the C-terminal 12-amino acid C1 peptide of GRIP1a/b. Additional GRIP1 splice variants may exist because of the presence (Dong et al., 1999; Yamazaki et al., 2001) or absence of other exons encoding 27 (I), 52 (II) and 15 (III) amino acid peptides found in some of the variants. Reproduced with permission from Charych et al., 2006.

1.7 Synapse Balance and Excitatory/Inhibitory Ratio

1.7.1 The Ratio of Excitatory to Inhibitory Synapses and Neuronal Activity

Processing of neural information is thought to occur by integration of excitatory and inhibitory synaptic inputs (Schummers et al., 2002). Almost all brain functions, ranging from motor control and processing of sensory information, to sleep and cognitive processes, require a precise balance between excitation and inhibition. Indeed, normal brain function is disrupted when imbalances in such integration processes occur. For instance, GABA-mediated inhibition is important for proper spatial and temporal control of neuronal signaling patterns in many areas of the mammalian brain (reviewed in (Markram et al., 2004)). In the case of epilepsy, reduced levels of inhibitory signaling are thought to be an underlying basis for seizures, whereby reduced GABA-mediated transmission leads to the spread of fast excitatory discharges throughout a particular brain region (Dichter, 2009). In the case of Parkinson's disease, hyperactive pallidial input onto the motor thalamus results in excessive inhibition, which in turn leads to thalamic cell hyperpolarization, and ultimately to thalamocortical dysrhythmia associated with this disease (Llinas et al., 1999). Synaptic imbalance has also been associated with neurological diseases such as autism (see below) and schizophrenia (Wassef et al., 2003).

The number and distribution of excitatory versus inhibitory contacts that a single neuron receives dictates neuronal excitability and function (Gulledge et al., 2005). However, neurons typically have vast and elaborate dendritic arbours that can receive thousands of synaptic inputs from a broad array of neuronal subtypes. Thus, precise control systems must be established in each neuron to maintain appropriate numbers of excitatory and inhibitory synapses. As such, neurons take exquisite care in outfitting each synapse type with the appropriate structural and neurochemical features (Kim and Sheng, 2004; Luscher and Keller, 2004). Thus, factors that trigger the transformation of initial sites of contact to either excitatory or inhibitory synapses are critical for understanding the processes involved in control of synaptic balance.

1.7.2 Synaptic Imbalance and Neuroligins in Neuropsychiatric Disorders

Mechanisms relating to the formation of excitatory versus inhibitory synapses have important implications in neurodevelopmental psychiatric disorders such as autism spectrum disorders

(ASDs) and some forms of mental retardation, in which an imbalance in E/I ratio is thought to occur (Rubenstein and Merzenich, 2003). In particular, it has been proposed that altered neuronal circuitry due to enhanced excitability in key regions of the brain underlies the expression of abnormal social behaviour and cognitive processes characteristic of ASDs. In support of this idea, a series of recent genetic studies have linked various synaptic proteins to ASDs and other neuropsychiatric disorders (Table 1.1).

ASDs, which include autism and Asperger's syndrome, fall into a group of syndromes referred to as pervasive developmental disorders, and are typically characterized by deficits in communication ability, social interaction and information processing, and are associated with repetitive, stereotyped behavioural patterns and high incidence of seizure (Association, 1994). Chromosomal abnormalities in the regions containing the genes for NL1, NL2, PSD-95 and GABARAP, along with mutations in NL3 and NL4, as well as mutations and deletions in neurexin 1, have been identified in a small number of patients with familial autism (Table 1.1) (Mariner et al., 1986; Konstantareas and Homatidis, 1999; Risch et al., 1999; Thomas et al., 1999; Auranen et al., 2002; Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2004; Feng et al., 2006; Szatmari and al., 2007; Kim et al., 2008; Marshall et al., 2008). A deletion associated with protocadherin 10 has also been linked to ASDs (Morrow et al., 2008). In the case of NL3, an Arg451Cys mutation was identified in two Swedish brothers with ASDs (Jamain et al., 2003); the presence of this mutation results in impaired cell surface trafficking of NL3 protein (Comoletti et al., 2004; Tabuchi et al., 2007; De Jaco et al., 2008). Since the small proportion of NL3 that is successfully trafficked to the surface appears to be functional (Chih et al., 2004; Chubykin et al., 2005), lack of surface-exposed NL3 is thought to be the primary underlying defect in autistic patients carrying this mutation. In further support of a link between this mutation in NL3 and autism, NL3 Arg451Cys knock-in mice showed abnormal social interactions correlated with increased inhibitory synaptic transmission (Tabuchi et al., 2007). Similarly, a more recent study looking at the effects of NL3 knockout in mice found deficits in vocal communication and social memory (Radyushkin et al., 2009). However, behavioural changes associated with loss of NL3 were somewhat limited, in that many of the behaviours typically associated with ASDs in humans were not observed in these mice. In contrast with these studies, however, analysis of a second, independent NL3 Arg451Cys knock-in mouse line

revealed no significant behavioural changes reminiscent of ASDs (Chadman et al., 2008). Thus, although it appears that neuroligin mutations can underlie phenotypes associated with ASDs, a clear understanding of the role of this family in the etiology of these diseases is lacking.

Protein	Link to Autism Spectrum Disorders	References
PSD-95	Linkage in siblings affected with ASD	(Risch et al., 1999)
NL1	Chromosomal abnormality detected in children with autism disorder (replicated at least once in family members with ASDs)	(Konstantareas and Homatidis, 1999; Auranen et al., 2002; Auranen et al., 2003)
NL2	Chromosomal abnormalities detected in patients with autism disorder or mental retardation Linkage in siblings affected with ASD	(Mariner et al., 1986; Risch et al., 1999)
NL3	Point mutation (R451C) identified in two brothers with ASDs	(Jamain et al., 2003)
NL4	De novo chromosomal deletions detected in three males with autism Two brothers with ASDs found with frame shift mutation (D396X) generating a stop codon Found 2-bp deletion (1253delAG) generating a stop codon Four missense mutations found in unrelated patients with ASDs (G99S, F378R, V403M, R704C)	(Thomas et al., 1999; Jamain et al., 2003; Laumonier et al., 2004; Yan et al., 2004; Lawson-Yuen et al., 2008; Marshall et al., 2008)
Neurexin 1	α and β , incomplete penetrance in two independent patients with autism Two putative missense structural variants were identified Hemizygous deletion eliminates coding exons	(Feng et al., 2006; Szatmari and al., 2007; Kim et al., 2008; Marshall et al., 2008)

Table 1.1. Neuroligin-related genetic mutations and chromosomal abnormalities associated with autism spectrum disorders.

1.8 Rationale and Hypothesis

Correct formation of neuronal circuits in the brain, and ultimately proper brain function, depends critically on normal development of synapses. Synaptic development involves a complex series of events, beginning with the initial establishment of a contact site between an axonal terminal and postsynaptic dendrite. Later stages involve the recruitment of a vast array of molecules on both the pre- and postsynaptic sides. In the presynaptic terminal, accumulation of synaptic vesicle machinery and cell adhesion molecules are hallmarks of a developing synapse. On the postsynaptic side, clustering of neurotransmitter receptors, cell adhesion molecules, scaffolding proteins and molecules important for signal transduction and cytoskeletal organization occurs in discrete morphological structures referred to as spines. A role for the neuroligin family of postsynaptic cell adhesion molecules and their presynaptic binding partners, the neuexins, has begun to emerge, implicating these proteins in various stages of CNS synapse development. Indeed, neuroligins have recently been shown to play a role in synaptic maturation and function *in vivo* (Varoqueaux et al., 2006). The roles of neuroligins in synapse development are potentially due to differential sorting of members of this family. Recent findings indeed show that specific members are localized mainly at glutamatergic synapses, while NL2 is enriched at GABAergic synapses (Song et al., 1999; Varoqueaux et al., 2004; Levinson et al., 2005; Varoqueaux et al., 2006). Thus, understanding mechanisms that control sorting of individual family members is not only important for clarifying mechanisms that control trafficking of these proteins to synaptic sites, but may also be important for elucidating mechanisms involved in maintaining the balance between excitation and inhibition. NL1 targeting to excitatory synapses has been shown to be modulated by association with neuexins, as well as with postsynaptic scaffolding molecules enriched at excitatory contacts, such as PSD-95 (Graf et al., 2004; Prange et al., 2004).

I hypothesize that neuroligin-neuexin interaction is important for the development of both glutamatergic and GABAergic synapses, and that differential postsynaptic, intracellular mechanisms may exist to regulate the synaptic sorting of different neuroligin family members. To investigate this hypothesis, I have taken a two-stage approach. In Chapter 2, I have addressed the roles of NL2 and NL3 in synapse formation, as well as examined the contribution of neuexin

to neuroligin-induced inhibitory contact formation. I have also explored the ability of PSD-95 to regulate the synaptic localization of different neuroligin family members. In the third chapter, I have asked the question: Can postsynaptic, intracellular events involving scaffolding molecules specific to glutamatergic or GABAergic contacts differentially control the delivery of neuroligins to distinct synaptic subtypes?

1.9 References

- Adesnik H, Nicoll RA, England PM (2005) Photoinactivation of native AMPA receptors reveals their real-time trafficking. *Neuron* 48:977-985.
- Ahmari SE, Buchanan J, Smith SJ (2000) Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat Neurosci* 3:445-451.
- Angata K, Long JM, Bukalo O, Lee W, Dityatev A, Wynshaw-Boris A, Schachner M, Fukuda M, Marth JD (2004) Sialyltransferase ST8Sia-II assembles a subset of polysialic acid that directs hippocampal axonal targeting and promotes fear behavior. *J Biol Chem* 279:32603-32613.
- Ango F, di Cristo G, Higashiyama H, Bennett V, Wu P, Huang ZJ (2004) Ankyrin-based subcellular gradient of neurofascin, an immunoglobulin family protein, directs GABAergic innervation at purkinje axon initial segment. *Cell* 119:257-272.
- Anne Stephenson F, Cousins SL, Kenny AV (2008) Assembly and forward trafficking of NMDA receptors (Review). *Molecular Membrane Biology* 25:311-320.
- Antonova I, Arancio O, Trillat AC, Wang HG, Zablow L, Udo H, Kandel ER, Hawkins RD (2001) Rapid increase in clusters of presynaptic proteins at onset of long-lasting potentiation. *Science* 294:1547-1550.
- Aoki-Suzuki M, Yamada K, Meerabux J, Iwayama-Shigeno Y, Ohba H, Iwamoto K, Takao H, Toyota T, Suto Y, Nakatani N, Dean B, Nishimura S, Seki K, Kato T, Itohara S, Nishikawa T, Yoshikawa T (2005) A family-based association study and gene expression analyses of netrin-G1 and -G2 genes in schizophrenia. *Biol Psychiatry* 57:382-393.
- Apperson ML, Moon IS, Kennedy MB (1996) Characterization of densin-180, a new brain-specific synaptic protein of the O-sialoglycoprotein family. *J Neurosci* 16:6839-6852.
- Arac D, Boucard AA, Ozkan E, Strop P, Newell E, Sudhof TC, Brunger AT (2007) Structures of neuroligin-1 and the neuroligin-1/neurexin-1 beta complex reveal specific protein-protein and protein-Ca²⁺ interactions. *Neuron* 56:992-1003.
- Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci* 22:208-215.
- Arase N, Takeuchi A, Unno M, Hirano S, Yokosuka T, Arase H, Saito T (2005) Heterotypic interaction of CRTAM with Necl2 induces cell adhesion on activated NK cells and CD8⁺ T cells. *Int Immunol* 17:1227-1237.
- Arikkath J, Reichardt LF (2008) Cadherins and catenins at synapses: roles in synaptogenesis and synaptic plasticity. *Trends Neurosci* 31:487-494.
- Arstikaitis P, Gauthier-Campbell C, Carolina Gutierrez Herrera R, Huang K, Levinson JN, Murphy TH, Kilimann MW, Sala C, Colicos MA, El-Husseini A (2008) Paralemmin-1, a modulator of filopodia induction is required for spine maturation. *Mol Biol Cell* 19:2026-2038.
- Asada H, Kawamura Y, Maruyama K, Kume H, Ding R-G, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K (1997) Cleft palate and decreased brain ¹³C-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. *Proceedings of the National Academy of Sciences of the United States of America* 94:6496-6499.
- Ashby MC, Maier SR, Nishimune A, Henley JM (2006) Lateral diffusion drives constitutive exchange of AMPA receptors at dendritic spines and is regulated by spine morphology. *J Neurosci* 26:7046-7055.

- Ashton AC, Dolly JO (1988) Characterization of the inhibitory action of botulinum neurotoxin type A on the release of several transmitters from rat cerebrocortical synaptosomes. *J Neurochem* 50:1808-1816.
- Association AP (1994) Diagnostic and statistical manual of mental disorders. Washington D.C.: American Psychiatric Press.
- Auranen M, Vanhala R, Varilo T, Ayers K, Kempas E, Ylisaukko-Oja T, Sinsheimer JS, Peltonen L, Jarvela I (2002) A genomewide screen for autism-spectrum disorders: evidence for a major susceptibility locus on chromosome 3q25-27. *Am J Hum Genet* 71:777-790.
- Auranen M, Varilo T, Alen R, Vanhala R, Ayers K, Kempas E, Ylisaukko-Oja T, Peltonen L, Jarvela I (2003) Evidence for allelic association on chromosome 3q25-27 in families with autism spectrum disorders originating from a subisolate of Finland. *Mol Psychiatry* 8:879-884.
- Bamji SX, Shimazu K, Kimes N, Huelsken J, Birchmeier W, Lu B, Reichardt LF (2003) Role of [beta]-Catenin in Synaptic Vesicle Localization and Presynaptic Assembly. *Neuron* 40:719-731.
- Banker GA (1980) Trophic interactions between astroglial cells and hippocampal neurons in culture. *Science* 209:809-810.
- Barresi R, Campbell KP (2006) Dystroglycan: from biosynthesis to pathogenesis of human disease. *J Cell Sci* 119:199-207.
- Bats C, Groc L, Choquet D (2007) The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. *Neuron* 53:719-734.
- Bechade C, Colin I, Kirsch J, Betz H, Triller A (1996) Expression of glycine receptor alpha subunits and gephyrin in cultured spinal neurons. *Eur J Neurosci* 8:429-435.
- Becker CG, Artola A, Gerardy-Schahn R, Becker T, Welzl H, Schachner M (1996) The polysialic acid modification of the neural cell adhesion molecule is involved in spatial learning and hippocampal long-term potentiation. *J Neurosci Res* 45:143-152.
- Beggs HE, Soriano P, Maness PF (1994) NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn-minus mice. *J Cell Biol* 127:825-833.
- Beggs HE, Baragona SC, Hemperly JJ, Maness PF (1997) NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn). *J Biol Chem* 272:8310-8319.
- Bellocchio EE, Reimer RJ, Fremeau RT, Jr., Edwards RH (2000) Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science* 289:957-960.
- Ben-Ari Y (2002) Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci* 3:728-739.
- Benson DL, Tanaka H (1998) N-cadherin redistribution during synaptogenesis in hippocampal neurons. *J Neurosci* 18:6892-6904.
- Biederer T (2006) Bioinformatic characterization of the SynCAM family of immunoglobulin-like domain-containing adhesion molecules. *Genomics* 87:139-150.
- Biederer T, Sara Y, Mozhayeva M, Atasoy D, Liu X, Kavalali ET, Sudhof TC (2002) SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 297:1525-1531.
- Bliss T, Errington M, Fransen E, Godfraind JM, Kauer JA, Kooy RF, Maness PF, Furley AJ (2000) Long-term potentiation in mice lacking the neural cell adhesion molecule L1. *Curr Biol* 10:1607-1610.

- Boeckers TM, Winter C, Smalla KH, Kreutz MR, Bockmann J, Seidenbecher C, Garner CC, Gundelfinger ED (1999) Proline-rich synapse-associated proteins ProSAP1 and ProSAP2 interact with synaptic proteins of the SAPAP/GKAP family. *Biochem Biophys Res Commun* 264:247-252.
- Boles KS, Barchet W, Diacovo T, Cella M, Colonna M (2005) The tumor suppressor TSLC1/NECL-2 triggers NK-cell and CD8⁺ T-cell responses through the cell-surface receptor CRTAM. *Blood* 106:779-786.
- Bolliger MF, Frei K, Winterhalter KH, Gloor SM (2001) Identification of a novel neuroligin in humans which binds to PSD-95 and has a widespread expression. *Biochem J* 356:581-588.
- Bolliger MF, Pei J, Maxeiner S, Boucard AA, Grishin NV, Sudhof TC (2008) Unusually rapid evolution of Neuroligin-4 in mice. *Proc Natl Acad Sci U S A* 105:6421-6426.
- Borg I, Freude K, Kubart S, Hoffmann K, Menzel C, Laccone F, Firth H, Ferguson-Smith MA, Tommerup N, Ropers HH, Sargan D, Kalscheuer VM (2005) Disruption of Netrin G1 by a balanced chromosome translocation in a girl with Rett syndrome. *Eur J Hum Genet* 13:921-927.
- Boucard AA, Chubykin AA, Comoletti D, Taylor P, Sudhof TC (2005) A splice code for trans-synaptic cell adhesion mediated by binding of neuroligin 1 to alpha- and beta-neurexins. *Neuron* 48:229-236.
- Bourne J, Harris KM (2007) Do thin spines learn to be mushroom spines that remember? *Curr Opin Neurobiol* 17:381-386.
- Bowe MA, Fallon JR (1995) The role of agrin in synapse formation. *Annu Rev Neurosci* 18:443-462.
- Bozdagi O, Shan W, Tanaka H, Benson DL, Huntley GW (2000) Increasing numbers of synaptic puncta during late-phase LTP: N-cadherin is synthesized, recruited to synaptic sites, and required for potentiation. *Neuron* 28:245-259.
- Bozdagi O, Valcin M, Poskanzer K, Tanaka H, Benson DL (2004) Temporally distinct demands for classic cadherins in synapse formation and maturation. *Mol Cell Neurosci* 27:509-521.
- Bredt DS, Nicoll RA (2003) AMPA receptor trafficking at excitatory synapses. *Neuron* 40:361-379.
- Bresler T, Shapira M, Boeckers T, Dresbach T, Futter M, Garner CC, Rosenblum K, Gundelfinger ED, Ziv NE (2004) Postsynaptic density assembly is fundamentally different from presynaptic active zone assembly. *J Neurosci* 24:1507-1520.
- Bu DF, Erlander MG, Hitz BC, Tillakaratne NJ, Kaufman DL, Wagner-McPherson CB, Evans GA, Tobin AJ (1992) Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. *Proc Natl Acad Sci U S A* 89:2115-2119.
- Budreck EC, Scheiffele P (2007) Neuroligin-3 is a neuronal adhesion protein at GABAergic and glutamatergic synapses. *Eur J Neurosci* 26:1738-1748.
- Bukalo O, Fentrop N, Lee AY, Salmen B, Law JW, Wotjak CT, Schweizer M, Dityatev A, Schachner M (2004) Conditional ablation of the neural cell adhesion molecule reduces precision of spatial learning, long-term potentiation, and depression in the CA1 subfield of mouse hippocampus. *J Neurosci* 24:1565-1577.
- Burette A, Khatri L, Wyszynski M, Sheng M, Ziff EB, Weinberg RJ (2001) Differential cellular and subcellular localization of ampa receptor-binding protein and glutamate receptor-interacting protein. *J Neurosci* 21:495-503.
- Burgess RW, Nguyen QT, Son YJ, Lichtman JW, Sanes JR (1999) Alternatively spliced isoforms of nerve- and muscle-derived agrin: their roles at the neuromuscular junction. *Neuron* 23:33-44.
- Bushong EA, Martone ME, Jones YZ, Ellisman MH (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J Neurosci* 22:183-192.

- Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J Neurosci* 28:264-278.
- Calakos N, Scheller RH (1996) Synaptic vesicle biogenesis, docking, and fusion: a molecular description. *Physiol Rev* 76:1-29.
- Carlisle HJ, Kennedy MB (2005) Spine architecture and synaptic plasticity. *Trends Neurosci* 28:182-187.
- Chadman KK, Gong S, Scattoni ML, Boltuck SE, Gandhi SU, Heintz N, Crawley JN (2008) Minimal aberrant behavioral phenotypes of neuroligin-3 R451C knockin mice. *Autism Res* 1:147-158.
- Charych EI, Li R, Serwanski DR, Li X, Miralles CP, Pinal N, De Blas AL (2006) Identification and characterization of two novel splice forms of GRIP1 in the rat brain. *J Neurochem* 97:884-898.
- Charych EI, Yu W, Li R, Serwanski DR, Miralles CP, Li X, Yang BY, Pinal N, Walikonis R, De Blas AL (2004) A four PDZ domain-containing splice variant form of GRIP1 is localized in GABAergic and glutamatergic synapses in the brain. *J Biol Chem* 279:38978-38990.
- Chattopadhyaya B, Di Cristo G, Wu CZ, Knott G, Kuhlman S, Fu Y, Palmiter RD, Huang ZJ (2007) GAD67-Mediated GABA Synthesis and Signaling Regulate Inhibitory Synaptic Innervation in the Visual Cortex. *Neuron* 54:889-903.
- Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Brecht DS, Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* 408:936-943.
- Chen X, Vinade L, Leapman RD, Petersen JD, Nakagawa T, Phillips TM, Sheng M, Reese TS (2005) Mass of the postsynaptic density and enumeration of three key molecules. *Proc Natl Acad Sci U S A* 102:11551-11556.
- Chen Y, Aulia S, Li L, Tang BL (2006) AMIGO and friends: an emerging family of brain-enriched, neuronal growth modulating, type I transmembrane proteins with leucine-rich repeats (LRR) and cell adhesion molecule motifs. *Brain Res Rev* 51:265-274.
- Cheng D, Hoogenraad CC, Rush J, Ramm E, Schlager MA, Duong DM, Xu P, Wijayawardana SR, Hanfelt J, Nakagawa T, Sheng M, Peng J (2006) Relative and absolute quantification of postsynaptic density proteome isolated from rat forebrain and cerebellum. *Mol Cell Proteomics* 5:1158-1170.
- Chetkovich DM, Bunn RC, Kuo SH, Kawasaki Y, Kohwi M, Brecht DS (2002) Postsynaptic targeting of alternative postsynaptic density-95 isoforms by distinct mechanisms. *J Neurosci* 22:6415-6425.
- Chih B, Engelman H, Scheiffele P (2005) Control of excitatory and inhibitory synapse formation by neuroligins. *Science* 307:1324-1328.
- Chih B, Gollan L, Scheiffele P (2006) Alternative splicing controls selective trans-synaptic interactions of the neuroligin-neurexin complex. *Neuron* 51:171-178.
- Chih B, Afridi SK, Clark L, Scheiffele P (2004) Disorder-associated mutations lead to functional inactivation of neuroligins. *Hum Mol Genet* 13:1471-1477.
- Christopherson KS, Hillier BJ, Lim WA, Brecht DS (1999) PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. *J Biol Chem* 274:27467-27473.
- Christopherson KS, Sweeney NT, Craven SE, Kang R, El-Husseini Ael D, Brecht DS (2003) Lipid- and protein-mediated multimerization of PSD-95: implications for receptor clustering and assembly of synaptic protein networks. *J Cell Sci* 116:3213-3219.

- Christopherson KS, Ullian EM, Stokes CC, MULLowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120:421-433.
- Chubykin AA, Liu X, Comoletti D, Tsigelny I, Taylor P, Sudhof TC (2005) Dissection of synapse induction by neuroligins: effect of a neuroligin mutation associated with autism. *J Biol Chem* 280:22365-22374.
- Cline HT (2001) Dendritic arbor development and synaptogenesis. *Curr Opin Neurobiol* 11:118-126.
- Cohen I, Rimer M, Lomo T, McMahan UJ (1997) Agrin-induced postsynaptic-like apparatus in skeletal muscle fibers in vivo. *Mol Cell Neurosci* 9:237-253.
- Colicos MA, Collins BE, Sailor MJ, Goda Y (2001) Remodeling of synaptic actin induced by photoconductive stimulation. *Cell* 107:605-616.
- Collingridge GL, Isaac JT, Wang YT (2004) Receptor trafficking and synaptic plasticity. *Nat Rev Neurosci* 5:952-962.
- Colon-Ramos DA, Margeta MA, Shen K (2007) Glia promote local synaptogenesis through UNC-6 (netrin) signaling in *C. elegans*. *Science* 318:103-106.
- Comoletti D, Grishaev A, Whitten AE, Tsigelny I, Taylor P, Trehwella J (2007) Synaptic Arrangement of the Neuroligin/beta-Neurexin Complex Revealed by X-Ray and Neutron Scattering. *Structure* 15:693-705.
- Comoletti D, De Jaco A, Jennings LL, Flynn RE, Gaietta G, Tsigelny I, Ellisman MH, Taylor P (2004) The Arg451Cys-neuroligin-3 mutation associated with autism reveals a defect in protein processing. *J Neurosci* 24:4889-4893.
- Connolly CN, Wafford KA (2004) The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. *Biochem Soc Trans* 32:529-534.
- Covault J, Sanes JR (1986) Distribution of N-CAM in synaptic and extrasynaptic portions of developing and adult skeletal muscle. *J Cell Biol* 102:716-730.
- Craig AM, Banker G (1994) Neuronal polarity. *Annu Rev Neurosci* 17:267-310.
- Craven SE, El-Husseini AE, Brecht DS (1999) Synaptic targeting of the postsynaptic density protein PSD-95 mediated by lipid and protein motifs. *Neuron* 22:497-509.
- Cremer H, Chazal G, Carleton A, Goridis C, Vincent JD, Lledo PM (1998) Long-term but not short-term plasticity at mossy fiber synapses is impaired in neural cell adhesion molecule-deficient mice. *Proc Natl Acad Sci U S A* 95:13242-13247.
- Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR, et al. (1991) Cloning of the gamma-aminobutyric acid (GABA) rho 1 cDNA: a GABA receptor subunit highly expressed in the retina. *Proc Natl Acad Sci U S A* 88:2673-2677.
- Dai Z, Peng HB (1996) Dynamics of synaptic vesicles in cultured spinal cord neurons in relationship to synaptogenesis. *Mol Cell Neurosci* 7:443-452.
- Dailey ME, Smith SJ (1996) The dynamics of dendritic structure in developing hippocampal slices. *J Neurosci* 16:2983-2994.
- Dalva MB, McClelland AC, Kayser MS (2007) Cell adhesion molecules: signalling functions at the synapse. *Nat Rev Neurosci* 8:206-220.

- de Arruda M, Watson S, Lin C, Leavitt J, Matsudaira P (1990) Fimbrin is a homologue of the cytoplasmic phosphoprotein plastin and has domains homologous with calmodulin and actin gelation proteins. *J Cell Biol* 111:1069-1079.
- De Jaco A, Comoletti D, King CC, Taylor P (2008) Trafficking of cholinesterases and neuroligins mutant proteins. An association with autism. *Chem Biol Interact* 175:349-351.
- Dean C, Dresbach T (2006) Neuroligins and neuroligins: linking cell adhesion, synapse formation and cognitive function. *Trends Neurosci* 29:21-29.
- Dean C, Scholl FG, Choih J, DeMaria S, Berger J, Isacoff E, Scheiffele P (2003) Neuroligin mediates the assembly of presynaptic terminals. *Nat Neurosci* 6:708-716.
- DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glass DJ, Burden SJ, Yancopoulos GD (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* 85:501-512.
- Dichter MA (2009) Emerging Concepts in the Pathogenesis of Epilepsy and Epileptogenesis. *Arch Neurol* 66:443-447.
- Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol Rev* 51:7-61.
- Dityatev A, Bukalo O, Schachner M (2008) Modulation of synaptic transmission and plasticity by cell adhesion and repulsion molecules. *Neuron Glia Biol* 4:197-209.
- Dityatev A, Dityateva G, Sytnyk V, Delling M, Toni N, Nikonenko I, Muller D, Schachner M (2004) Polysialylated neural cell adhesion molecule promotes remodeling and formation of hippocampal synapses. *J Neurosci* 24:9372-9382.
- Doherty P, Williams G, Williams EJ (2000) CAMs and axonal growth: a critical evaluation of the role of calcium and the MAPK cascade. *Mol Cell Neurosci* 16:283-295.
- Dong H, O'Brien RJ, Fung ET, Lanahan AA, Worley PF, Huganir RL (1997) GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors. *Nature* 386:279-284.
- Dong H, Zhang P, Song I, Petralia RS, Liao D, Huganir RL (1999) Characterization of the glutamate receptor-interacting proteins GRIP1 and GRIP2. *J Neurosci* 19:6930-6941.
- Duclert A, Changeux JP (1995) Acetylcholine receptor gene expression at the developing neuromuscular junction. *Physiol Rev* 75:339-368.
- Eckhardt M, Bukalo O, Chazal G, Wang L, Goridis C, Schachner M, Gerardy-Schahn R, Cremer H, Dityatev A (2000) Mice deficient in the polysialyltransferase ST8SiaIV/PST-1 allow discrimination of the roles of neural cell adhesion molecule protein and polysialic acid in neural development and synaptic plasticity. *J Neurosci* 20:5234-5244.
- Ehrlich I, Klein M, Rumpel S, Malinow R (2007) PSD-95 is required for activity-driven synapse stabilization. *Proc Natl Acad Sci U S A* 104:4176-4181.
- El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Brecht DS (2000a) PSD-95 involvement in maturation of excitatory synapses. *Science* 290:1364-1368.
- El-Husseini AE, Topinka JR, Lehrer-Graiwer JE, Firestein BL, Craven SE, Aoki C, Brecht DS (2000b) Ion channel clustering by membrane-associated guanylate kinases. Differential regulation by N-terminal lipid and metal binding motifs. *J Biol Chem* 275:23904-23910.

- Elias GM, Funke L, Stein V, Grant SG, Brecht DS, Nicoll RA (2006) Synapse-Specific and Developmentally Regulated Targeting of AMPA Receptors by a Family of MAGUK Scaffolding Proteins. *Neuron* 52:307-320.
- Elste AM, Benson DL (2006) Structural basis for developmentally regulated changes in cadherin function at synapses. *J Comp Neurol* 495:324-335.
- Engert F, Bonhoeffer T (1999) Dendritic spine changes associated with hippocampal long-term synaptic plasticity. *Nature* 399:66-70.
- Eroglu C (2009) The role of astrocyte-secreted extracellular matrix proteins in central nervous system development and function. *J Cell Commun Signal*.
- Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin. *Nat Neurosci* 1:563-571.
- Ethell IM, Pasquale EB (2005) Molecular mechanisms of dendritic spine development and remodeling. *Prog Neurobiol* 75:161-205.
- Fabrichny IP, Leone P, Sulzenbacher G, Comoletti D, Miller MT, Taylor P, Bourne Y, Marchot P (2007) Structural analysis of the synaptic protein neuroligin and its beta-neurexin complex: determinants for folding and cell adhesion. *Neuron* 56:979-991.
- Feng G, Tintrup H, Kirsch J, Nichol MC, Kuhse J, Betz H, Sanes JR (1998) Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. *Science* 282:1321-1324.
- Feng J, Schroer R, Yan J, Song W, Yang C, Bockholt A, Cook EH, Jr., Skinner C, Schwartz CE, Sommer SS (2006) High frequency of neurexin 1beta signal peptide structural variants in patients with autism. *Neurosci Lett* 409:10-13.
- Fiala JC, Feinberg M, Popov V, Harris KM (1998) Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *J Neurosci* 18:8900-8911.
- Filbin MT (2003) Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat Rev Neurosci* 4:703-713.
- Flucher BE, Daniels MP (1989) Distribution of Na⁺ channels and ankyrin in neuromuscular junctions is complementary to that of acetylcholine receptors and the 43 kd protein. *Neuron* 3:163-175.
- Fogel AI, Akins MR, Krupp AJ, Stagi M, Stein V, Biederer T (2007) SynCAMs organize synapses through heterophilic adhesion. *J Neurosci* 27:12516-12530.
- Fremeau RT, Jr., Burman J, Qureshi T, Tran CH, Proctor J, Johnson J, Zhang H, Sulzer D, Copenhagen DR, Storm-Mathisen J, Reimer RJ, Chaudhry FA, Edwards RH (2002) The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proc Natl Acad Sci U S A* 99:14488-14493.
- Friedman HV, Bresler T, Garner CC, Ziv NE (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron* 27:57-69.
- Fujita E, Kouroku Y, Ozeki S, Tanabe Y, Toyama Y, Maekawa M, Kojima N, Senoo H, Toshimori K, Momoi T (2006) Oligo-astheno-teratozoospermia in mice lacking RA175/TSLC1/SynCAM/IGSF4A, a cell adhesion molecule in the immunoglobulin superfamily. *Mol Cell Biol* 26:718-726.
- Furuno T, Ito A, Koma Y, Watabe K, Yokozaki H, Bienenstock J, Nakanishi M, Kitamura Y (2005) The spermatogenic Ig superfamily/synaptic cell adhesion molecule mast-cell adhesion molecule promotes interaction with nerves. *J Immunol* 174:6934-6942.
- Gasnier B (2000) The loading of neurotransmitters into synaptic vesicles. *Biochimie* 82:327-337.

- Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85:525-535.
- Gerrow K, El-Husseini A (2006) Cell adhesion molecules at the synapse. *Front Biosci* 11:2400-2419.
- Gerrow K, Romorini S, Nabi SM, Colicos MA, Sala C, El-Husseini A (2006) A preformed complex of postsynaptic proteins is involved in excitatory synapse development. *Neuron* 49:547-562.
- Gesemann M, Denzer AJ, Ruegg MA (1995) Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. *J Cell Biol* 128:625-636.
- Giesemann T, Schwarz G, Nawrotzki R, Berhorster K, Rothkegel M, Schluter K, Schrader N, Schindelin H, Mendel RR, Kirsch J, Jockusch BM (2003) Complex formation between the postsynaptic scaffolding protein gephyrin, profilin, and Mena: a possible link to the microfilament system. *J Neurosci* 23:8330-8339.
- Gilbert M, Smith J, Roskams AJ, Auld VJ (2001) Neuroligin 3 is a vertebrate gliotactin expressed in the olfactory ensheathing glia, a growth-promoting class of macroglia. *Glia* 34:151-164.
- Gilbert MM, Auld VJ (2005) Evolution of clams (cholinesterase-like adhesion molecules): structure and function during development. *Front Biosci* 10:2177-2192.
- Goda Y, Davis GW (2003) Mechanisms of Synapse Assembly and Disassembly. *Neuron* 40:243-264.
- Graf ER, Kang Y, Hauner AM, Craig AM (2006) Structure function and splice site analysis of the synaptogenic activity of the neurexin-1 beta LNS domain. *J Neurosci* 26:4256-4265.
- Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM (2004) Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 119:1013-1026.
- Groc L, Heine M, Cognet L, Brickley K, Stephenson FA, Lounis B, Choquet D (2004) Differential activity-dependent regulation of the lateral mobilities of AMPA and NMDA receptors. *Nat Neurosci* 7:695-696.
- Gulledge AT, Kampa BM, Stuart GJ (2005) Synaptic integration in dendritic trees. *J Neurobiol* 64:75-90.
- Hall ZW, Sanes JR (1993) Synaptic structure and development: the neuromuscular junction. *Cell* 72 Suppl:99-121.
- Hammond MS, Sims C, Parameshwaran K, Suppiramaniam V, Schachner M, Dityatev A (2006) Neural cell adhesion molecule-associated polysialic acid inhibits NR2B-containing N-methyl-D-aspartate receptors and prevents glutamate-induced cell death. *J Biol Chem* 281:34859-34869.
- Harvey K, Duguid IC, Alldred MJ, Beatty SE, Ward H, Keep NH, Lingenfelter SE, Pearce BR, Lundgren J, Owen MJ, Smart TG, Luscher B, Rees MI, Harvey RJ (2004) The GDP-GTP exchange factor collybistin: an essential determinant of neuronal gephyrin clustering. *J Neurosci* 24:5816-5826.
- Hell JW, Maycox PR, Jahn R (1990) Energy dependence and functional reconstitution of the gamma-aminobutyric acid carrier from synaptic vesicles. *J Biol Chem* 265:2111-2117.
- Hennou S, Khalilov I, Diabira D, Ben-Ari Y, Gozlan H (2002) Early sequential formation of functional GABAA and glutamatergic synapses on CA1 interneurons of the rat foetal hippocampus. *European Journal of Neuroscience* 16:197-208.
- Hering H, Sheng M (2001) Dendritic spines: structure, dynamics and regulation. *Nat Rev Neurosci* 2:880-888.
- Herzog E, Gilchrist J, Gras C, Muzerelle A, Ravassard P, Giros B, Gaspar P, El Mestikawy S (2004) Localization of VGLUT3, the vesicular glutamate transporter type 3, in the rat brain. *Neuroscience* 123:983-1002.
- Higginson JR, Winder SJ (2005) Dystroglycan: a multifunctional adaptor protein. *Biochem Soc Trans* 33:1254-1255.

- Hirao K, Hata Y, Ide N, Takeuchi M, Irie M, Yao I, Deguchi M, Toyoda A, Sudhof TC, Takai Y (1998) A novel multiple PDZ domain-containing molecule interacting with N-methyl-D-aspartate receptors and neuronal cell adhesion proteins. *J Biol Chem* 273:21105-21110.
- Hoch W, Campanelli JT, Harrison S, Scheller RH (1994) Structural domains of agrin required for clustering of nicotinic acetylcholine receptors. *Embo J* 13:2814-2821.
- Hoffman JR, Dixit VM, O'Shea KS (1994) Expression of thrombospondin in the adult nervous system. *J Comp Neurol* 340:126-139.
- Honjo Y, Nakagawa S, Takeichi M (2000) Blockade of cadherin-6B activity perturbs the distribution of PSD-95 family proteins in retinal neurones. *Genes Cells* 5:309-318.
- Hoogenraad CC, Milstein AD, Ethell IM, Henkemeyer M, Sheng M (2005) GRIP1 controls dendrite morphogenesis by regulating EphB receptor trafficking. *Nat Neurosci*.
- Hsueh YP, Sheng M (1999) Requirement of N-terminal cysteines of PSD-95 for PSD-95 multimerization and ternary complex formation, but not for binding to potassium channel Kv1.4. *J Biol Chem* 274:532-536.
- Huang YZ, Wang Q, Xiong WC, Mei L (2001) Erbin is a protein concentrated at postsynaptic membranes that interacts with PSD-95. *J Biol Chem* 276:19318-19326.
- Ichtchenko K, Nguyen T, Sudhof TC (1996) Structures, alternative splicing, and neuroligin binding of multiple neuroligins. *J Biol Chem* 271:2676-2682.
- Ichtchenko K, Hata Y, Nguyen T, Ullrich B, Missler M, Moomaw C, Sudhof TC (1995) Neuroligin 1: a splice site-specific ligand for beta-neurexins. *Cell* 81:435-443.
- Iida J, Hirabayashi S, Sato Y, Hata Y (2004) Synaptic scaffolding molecule is involved in the synaptic clustering of neuroligin. *Mol Cell Neurosci* 27:497-508.
- Ilsley JL, Sudol M, Winder SJ (2002) The WW domain: Linking cell signalling to the membrane cytoskeleton. *Cellular Signalling* 14:183-189.
- Inuzuka H, Miyatani S, Takeichi M (1991) R-cadherin: a novel Ca(2+)-dependent cell-cell adhesion molecule expressed in the retina. *Neuron* 7:69-79.
- Iretton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy F, Reynolds AB (2002) A novel role for p120 catenin in E-cadherin function. *J Cell Biol* 159:465-476.
- Izawa I, Nishizawa M, Ohtakara K, Inagaki M (2002) Densin-180 interacts with delta-catenin/neural plakophilin-related armadillo repeat protein at synapses. *J Biol Chem* 277:5345-5350.
- Jamain S, Quach H, Betancur C, Rastam M, Colineaux C, Gillberg IC, Soderstrom H, Giros B, Leboyer M, Gillberg C, Bourgeron T (2003) Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat Genet* 34:27-29.
- Ji F, Kanbara N, Obata K (1999) GABA and histogenesis in fetal and neonatal mouse brain lacking both the isoforms of glutamic acid decarboxylase. *Neuroscience Research* 33:187-194.
- Johnson CP, Fujimoto I, Rutishauser U, Leckband DE (2005) Direct evidence that neural cell adhesion molecule (NCAM) polysialylation increases intermembrane repulsion and abrogates adhesion. *J Biol Chem* 280:137-145.
- Jonas P (2000) The Time Course of Signaling at Central Glutamatergic Synapses. *News Physiol Sci* 15:83-89.
- Jontes JD, Smith SJ (2000) Filopodia, spines, and the generation of synaptic diversity. *Neuron* 27:11-14.

- Jontes JD, Buchanan J, Smith SJ (2000) Growth cone and dendrite dynamics in zebrafish embryos: early events in synaptogenesis imaged in vivo. *Nat Neurosci* 3:231-237.
- Jontes JD, Emond MR, Smith SJ (2004) In vivo trafficking and targeting of N-cadherin to nascent presynaptic terminals. *J Neurosci* 24:9027-9034.
- Jung D, Yang B, Meyer J, Chamberlain JS, Campbell KP (1995) Identification and Characterization of the Dystrophin Anchoring Site on -Dystroglycan. *Journal of Biological Chemistry* 270:27305-27310.
- Kaether C, Skehel P, Dotti CG (2000) Axonal membrane proteins are transported in distinct carriers: a two-color video microscopy study in cultured hippocampal neurons. *Mol Biol Cell* 11:1213-1224.
- Kakunaga S, Ikeda W, Itoh S, Deguchi-Tawarada M, Ohtsuka T, Mizoguchi A, Takai Y (2005) Nectin-like molecule-1/TSLL1/SynCAM3: a neural tissue-specific immunoglobulin-like cell-cell adhesion molecule localizing at non-junctional contact sites of presynaptic nerve terminals, axons and glia cell processes. *J Cell Sci* 118:1267-1277.
- Katz B, ed (1966) *Nerve, muscle and synapse*. New York: McGraw-Hill.
- Kennedy MB (1997) The postsynaptic density at glutamatergic synapses. *Trends Neurosci* 20:264-268.
- Kim E, Sheng M (2004) PDZ domain proteins of synapses. *Nat Rev Neurosci* 5:771-781.
- Kim E, Niethammer M, Rothschild A, Jan YN, Sheng M (1995) Clustering of Shaker-type K⁺ channels by interaction with a family of membrane-associated guanylate kinases. *Nature* 378:85-88.
- Kim E, Naisbitt S, Hsueh YP, Rao A, Rothschild A, Craig AM, Sheng M (1997) GKAP, a novel synaptic protein that interacts with the guanylate kinase-like domain of the PSD-95/SAP90 family of channel clustering molecules. *J Cell Biol* 136:669-678.
- Kim HG et al. (2008) Disruption of neurexin 1 associated with autism spectrum disorder. *Am J Hum Genet* 82:199-207.
- Kim JH, Huganir RL (1999) Organization and regulation of proteins at synapses. *Curr Opin Cell Biol* 11:248-254.
- Kim JH, Liao D, Lau LF, Huganir RL (1998) SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. *Neuron* 20:683-691.
- Kim S, Burette A, Chung HS, Kwon SK, Woo J, Lee HW, Kim K, Kim H, Weinberg RJ, Kim E (2006) NGL family PSD-95-interacting adhesion molecules regulate excitatory synapse formation. *Nat Neurosci* 9:1294-1301.
- Kins S, Betz H, Kirsch J (2000) Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. *Nat Neurosci* 3:22-29.
- Kirsch J, Betz H (1995) The postsynaptic localization of the glycine receptor-associated protein gephyrin is regulated by the cytoskeleton. *J Neurosci* 15:4148-4156.
- Kirsch J, Malosio ML, Wolters I, Betz H (1993) Distribution of gephyrin transcripts in the adult and developing rat brain. *Eur J Neurosci* 5:1109-1117.
- Kirsch J, Langosch D, Prior P, Littauer UZ, Schmitt B, Betz H (1991) The 93-kDa glycine receptor-associated protein binds to tubulin. *J Biol Chem* 266:22242-22245.
- Kistner U, Garner CC, Linial M (1995) Nucleotide binding by the synapse associated protein SAP90. *FEBS Lett* 359:159-163.
- Kneussel M (2002) Dynamic regulation of GABA(A) receptors at synaptic sites. *Brain Res Brain Res Rev* 39:74-83.

- Kneussel M, Betz H (2000) Receptors, gephyrin and gephyrin-associated proteins: novel insights into the assembly of inhibitory postsynaptic membrane specializations. *J Physiol* 525 Pt 1:1-9.
- Kneussel M, Loeblich S (2007) Trafficking and synaptic anchoring of ionotropic inhibitory neurotransmitter receptors. *Biol Cell* 99:297-309.
- Kneussel M, Hermann A, Kirsch J, Betz H (1999a) Hydrophobic interactions mediate binding of the glycine receptor beta-subunit to gephyrin. *J Neurochem* 72:1323-1326.
- Kneussel M, Brandstatter JH, Laube B, Stahl S, Muller U, Betz H (1999b) Loss of postsynaptic GABA(A) receptor clustering in gephyrin-deficient mice. *J Neurosci* 19:9289-9297.
- Kneussel M, Brandstatter JH, Gasnier B, Feng G, Sanes JR, Betz H (2001) Gephyrin-independent clustering of postsynaptic GABA(A) receptor subtypes. *Mol Cell Neurosci* 17:973-982.
- Knott GW, Holtmaat A, Wilbrecht L, Welker E, Svoboda K (2006) Spine growth precedes synapse formation in the adult neocortex in vivo. *Nat Neurosci* 9:1117-1124.
- Ko J, Kim E (2007) Leucine-rich repeat proteins of synapses. *J Neurosci Res*.
- Ko J, Kim S, Chung HS, Kim K, Han K, Kim H, Jun H, Kaang BK, Kim E (2006) SALM synaptic cell adhesion-like molecules regulate the differentiation of excitatory synapses. *Neuron* 50:233-245.
- Kobe B, Kajava AV (2001) The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol* 11:725-732.
- Komiyama NH, Watabe AM, Carlisle HJ, Porter K, Charlesworth P, Monti J, Strathdee DJ, O'Carroll CM, Martin SJ, Morris RG, O'Dell TJ, Grant SG (2002) SynGAP regulates ERK/MAPK signaling, synaptic plasticity, and learning in the complex with postsynaptic density 95 and NMDA receptor. *J Neurosci* 22:9721-9732.
- Konstantareas MM, Homatidis S (1999) Chromosomal abnormalities in a series of children with autistic disorder. *J Autism Dev Disord* 29:275-285.
- Kornau HC, Seeburg PH, Kennedy MB (1997) Interaction of ion channels and receptors with PDZ domain proteins. *Curr Opin Neurobiol* 7:368-373.
- Kosik KS, Donahue CP, Israely I, Liu X, Ochiishi T (2005) Delta-catenin at the synaptic-adherens junction. *Trends Cell Biol* 15:172-178.
- Kraszewski K, Mundigl O, Daniell L, Verderio C, Matteoli M, De Camilli P (1995) Synaptic vesicle dynamics in living cultured hippocampal neurons visualized with CY3-conjugated antibodies directed against the lumenal domain of synaptotagmin. *J Neurosci* 15:4328-4342.
- Kuramochi M, Fukuhara H, Nobukuni T, Kanbe T, Maruyama T, Ghosh HP, Pletcher M, Isomura M, Onizuka M, Kitamura T, Sekiya T, Reeves RH, Murakami Y (2001) TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. *Nat Genet* 27:427-430.
- Lardi-Studler B, Fritschy J-M (2007) Matching of Pre- and Postsynaptic Specializations during Synaptogenesis. *Neuroscientist* 13:115-126.
- Laumonnier F, Bonnet-Brilhault F, Gomot M, Blanc R, David A, Moizard MP, Raynaud M, Ronce N, Lemonnier E, Calvas P, Laudier B, Chelly J, Fryns JP, Ropers HH, Hamel BC, Andres C, Barthelemy C, Moraine C, Briault S (2004) X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *Am J Hum Genet* 74:552-557.
- Lauren J, Airaksinen MS, Saarma M, Timmusk T (2003) A novel gene family encoding leucine-rich repeat transmembrane proteins differentially expressed in the nervous system. *Genomics* 81:411-421.

- Lawson-Yuen A, Saldivar JS, Sommer S, Picker J (2008) Familial deletion within NLGN4 associated with autism and Tourette syndrome. *Eur J Hum Genet* 16:614-618.
- Leal-Ortiz S, Waites CL, Terry-Lorenzo R, Zamorano P, Gundelfinger ED, Garner CC (2008) Piccolo modulation of Synapsin1a dynamics regulates synaptic vesicle exocytosis. *J Cell Biol* 181:831-846.
- Lee SH, Sheng M (2000) Development of neuron-neuron synapses. *Curr Opin Neurobiol* 10:125-131.
- Levi S, Logan SM, Tovar KR, Craig AM (2004) Gephyrin is critical for glycine receptor clustering but not for the formation of functional GABAergic synapses in hippocampal neurons. *J Neurosci* 24:207-217.
- Levi S, Grady RM, Henry MD, Campbell KP, Sanes JR, Craig AM (2002) Dystroglycan is selectively associated with inhibitory GABAergic synapses but is dispensable for their differentiation. *J Neurosci* 22:4274-4285.
- Levinson JN, El-Husseini A (2005) Building excitatory and inhibitory synapses: balancing neuroligin partnerships. *Neuron* 48:171-174.
- Levinson JN, Chery N, Huang K, Wong TP, Gerrow K, Kang R, Prange O, Wang YT, El-Husseini A (2005) Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. *J Biol Chem* 280:17312-17319.
- Li B, Otsu Y, Murphy TH, Raymond LA (2003) Developmental decrease in NMDA receptor desensitization associated with shift to synapse and interaction with postsynaptic density-95. *J Neurosci* 23:11244-11254.
- Li RW, Serwanski DR, Miralles CP, Li X, Charych E, Riquelme R, Huganir RL, de Blas AL (2005) GRIP1 in GABAergic synapses. *J Comp Neurol* 488:11-27.
- Lin JC, Ho WH, Gurney A, Rosenthal A (2003) The netrin-G1 ligand NGL-1 promotes the outgrowth of thalamocortical axons. *Nat Neurosci* 6:1270-1276.
- Lin Y, Skeberdis VA, Francesconi A, Bennett MV, Zukin RS (2004) Postsynaptic density protein-95 regulates NMDA channel gating and surface expression. *J Neurosci* 24:10138-10148.
- Linhoff MW, Lauren J, Cassidy RM, Dobie FA, Takahashi H, Nygaard HB, Airaksinen MS, Strittmatter SM, Craig AM (2009) An unbiased expression screen for synaptogenic proteins identifies the LRRTM protein family as synaptic organizers. *Neuron* 61:734-749.
- Llinas RR, Ribary U, Jeanmonod D, Kronberg E, Mitra PP (1999) Thalamocortical dysrhythmia: A neurological and neuropsychiatric syndrome characterized by magnetoencephalography. *Proc Natl Acad Sci U S A* 96:15222-15227.
- Lu Q, Paredes M, Medina M, Zhou J, Cavallo R, Peifer M, Orecchio L, Kosik KS (1999) delta-catenin, an adhesive junction-associated protein which promotes cell scattering. *J Cell Biol* 144:519-532.
- Lukasiewicz PD (1996) GABAC receptors in the vertebrate retina. *Mol Neurobiol* 12:181-194.
- Luscher B, Keller CA (2004) Regulation of GABAA receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. *Pharmacol Ther* 102:195-221.
- Luthl A, Laurent JP, Figuero A, Muller D, Schachner M (1994) Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM. *Nature* 372:777-779.
- Malenka RC, Nicoll RA (1999) Long-term potentiation--a decade of progress? *Science* 285:1870-1874.
- Maletic-Savatic M, Malinow R, Svoboda K (1999) Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science* 283:1923-1927.

- Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* 25:103-126.
- Malinow R, Mainen ZF, Hayashi Y (2000) LTP mechanisms: from silence to four-lane traffic. *Curr Opin Neurobiol* 10:352-357.
- Manabe T, Togashi H, Uchida N, Suzuki SC, Hayakawa Y, Yamamoto M, Yoda H, Miyakawa T, Takeichi M, Chisaka O (2000) Loss of cadherin-11 adhesion receptor enhances plastic changes in hippocampal synapses and modifies behavioral responses. *Mol Cell Neurosci* 15:534-546.
- Mariner R, Jackson AW, 3rd, Levitas A, Hagerman RJ, Braden M, McBogg PM, Smith AC, Berry R (1986) Autism, mental retardation, and chromosomal abnormalities. *J Autism Dev Disord* 16:425-440.
- Markram H, Toledo-Rodriguez M, Wang Y, Gupta A, Silberberg G, Wu C (2004) Interneurons of the neocortical inhibitory system. *Nat Rev Neurosci* 5:793-807.
- Marrs GS, Green SH, Dailey ME (2001) Rapid formation and remodeling of postsynaptic densities in developing dendrites. *Nat Neurosci* 4:1006-1013.
- Marshall CR et al. (2008) Structural variation of chromosomes in autism spectrum disorder. *Am J Hum Genet* 82:477-488.
- Matsunami H, Miyatani S, Inoue T, Copeland NG, Gilbert DJ, Jenkins NA, Takeichi M (1993) Cell binding specificity of mouse R-cadherin and chromosomal mapping of the gene. *J Cell Sci* 106 (Pt 1):401-409.
- Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M, Kasai H (2001) Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. *Nat Neurosci* 4:1086-1092.
- Maurel P, Einheber S, Galinska J, Thaker P, Lam I, Rubin MB, Scherer SS, Murakami Y, Gutmann DH, Salzer JL (2007) Nectin-like proteins mediate axon Schwann cell interactions along the internode and are essential for myelination. *J Cell Biol* 178:861-874.
- McAllister AK (2007) Dynamic aspects of CNS synapse formation. *Annu Rev Neurosci* 30:425-450.
- McGee AW, Dakoji SR, Olsen O, Bredt DS, Lim WA, Prehoda KE (2001) Structure of the SH3-guanylate kinase module from PSD-95 suggests a mechanism for regulated assembly of MAGUK scaffolding proteins. *Mol Cell* 8:1291-1301.
- McIntire SL, Jorgensen E, Horvitz HR (1993) Genes required for GABA function in *Caenorhabditis elegans*. *Nature* 364:334-337.
- McIntire SL, Reimer RJ, Schuske K, Edwards RH, Jorgensen EM (1997) Identification and characterization of the vesicular GABA transporter. *Nature* 389:870-876.
- McMahan UJ (1990) The agrin hypothesis. *Cold Spring Harb Symp Quant Biol* 55:407-418.
- Meier T, Hauser DM, Chiquet M, Landmann L, Ruegg MA, Brenner HR (1997) Neural agrin induces ectopic postsynaptic specializations in innervated muscle fibers. *J Neurosci* 17:6534-6544.
- Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA (1995) Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron* 15:805-819.
- Meyer G, Kirsch J, Betz H, Langosch D (1995) Identification of a gephyrin binding motif on the glycine receptor beta subunit. *Neuron* 15:563-572.
- Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RG, Morrison JH, O'Dell TJ, Grant SG (1998) Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* 396:433-439.

- Miller M, Peters A (1981) Maturation of rat visual cortex. II. A combined Golgi-electron microscope study of pyramidal neurons. *J Comp Neurol* 203:555-573.
- Mirsky R, Jessen KR (1996) Schwann cell development, differentiation and myelination. *Curr Opin Neurobiol* 6:89-96.
- Misgeld T, Burgess RW, Lewis RM, Cunningham JM, Lichtman JW, Sanes JR (2002) Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. *Neuron* 36:635-648.
- Missler M, Sudhof TC (1998) Neurexins: three genes and 1001 products. *Trends Genet* 14:20-26.
- Missler M, Fernandez-Chacon R, Sudhof TC (1998) The making of neurexins. *J Neurochem* 71:1339-1347.
- Montanaro F, Carbonetto S (2003) Targeting dystroglycan in the brain. *Neuron* 37:193-196.
- Moore SA, Saito F, Chen J, Michele DE, Henry MD, Messing A, Cohn RD, Ross-Barta SE, Westra S, Williamson RA, Hoshi T, Campbell KP (2002) Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. *Nature* 418:422-425.
- Morimura N, Inoue T, Katayama K, Aruga J (2006) Comparative analysis of structure, expression and PSD95-binding capacity of Lrln, a novel family of neuronal transmembrane proteins. *Gene* 380:72-83.
- Morrow EM et al. (2008) Identifying autism loci and genes by tracing recent shared ancestry. *Science* 321:218-223.
- Moss SJ, Smart TG (2001) Constructing inhibitory synapses. *Nat Rev Neurosci* 2:240-250.
- Muller D, Wang C, Skibo G, Toni N, Cremer H, Calaora V, Rougon G, Kiss JZ (1996) PSA-NCAM is required for activity-induced synaptic plasticity. *Neuron* 17:413-422.
- Murase S, Mosser E, Schuman EM (2002) Depolarization drives beta-Catenin into neuronal spines promoting changes in synaptic structure and function. *Neuron* 35:91-105.
- Murphy-Erdosh C, Yoshida CK, Paradies N, Reichardt LF (1995) The cadherin-binding specificities of B-cadherin and LCAM. *J Cell Biol* 129:1379-1390.
- Murray BA, Hemperly JJ, Prediger EA, Edelman GM, Cunningham BA (1986) Alternatively spliced mRNAs code for different polypeptide chains of the chicken neural cell adhesion molecule (N-CAM). *J Cell Biol* 102:189-193.
- Naisbitt S, Kim E, Weinberg RJ, Rao A, Yang FC, Craig AM, Sheng M (1997) Characterization of guanylate kinase-associated protein, a postsynaptic density protein at excitatory synapses that interacts directly with postsynaptic density-95/synapse-associated protein 90. *J Neurosci* 17:5687-5696.
- Naisbitt S, Kim E, Tu JC, Xiao B, Sala C, Valtschanoff J, Weinberg RJ, Worley PF, Sheng M (1999) Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin. *Neuron* 23:569-582.
- Nakagawa S, Takeichi M (1995) Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development* 121:1321-1332.
- Nakagawa T, Futai K, Lashuel HA, Lo I, Okamoto K, Walz T, Hayashi Y, Sheng M (2004) Quaternary structure, protein dynamics, and synaptic function of SAP97 controlled by L27 domain interactions. *Neuron* 44:453-467.
- Nakashiba T, Nishimura S, Ikeda T, Itohara S (2002) Complementary expression and neurite outgrowth activity of netrin-G subfamily members. *Mech Dev* 111:47-60.

- Nakashiba T, Ikeda T, Nishimura S, Tashiro K, Honjo T, Culotti JG, Itohara S (2000) Netrin-G1: a novel glycosyl phosphatidylinositol-linked mammalian netrin that is functionally divergent from classical netrins. *J Neurosci* 20:6540-6550.
- Nakata T, Terada S, Hirokawa N (1998) Visualization of the dynamics of synaptic vesicle and plasma membrane proteins in living axons. *J Cell Biol* 140:659-674.
- Nam CI, Chen L (2005) Postsynaptic assembly induced by neurexin-neurologin interaction and neurotransmitter. *Proc Natl Acad Sci U S A* 102:6137-6142.
- Nehring RB, Wischmeyer E, Doring F, Veh RW, Sheng M, Karschin A (2000) Neuronal inwardly rectifying K(+) channels differentially couple to PDZ proteins of the PSD-95/SAP90 family. *J Neurosci* 20:156-162.
- Nguyen QT, Son YJ, Sanes JR, Lichtman JW (2000) Nerve terminals form but fail to mature when postsynaptic differentiation is blocked: in vivo analysis using mammalian nerve-muscle chimeras. *J Neurosci* 20:6077-6086.
- Niell CM, Meyer MP, Smith SJ (2004) In vivo imaging of synapse formation on a growing dendritic arbor. *Nat Neurosci* 7:254-260.
- Niethammer M, Kim E, Sheng M (1996) Interaction between the C terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases. *J Neurosci* 16:2157-2163.
- Nishimura EK, Yoshida H, Kunisada T, Nishikawa SI (1999) Regulation of E- and P-cadherin expression correlated with melanocyte migration and diversification. *Dev Biol* 215:155-166.
- Nishimura W, Yao I, Iida J, Tanaka N, Hata Y (2002) Interaction of synaptic scaffolding molecule and Beta-catenin. *J Neurosci* 22:757-765.
- Nitkin RM, Smith MA, Magill C, Fallon JR, Yao YM, Wallace BG, McMahan UJ (1987) Identification of agrin, a synaptic organizing protein from Torpedo electric organ. *J Cell Biol* 105:2471-2478.
- Noguchi J, Matsuzaki M, Ellis-Davies GC, Kasai H (2005) Spine-neck geometry determines NMDA receptor-dependent Ca²⁺ signaling in dendrites. *Neuron* 46:609-622.
- Nollet F, Kools P, van Roy F (2000) Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J Mol Biol* 299:551-572.
- Nusser Z (2000) AMPA and NMDA receptors: similarities and differences in their synaptic distribution. *Curr Opin Neurobiol* 10:337-341.
- Nusser Z, Lujan R, Laube G, Roberts JD, Molnar E, Somogyi P (1998) Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. *Neuron* 21:545-559.
- O'Shea KS, Dixit VM (1988) Unique distribution of the extracellular matrix component thrombospondin in the developing mouse embryo. *J Cell Biol* 107:2737-2748.
- O'Shea KS, Rheinheimer JS, Dixit VM (1990a) Deposition and role of thrombospondin in the histogenesis of the cerebellar cortex. *J Cell Biol* 110:1275-1283.
- O'Shea KS, Liu LH, Kinnunen LH, Dixit VM (1990b) Role of the extracellular matrix protein thrombospondin in the early development of the mouse embryo. *J Cell Biol* 111:2713-2723.
- Ogata T (1988) Structure of motor endplates in the different fiber types of vertebrate skeletal muscles. *Arch Histol Cytol* 51:385-424.

- Ohtakara K, Nishizawa M, Izawa I, Hata Y, Matsushima S, Taki W, Inada H, Takai Y, Inagaki M (2002) Densin-180, a synaptic protein, links to PSD-95 through its direct interaction with MAGUI-1. *Genes Cells* 7:1149-1160.
- Okabe S (2007) Molecular anatomy of the postsynaptic density. *Mol Cell Neurosci* 34:503-518.
- Okabe S, Miwa A, Okado H (2001) Spine formation and correlated assembly of presynaptic and postsynaptic molecules. *J Neurosci* 21:6105-6114.
- Okamura K, Tanaka H, Yagita Y, Saeki Y, Taguchi A, Hiraoka Y, Zeng LH, Colman DR, Miki N (2004) Cadherin activity is required for activity-induced spine remodeling. *J Cell Biol* 167:961-972.
- Okuda T, Yu LM, Cingolani LA, Kemler R, Goda Y (2007) beta-Catenin regulates excitatory postsynaptic strength at hippocampal synapses. *Proc Natl Acad Sci U S A* 104:13479-13484.
- Overduin M, Harvey TS, Bagby S, Tong KI, Yau P, Takeichi M, Ikura M (1995) Solution structure of the epithelial cadherin domain responsible for selective cell adhesion. *Science* 267:386-389.
- Paarmann I, Saiyed T, Schmitt B, Betz H (2006) Gephyrin: does splicing affect its function? *Biochem Soc Trans* 34:45-47.
- Pak DT, Sheng M (2003) Targeted protein degradation and synapse remodeling by an inducible protein kinase. *Science* 302:1368-1373.
- Palmer CL, Cotton L, Henley JM (2005) The molecular pharmacology and cell biology of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Pharmacol Rev* 57:253-277.
- Paradis S, Harrar DB, Lin Y, Koon AC, Hauser JL, Griffith EC, Zhu L, Brass LF, Chen C, Greenberg ME (2007) An RNAi-based approach identifies molecules required for glutamatergic and GABAergic synapse development. *Neuron* 53:217-232.
- Paraoanu LE, Becker-Roeck M, Christ E, Layer PG (2006) Expression patterns of neurexin-1 and neuroligins in brain and retina of the chick embryo: Neuroligin-3 is absent in retina. *Neurosci Lett* 395:114-117.
- Passafaro M, Sala C, Niethammer M, Sheng M (1999) Microtubule binding by CRIPT and its potential role in the synaptic clustering of PSD-95. *Nat Neurosci* 2:1063-1069.
- Passafaro M, Nakagawa T, Sala C, Sheng M (2003) Induction of dendritic spines by an extracellular domain of AMPA receptor subunit GluR2. *Nature* 424:677-681.
- Patel SD, Ciatto C, Chen CP, Bahna F, Rajebhosale M, Arkus N, Schieren I, Jessell TM, Honig B, Price SR, Shapiro L (2006) Type II cadherin ectodomain structures: implications for classical cadherin specificity. *Cell* 124:1255-1268.
- Patton BL (2003) Basal lamina and the organization of neuromuscular synapses. *J Neurocytol* 32:883-903.
- Peifer M, Berg S, Reynolds AB (1994) A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell* 76:789-791.
- Persohn E, Pollerberg GE, Schachner M (1989) Immunoelectron-microscopic localization of the 180 kD component of the neural cell adhesion molecule N-CAM in postsynaptic membranes. *J Comp Neurol* 288:92-100.
- Peters A, Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. *Am J Anat* 127:321-355.
- Petersen JD, Chen X, Vinade L, Dosemeci A, Lisman JE, Reese TS (2003) Distribution of postsynaptic density (PSD)-95 and Ca²⁺/calmodulin-dependent protein kinase II at the PSD. *J Neurosci* 23:11270-11278.

- Pfeiffer F, Graham D, Betz H (1982) Purification by affinity chromatography of the glycine receptor of rat spinal cord. *J Biol Chem* 257:9389-9393.
- Pfriegeer FW, Barres BA (1997) Synaptic efficacy enhanced by glial cells in vitro. *Science* 277:1684-1687.
- Philibert RA, Winfield SL, Sandhu HK, Martin BM, Ginns EI (2000) The structure and expression of the human neuroligin-3 gene. *Gene* 246:303-310.
- Phillips GR, Huang JK, Wang Y, Tanaka H, Shapiro L, Zhang W, Shan WS, Arndt K, Frank M, Gordon RE, Gawinowicz MA, Zhao Y, Colman DR (2001) The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. *Neuron* 32:63-77.
- Pinal CS, Tobin AJ (1998) Uniqueness and redundancy in GABA production. *Perspect Dev Neurobiol* 5:109-118.
- Portera-Cailliau C, Pan DT, Yuste R (2003) Activity-regulated dynamic behavior of early dendritic protrusions: evidence for different types of dendritic filopodia. *J Neurosci* 23:7129-7142.
- Prange O, Wong TP, Gerrow K, Wang YT, El-Husseini A (2004) A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. *Proc Natl Acad Sci U S A* 101:13915-13920.
- Puschel AW, Betz H (1995) Neurexins are differentially expressed in the embryonic nervous system of mice. *J Neurosci* 15:2849-2856.
- Quitsch A, Berhorster K, Liew CW, Richter D, Kreienkamp HJ (2005) Postsynaptic shank antagonizes dendrite branching induced by the leucine-rich repeat protein Densin-180. *J Neurosci* 25:479-487.
- Radyushkin K, Hammerschmidt K, Boretius S, Varoqueaux F, El-Kordi A, Ronnenberg A, Winter D, Frahm J, Fischer J, Brose N, Ehrenreich H (2009) Neuroligin-3-deficient mice: model of a monogenic heritable form of autism with an olfactory deficit. *Genes, Brain and Behavior* 8:416-425.
- Ramon y Cajal S (1888) Estructura de los centros nerviosos de las aves. *Rev Trim Histol Norm Patol* 1:1-10.
- Ramon y Cajal S, ed (1928) Degeneration and regeneration of the nervous system. London: Oxford University Press.
- Rao A, Kim E, Sheng M, Craig AM (1998) Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. *J Neurosci* 18:1217-1229.
- Redies C, Takeichi M (1996) Cadherins in the developing central nervous system: an adhesive code for segmental and functional subdivisions. *Dev Biol* 180:413-423.
- Regalado MP, Terry-Lorenzo RT, Waites CL, Garner CC, Malenka RC (2006) Transsynaptic signaling by postsynaptic synapse-associated protein 97. *J Neurosci* 26:2343-2357.
- Risch N et al. (1999) A genomic screen of autism: evidence for a multilocus etiology. *Am J Hum Genet* 65:493-507.
- Ritzenthaler S, Suzuki E, Chiba A (2000) Postsynaptic filopodia in muscle cells interact with innervating motoneuron axons. *Nat Neurosci* 3:1012-1017.
- Robison AJ, Bass MA, Jiao Y, MacMillan LB, Carmody LC, Bartlett RK, Colbran RJ (2005) Multivalent interactions of calcium/calmodulin-dependent protein kinase II with the postsynaptic density proteins NR2B, densin-180, and alpha-actinin-2. *J Biol Chem* 280:35329-35336.
- Rubenstein JL, Merzenich MM (2003) Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav* 2:255-267.
- Rumbaugh G, Sia GM, Garner CC, Huganir RL (2003) Synapse-associated protein-97 isoform-specific regulation of surface AMPA receptors and synaptic function in cultured neurons. *J Neurosci* 23:4567-4576.

- Sabo SL, Gomes RA, McAllister AK (2006) Formation of presynaptic terminals at predefined sites along axons. *J Neurosci* 26:10813-10825.
- Saffell JL, Williams EJ, Mason IJ, Walsh FS, Doherty P (1997) Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs. *Neuron* 18:231-242.
- Saghatelyan AK, Nikonenko AG, Sun M, Rolf B, Putthoff P, Kutsche M, Bartsch U, Dityatev A, Schachner M (2004) Reduced GABAergic transmission and number of hippocampal perisomatic inhibitory synapses in juvenile mice deficient in the neural cell adhesion molecule L1. *Mol Cell Neurosci* 26:191-203.
- Saglietti L, Dequidt C, Kamieniarz K, Rousset MC, Valnegri P, Thoumine O, Beretta F, Fagni L, Choquet D, Sala C, Sheng M, Passafaro M (2007) Extracellular interactions between GluR2 and N-cadherin in spine regulation. *Neuron* 54:461-477.
- Sagne C, El Mestikawy S, Isambert MF, Hamon M, Henry JP, Giros B, Gasnier B (1997) Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases. *FEBS Lett* 417:177-183.
- Saito Y, Song WJ, Murakami F (1997) Preferential termination of corticorubral axons on spine-like dendritic protrusions in developing cat. *J Neurosci* 17:8792-8803.
- Sala C, Piech V, Wilson NR, Passafaro M, Liu G, Sheng M (2001) Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* 31:115-130.
- Salpeter MM, Loring RH (1985) Nicotinic acetylcholine receptors in vertebrate muscle: Properties, distribution and neural control. *Progress in Neurobiology* 25:297-325.
- Sanes JR, Lichtman JW (1999) Development of the vertebrate neuromuscular junction. *Annual Review of Neuroscience* 22:389-442.
- Sanes JR, Yamagata M (1999) Formation of lamina-specific synaptic connections. *Curr Opin Neurobiol* 9:79-87.
- Sanes JR, Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci* 2:791-805.
- Sans N, Petralia RS, Wang YX, Blahos J, 2nd, Hell JW, Wenthold RJ (2000) A developmental change in NMDA receptor-associated proteins at hippocampal synapses. *J Neurosci* 20:1260-1271.
- Sans N, Racca C, Petralia RS, Wang YX, McCallum J, Wenthold RJ (2001) Synapse-associated protein 97 selectively associates with a subset of AMPA receptors early in their biosynthetic pathway. *J Neurosci* 21:7506-7516.
- Sara Y, Biederer T, Atasoy D, Chubykin A, Mozhayeva MG, Sudhof TC, Kavalali ET (2005) Selective capability of SynCAM and neuroligin for functional synapse assembly. *J Neurosci* 25:260-270.
- Sasaki YF, Rothe T, Premkumar LS, Das S, Cui J, Talantova MV, Wong HK, Gong X, Chan SF, Zhang D, Nakanishi N, Sucher NJ, Lipton SA (2002) Characterization and comparison of the NR3A subunit of the NMDA receptor in recombinant systems and primary cortical neurons. *J Neurophysiol* 87:2052-2063.
- Satoh K, Yanai H, Senda T, Kohu K, Nakamura T, Okumura N, Matsumine A, Kobayashi S, Toyoshima K, Akiyama T (1997) DAP-1, a novel protein that interacts with the guanylate kinase-like domains of hDLG and PSD-95. *Genes Cells* 2:415-424.
- Sattler R, Xiong Z, Lu WY, Hafner M, MacDonald JF, Tymianski M (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science* 284:1845-1848.
- Schafer MK, Varoqui H, Defamie N, Weihe E, Erickson JD (2002) Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. *J Biol Chem* 277:50734-50748.

- Scheiffele P (2003) Cell-cell signaling during synapse formation in the CNS. *Annu Rev Neurosci* 26:485-508.
- Scheiffele P, Fan J, Choih J, Fetter R, Serafini T (2000) Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101:657-669.
- Schnell E, Sizemore M, Karimzadegan S, Chen L, Brecht DS, Nicoll RA (2002) Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. *Proc Natl Acad Sci U S A* 99:13902-13907.
- Schoch S, Gundelfinger ED (2006) Molecular organization of the presynaptic active zone. *Cell Tissue Res* 326:379-391.
- Schummers J, Marino J, Sur M (2002) Synaptic integration by V1 neurons depends on location within the orientation map. *Neuron* 36:969-978.
- Seeburg PH, Higuchi M, Sprengel R (1998) RNA editing of brain glutamate receptor channels: mechanism and physiology. *Brain Res Brain Res Rev* 26:217-229.
- Shaham S (2006) Glia-neuron interactions in the nervous system of *Caenorhabditis elegans*. *Curr Opin Neurobiol* 16:522-528.
- Shan W, Yoshida M, Wu XR, Huntley GW, Colman DR (2002) Neural (N-) cadherin, a synaptic adhesion molecule, is induced in hippocampal mossy fiber axonal sprouts by seizure. *J Neurosci Res* 69:292-304.
- Shapira M, Zhai RG, Dresbach T, Bresler T, Torres VI, Gundelfinger ED, Ziv NE, Garner CC (2003) Unitary assembly of presynaptic active zones from Piccolo-Bassoon transport vesicles. *Neuron* 38:237-252.
- Shapiro L, Kwong PD, Fannon AM, Colman DR, Hendrickson WA (1995a) Considerations on the folding topology and evolutionary origin of cadherin domains. *Proc Natl Acad Sci U S A* 92:6793-6797.
- Shapiro L, Fannon AM, Kwong PD, Thompson A, Lehmann MS, Grubel G, Legrand JF, Als-Nielsen J, Colman DR, Hendrickson WA (1995b) Structural basis of cell-cell adhesion by cadherins. *Nature* 374:327-337.
- Shen K, Fetter RD, Bargmann CI (2004) Synaptic specificity is generated by the synaptic guidepost protein SYG-2 and its receptor, SYG-1. *Cell* 116:869-881.
- Sheng M, Hoogenraad CC (2007) The Postsynaptic Architecture of Excitatory Synapses: A More Quantitative View. *Annual Review of Biochemistry* 76:823-847.
- Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, Malinow R (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science* 284:1811-1816.
- Shingai T, Ikeda W, Kakunaga S, Morimoto K, Takekuni K, Itoh S, Satoh K, Takeuchi M, Imai T, Monden M, Takai Y (2003) Implications of nectin-like molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in cell-cell adhesion and transmembrane protein localization in epithelial cells. *J Biol Chem* 278:35421-35427.
- Soghomonian J-J, Martin DL (1998) Two isoforms of glutamate decarboxylase: why? *J Neurosci* 19:500-505.
- Song I, Huganir RL (2002) Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci* 25:578-588.
- Song JY, Ichtchenko K, Sudhof TC, Brose N (1999) Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc Natl Acad Sci U S A* 96:1100-1105.
- Sperry RW (1963) Chemoaffinity in the Orderly Growth of Nerve Fiber Patterns and Connections. *Proc Natl Acad Sci U S A* 50:703-710.
- Spiegel I, Adamsky K, Eshed Y, Milo R, Sabanay H, Sarig-Nadir O, Horresh I, Scherer SS, Rasband MN, Peles E (2007) A central role for Necl4 (SynCAM4) in Schwann cell-axon interaction and myelination. *Nat Neurosci* 10:861-869.

- Sprengel R, Suchanek B, Amico C, Brusa R, Burnashev N, Rozov A, Hvalby O, Jensen V, Paulsen O, Andersen P, Kim JJ, Thompson RF, Sun W, Webster LC, Grant SG, Eilers J, Konnerth A, Li J, McNamara JO, Seeburg PH (1998) Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. *Cell* 92:279-289.
- Srivastava S, Ziff EB (1999) ABP: a novel AMPA receptor binding protein. *Ann N Y Acad Sci* 868:561-564.
- Sudhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* 375:645-653.
- Sugiyama Y, Kawabata I, Sobue K, Okabe S (2005) Determination of absolute protein numbers in single synapses by a GFP-based calibration technique. *Nat Methods* 2:677-684.
- Sumita K, Sato Y, Iida J, Kawata A, Hamano M, Hirabayashi S, Ohno K, Peles E, Hata Y (2006) Synaptic scaffolding molecule (S-SCAM) membrane-associated guanylate kinase with inverted organization (MAGI)-2 is associated with cell adhesion molecules at inhibitory synapses in rat hippocampal neurons. *J Neurochem* 100:154-166.
- Sun Y, Aiga M, Yoshida E, Humbert PO, Bamji SX (2009) Scribble interacts with beta-catenin to localize synaptic vesicles to synapses. *Mol Biol Cell* 20:3390-3400.
- Sunada Y, Bernier SM, Kozak CA, Yamada Y, Campbell KP (1994) Deficiency of merosin in dystrophic dy mice and genetic linkage of laminin M chain gene to dy locus. *Journal of Biological Chemistry* 269:13729-13732.
- Suzuki A, Yoshida M, Hayashi K, Mizuno Y, Hagiwara Y, Ozawa E (1994) Molecular organization at the glycoprotein-complex-binding site of dystrophin. *European Journal of Biochemistry* 220:283-292.
- Swayze RD, Lise MF, Levinson JN, Phillips A, El-Husseini A (2004) Modulation of dopamine mediated phosphorylation of AMPA receptors by PSD-95 and AKAP79/150. *Neuropharmacology* 47:764-778.
- Szatmari P, al. E (2007) Mapping autism risk loci using genetic linkage and chromosomal rearrangements. *Nat Genet* 39:319-328.
- Tabuchi K, Sudhof TC (2002) Structure and evolution of neurexin genes: insight into the mechanism of alternative splicing. *Genomics* 79:849-859.
- Tabuchi K, Blundell J, Etherton MR, Hammer RE, Liu X, Powell CM, Sudhof TC (2007) A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science* 318:71-76.
- Takahashi H, Sekino Y, Tanaka S, Mizui T, Kishi S, Shirao T (2003) Drebrin-dependent actin clustering in dendritic filopodia governs synaptic targeting of postsynaptic density-95 and dendritic spine morphogenesis. *J Neurosci* 23:6586-6595.
- Takamori S (2006) VGLUTs: []Exciting' times for glutamatergic research? *Neuroscience Research* 55:343-351.
- Takamori S, Rhee JS, Rosenmund C, Jahn R (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* 407:189-194.
- Takeuchi M, Hata Y, Hirao K, Toyoda A, Irie M, Takai Y (1997) SAPAPs. A family of PSD-95/SAP90-associated proteins localized at postsynaptic density. *J Biol Chem* 272:11943-11951.
- Takumi Y, Ramirez-Leon V, Laake P, Rinvik E, Ottersen OP (1999) Different modes of expression of AMPA and NMDA receptors in hippocampal synapses. *Nat Neurosci* 2:618-624.
- Tanaka H, Shan W, Phillips GR, Arndt K, Bozdagi O, Shapiro L, Huntley GW, Benson DL, Colman DR (2000) Molecular modification of N-cadherin in response to synaptic activity. *Neuron* 25:93-107.

- Tang L, Hung CP, Schuman EM (1998) A role for the cadherin family of cell adhesion molecules in hippocampal long-term potentiation. *Neuron* 20:1165-1175.
- Tavares GA, Panepucci EH, Brunger AT (2001) Structural characterization of the intramolecular interaction between the SH3 and guanylate kinase domains of PSD-95. *Mol Cell* 8:1313-1325.
- Thierry JP, Brackenbury R, Rutishauser U, Edelman GM (1977) Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. *J Biol Chem* 252:6841-6845.
- Thomas LA, Akins MR, Biederer T (2008) Expression and adhesion profiles of SynCAM molecules indicate distinct neuronal functions. *J Comp Neurol* 510:47-67.
- Thomas NS, Sharp AJ, Browne CE, Skuse D, Hardie C, Dennis NR (1999) Xp deletions associated with autism in three females. *Hum Genet* 104:43-48.
- Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK, Reynolds AB (2000) Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. *J Cell Biol* 148:189-202.
- Togashi H, Abe K, Mizoguchi A, Takaoka K, Chisaka O, Takeichi M (2002) Cadherin Regulates Dendritic Spine Morphogenesis. *Neuron* 35:77-89.
- Tomita S, Chen L, Kawasaki Y, Petralia RS, Wenthold RJ, Nicoll RA, Brecht DS (2003) Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins. *J Cell Biol* 161:805-816.
- Toni N, Buchs PA, Nikonenko I, Bron CR, Muller D (1999) LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. *Nature* 402:421-425.
- Topinka JR, Brecht DS (1998) N-terminal palmitoylation of PSD-95 regulates association with cell membranes and interaction with K⁺ channel Kv1.4. *Neuron* 20:125-134.
- Townsend M, Yoshii A, Mishina M, Constantine-Paton M (2003) Developmental loss of miniature N-methyl-D-aspartate receptor currents in NR2A knockout mice. *Proc Natl Acad Sci U S A* 100:1340-1345.
- Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, Svoboda K (2002) Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* 420:788-794.
- Tretter V, Jacob TC, Mukherjee J, Fritschy JM, Pangalos MN, Moss SJ (2008) The clustering of GABA(A) receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor alpha 2 subunits to gephyrin. *J Neurosci* 28:1356-1365.
- Tsen G, Halfter W, Kroger S, Cole GJ (1995) Agrin is a heparan sulfate proteoglycan. *J Biol Chem* 270:3392-3399.
- Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M, Worley PF (1999) Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron* 23:583-592.
- Ullian EM, Sapperstein SK, Christopherson KS, Barres BA (2001) Control of synapse number by glia. *Science* 291:657-661.
- Ullrich B, Ushkaryov YA, Sudhof TC (1995) Cartography of neuroligins: more than 1000 isoforms generated by alternative splicing and expressed in distinct subsets of neurons. *Neuron* 14:497-507.
- Unwin N (1998) The nicotinic acetylcholine receptor of the Torpedo electric ray. *J Struct Biol* 121:181-190.

- Urase K, Soyama A, Fujita E, Momoi T (2001) Expression of RA175 mRNA, a new member of the immunoglobulin superfamily, in developing mouse brain. *Neuroreport* 12:3217-3221.
- Ushkaryov YA, Petrenko AG, Geppert M, Sudhof TC (1992) Neurexins: synaptic cell surface proteins related to the alpha-latrotoxin receptor and laminin. *Science* 257:50-56.
- Ushkaryov YA, Hata Y, Ichtchenko K, Moomaw C, Afendis S, Slaughter CA, Sudhof TC (1994) Conserved domain structure of beta-neurexins. Unusual cleaved signal sequences in receptor-like neuronal cell-surface proteins. *J Biol Chem* 269:11987-11992.
- Vaithianathan T, Matthias K, Bahr B, Schachner M, Suppiramaniam V, Dityatev A, Steinhauser C (2004) Neural cell adhesion molecule-associated polysialic acid potentiates alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor currents. *J Biol Chem* 279:47975-47984.
- Valente L, Nishikura K (2005) ADAR gene family and A-to-I RNA editing: diverse roles in posttranscriptional gene regulation. *Prog Nucleic Acid Res Mol Biol* 79:299-338.
- Valenzuela DM, Stitt TN, DiStefano PS, Rojas E, Mattsson K, Compton DL, Nunez L, Park JS, Stark JL, Gies DR, et al. (1995) Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* 15:573-584.
- Valtschanoff JG, Weinberg RJ (2001) Laminar organization of the NMDA receptor complex within the postsynaptic density. *J Neurosci* 21:1211-1217.
- Valtschanoff JG, Burette A, Davare MA, Leonard AS, Hell JW, Weinberg RJ (2000) SAP97 concentrates at the postsynaptic density in cerebral cortex. *Eur J Neurosci* 12:3605-3614.
- Van Aken E, Papeleu P, De Potter P, De Laey JJ, Mareel M (2000) Cadherin expression in the eye. *Bull Soc Belge Ophthalmol*:55-59.
- Varoqueaux F, Jamain S, Brose N (2004) Neuroligin 2 is exclusively localized to inhibitory synapses. *Eur J Cell Biol* 83:449-456.
- Varoqueaux F, Aramuni G, Rawson RL, Mohrmann R, Missler M, Gottmann K, Zhang W, Sudhof TC, Brose N (2006) Neuroligins determine synapse maturation and function. *Neuron* 51:741-754.
- Vaughn JE, Henrikson CK, Grieshaber JA (1974) A quantitative study of synapses on motor neuron dendritic growth cones in developing mouse spinal cord. *J Cell Biol* 60:664-672.
- Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T, Rossetto O, Frassoni C, Matteoli M (2004) SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron* 41:599-610.
- Waites CL, Craig AM, Garner CC (2005) Mechanisms of vertebrate synaptogenesis. *Annu Rev Neurosci* 28:251-274.
- Walikonis RS, Oguni A, Khorosheva EM, Jeng CJ, Asuncion FJ, Kennedy MB (2001) Densin-180 forms a ternary complex with the (alpha)-subunit of Ca²⁺/calmodulin-dependent protein kinase II and (alpha)-actinin. *J Neurosci* 21:423-433.
- Wallace BG (1989) Agrin-induced specializations contain cytoplasmic, membrane, and extracellular matrix-associated components of the postsynaptic apparatus. *J Neurosci* 9:1294-1302.
- Wang CY, Chang K, Petralia RS, Wang YX, Seabold GK, Wenthold RJ (2006) A novel family of adhesion-like molecules that interacts with the NMDA receptor. *J Neurosci* 26:2174-2183.
- Wang J, Arora A, Yang L, Parelkar N, Zhang G, Liu X, Choe E, Mao L (2005) Phosphorylation of AMPA receptors. *Molecular Neurobiology* 32:237-249.

- Washbourne P, Bennett JE, McAllister AK (2002) Rapid recruitment of NMDA receptor transport packets to nascent synapses. *Nat Neurosci* 5:751-759.
- Washbourne P, Dityatev A, Scheiffle P, Biederer T, Weiner JA, Christopherson KS, El-Husseini A (2004) Cell adhesion molecules in synapse formation. *J Neurosci* 24:9244-9249.
- Wassef A, Baker J, Kochan LD (2003) GABA and schizophrenia: a review of basic science and clinical studies. *J Clin Psychopharmacol* 23:601-640.
- Weiner JA, Wang X, Tapia JC, Sanes JR (2005) Gamma protocadherins are required for synaptic development in the spinal cord. *Proc Natl Acad Sci U S A* 102:8-14.
- Whiting PJ, Bonnert TP, McKernan RM, Farrar S, Le Bourdelles B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, Thompson SA, Wafford KA (1999) Molecular and functional diversity of the expanding GABA-A receptor gene family. *Ann N Y Acad Sci* 868:645-653.
- Wong K, Park HT, Wu JY, Rao Y (2002) Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr Opin Genet Dev* 12:583-591.
- Wood SJ, Slater CR (1997) The contribution of postsynaptic folds to the safety factor for neuromuscular transmission in rat fast- and slow-twitch muscles. *J Physiol* 500 (Pt 1):165-176.
- Wu GY, Zou DJ, Rajan I, Cline H (1999) Dendritic dynamics in vivo change during neuronal maturation. *J Neurosci* 19:4472-4483.
- Wyszynski M, Valtschanoff JG, Naisbitt S, Dunah AW, Kim E, Standaert DG, Weinberg R, Sheng M (1999) Association of AMPA receptors with a subset of glutamate receptor-interacting protein in vivo. *J Neurosci* 19:6528-6537.
- Xiao B, Tu JC, Worley PF (2000) Homer: a link between neural activity and glutamate receptor function. *Curr Opin Neurobiol* 10:370-374.
- Yagi T, Takeichi M (2000) Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. *Genes Dev* 14:1169-1180.
- Yamagata M, Sanes JR, Weiner JA (2003) Synaptic adhesion molecules. *Curr Opin Cell Biol* 15:621-632.
- Yamazaki M, Fukaya M, Abe M, Ikeno K, Kakizaki T, Watanabe M, Sakimura K (2001) Differential palmitoylation of two mouse glutamate receptor interacting protein 1 forms with different N-terminal sequences. *Neurosci Lett* 304:81-84.
- Yan J, Oliveira G, Coutinho A, Yang C, Feng J, Katz C, Sram J, Bockholt A, Jones IR, Craddock N, Cook EH, Vicente A, Sommer SS (2004) Analysis of the neurexins 3 and 4 genes in autism and other neuropsychiatric patients. *Mol Psychiatry*.
- Yang X, Li W, Prescott ED, Burden SJ, Wang JC (2000) DNA topoisomerase IIbeta and neural development. *Science* 287:131-134.
- Yang X, Arber S, William C, Li L, Tanabe Y, Jessell TM, Birchmeier C, Burden SJ (2001) Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron* 30:399-410.
- Yin Y, Miner JH, Sanes JR (2002) Laminins: laminin- and netrin-related genes expressed in distinct neuronal subsets. *Mol Cell Neurosci* 19:344-358.
- Yoshida M, Suzuki A, Yamamoto H, Noguchi S, Mizuno Y, Ozawa E (1994) Dissociation of the complex of dystrophin and its associated proteins into several unique groups by *n*-octyl- β -D-glucoside. *European Journal of Biochemistry* 222:1055-1061.

- Yuste R, Bonhoeffer T (2004) Genesis of dendritic spines: insights from ultrastructural and imaging studies. *Nat Rev Neurosci* 5:24-34.
- Zampighi GA, Fain N, Zampighi LM, Cantele F, Lanzavecchia S, Wright EM (2008) Conical electron tomography of a chemical synapse: polyhedral cages dock vesicles to the active zone. *J Neurosci* 28:4151-4160.
- Zhai RG, Vardinon-Friedman H, Cases-Langhoff C, Becker B, Gundelfinger ED, Ziv NE, Garner CC (2001) Assembling the presynaptic active zone: a characterization of an active one precursor vesicle. *Neuron* 29:131-143.
- Zhang Q, Wang J, Fan S, Wang L, Cao L, Tang K, Peng C, Li Z, Li W, Gan K, Liu Z, Li X, Shen S, Li G (2005) Expression and functional characterization of LRRC4, a novel brain-specific member of the LRR superfamily. *FEBS Lett* 579:3674-3682.
- Zhu JJ, Qin Y, Zhao M, Van Aelst L, Malinow R (2002) Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell* 110:443-455.
- Ziv NE, Smith SJ (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* 17:91-102.
- Zuo Y, Lin A, Chang P, Gan WB (2005a) Development of long-term dendritic spine stability in diverse regions of cerebral cortex. *Neuron* 46:181-189.
- Zuo Y, Yang G, Kwon E, Gan WB (2005b) Long-term sensory deprivation prevents dendritic spine loss in primary somatosensory cortex. *Nature* 436:261-265.

2. Neuroligins Mediate Excitatory and Inhibitory Synapse Formation: Involvement of PSD-95 and Neurexin-1 β in Neuroligin Induced Synaptic Specificity¹

2.1 Introduction

Synapse formation is a tightly regulated process that involves the recruitment of specific cell adhesion molecules and scaffolding proteins to newly formed contacts between an axon and a dendrite (Craig and Boudin, 2001; Kim and Sheng, 2004; Washbourne et al., 2004). In the brain, excitatory and inhibitory synaptic transmission is mainly mediated by two neurotransmitters: glutamate which is released at excitatory glutamatergic synaptic contacts, and GABA which is released at inhibitory GABAergic synapses. Initial transformation of a contact to either an excitatory or inhibitory synapse is thought to be controlled by spatial and temporal changes in protein content. This process is critical since an appropriate balance between excitatory and inhibitory synapses is required for proper neuronal excitability and function (Ziv and Smith, 1996; Lee and Sheng, 2000; Craig and Boudin, 2001; Ziv, 2001). However, molecular events that control differentiation of a contact into either an excitatory or inhibitory synapse remain unknown.

The postsynaptic density (PSD) protein, PSD-95, is a molecule that is exclusively localized to glutamatergic synapses and regulates clustering of AMPA receptors through association with stargazin (Chen et al., 2000; El-Husseini et al., 2000a). Through its third PSD-95/Dlg/ZO-1 homology (PDZ) domain, PSD-95 also recruits neuroligin-1 (NL1), a cell adhesion molecule involved in synapse formation (Ichtchenko et al., 1995; Irie et al., 1997; Brose, 1999). These findings indicate that association of PSD-95 with NL1 is involved in excitatory synapse development. Recent work by Prange et al. (2004) showed that NL1 can drive both excitatory and inhibitory presynaptic contact formation (Prange et al., 2004a). These results suggested that NLs are involved in inhibitory synapse formation. Our work also showed that the effects of NL1

¹ A version of this chapter has been published. **Levinson JN**, Chery N, Huang K, Wong TP, Gerrow K, Kang R, Prange O, Wang YT, El-Husseini A. 2005. Neuroligins Mediate Excitatory and Inhibitory Synapse Formation: Involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. *J Biol Chem* **280**:17312-17319.

on postsynaptic differentiation were less dramatic. Overexpression of NL1 modestly increased the total number of excitatory postsynaptic sites but did not enhance clustering of PSD-95 or AMPA receptors (Prange et al., 2004a). In contrast, coexpression of PSD-95 with NL1 coordinated the maturation of pre- and postsynaptic elements and the recruitment of AMPA receptors (Prange et al., 2004a). Moreover, enhanced expression of PSD-95 induced changes in the number of excitatory versus inhibitory synapses and resulted in an overall increase in the ratio of excitatory/inhibitory (E/I) synaptic currents (Prange et al., 2004a). These findings provided the first evidence that assembly of specific postsynaptic elements can regulate a balance between excitatory and inhibitory synapses. Thus, abnormalities in the expression of and/or interactions between these molecules may result in aberrant synapse formation and a change in E/I ratio which underlie complex psychiatric disorders (Rubenstein and Merzenich, 2003). The detection of mutations in NL3 and NL4 in autistic patients suggests that synaptic imbalance may underlie autism (Jamain et al., 2003).

NL1 has additional homologs in rat including NL2 and NL3, which also contain a PDZ-binding site (Irie et al., 1997). Thus, PSD-95 may similarly control the action of NL2 and NL3 in neurons. Binding of NLs to presynaptic neurexin-1 β , a specific isoform of β -neurexin, is required for synaptic contact formation through trans-synaptic heterophilic protein interactions (Ichtchenko et al., 1995; Missler and Sudhof, 1998; Brose, 1999; Rao et al., 2000; Scheiffele et al., 2000). However, it remains unclear whether association of NLs with β -neurexin is involved in the formation of both excitatory and inhibitory presynaptic contacts. Also, it remains unclear whether NLs are localized to both excitatory and inhibitory synapses and whether PSD-95 modulates E/I ratio through regulated distribution of these proteins.

To elucidate the events that regulate synaptic specificity, we have analyzed the role of NLs and PSD-95 in this process. We show that NL1, NL2 and NL3 are capable of inducing both excitatory and inhibitory presynaptic contact formation. The effect on inhibitory synapses was blocked by a soluble form of neurexin-1 β . Moreover, enhanced expression of PSD-95 induced clustering of NL2 and NL3 and shifted endogenous NL2 from inhibitory to excitatory synapses. These results demonstrate that members of the NL family are involved in establishing excitatory and inhibitory synapses, however association with postsynaptic scaffolding proteins regulates the distribution of NLs and control the type of synapses formed.

2.2 Materials and Methods

2.2.1 *cDNA Cloning and Mutagenesis*

The hemagglutinin (HA) tagged wild type NL1 (1ab splice variant) amplified from mouse cerebellum was a gift from Dr. Peter Scheiffele, Columbia University. Generation of GW1 PSD-95 fused to GFP was previously described (Craven et al., 1999; El-Husseini et al., 2000b). Generation of NL2 and NL3 constructs was carried out by PCR using oligos containing BglII and HindIII restriction sites (NL2: GGGCCCAGATCTCGGGGAGGAGGGGGTCCC and GGGCCCAAGCTTCTATAACCCGAGTGGTGGGA; NL3: GGGCCCAGATCTGCCAGTACCCAGGCCCCG and GGGCCCAAGCTTCTATAACACGGGTAGTGGGA) and subcloning the resulting fragments into pEGFP-C1 containing the NL1 signal sequence followed by GFP (Prange et al., 2004a). The final HA-tagged versions were made by removing GFP (using AgeI and BglII) and inserting an HA tag.

2.2.2 *Neuronal cell Culture and Transfections*

Dissociated primary neuronal cultures were prepared from hippocampi of E18/E19 Wistar rats. The hippocampi were dissociated by enzymatic papain digestion followed by brief mechanical trituration. Cells were plated on poly-D-lysine (Sigma) treated glass coverslips. Cultures were maintained in Neurobasal medium (Gibco) supplemented with B27, penicillin, streptomycin, and L-glutamine as described in (Brewer et al., 1993). Hippocampal cultures were transfected by lipid-mediated gene transfer using the Lipofectamine 2000 agent (Gibco) or by the calcium phosphate method (Clontech) at least two days prior to immunostaining.

2.2.3 *Immunocytochemistry*

Coverslips were removed from culture wells and fixed in -20°C methanol. Cells were washed three times with phosphate-buffered saline containing 0.3% Triton-X-100 (PBST) before each antibody incubation. The following primary antibodies were used (immunoreactivity and dilution as indicated): HA (mouse, 1:1000; BABCO), GFP (guinea pig, 1:1000), NL1 (mouse, 1:1000; gift from Dr. Nils. Brose), NL2 (goat, 1:50; Santa Cruz Biotechnology), VGluT1 (rabbit, 1:1000; Synaptic Systems), VGAT (rabbit, 1:1000; Synaptic Systems), PSD-95 (1:500; Affinity

BioReagents), and Shank (guinea pig, 1:500; gift from Dr. Morgan Sheng). Appropriate fluorescently conjugated secondary antibodies were used as previously described (Prange et al., 2004b). All antibody reactions were performed in blocking solution (2% normal goat or horse serum) for 1 hr at room temperature or overnight at 4°C. Coverslips were then mounted on slides (Frost Plus; Fisher) with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL).

2.2.4 Western Blotting and Application of Neurexin Fusion Protein

For immunoblotting, protein samples were harvested in lysis buffer containing 25 mM Tris, 150 mM NaCl, 3 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma), 0.8% Triton X-100, 0.2% SDS and 1 protease inhibitor cocktail tablet/10 ml (Roche). Samples were boiled for 10 min in loading buffer (62.5 mM Tris-HCl, 2% SDS, 1% β -mercaptoethanol, 7.5% glycerol, 15 μ M bromophenol blue), and proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Non-specific binding was blocked by incubating membranes with 3% BSA in Tris buffered saline with 0.1% Tween-20 (TBST) for 1 h. After three washes in TBST, primary antibodies (HA: mouse, 1:1000, NL2: goat, 1:250) were diluted in 3% BSA in TBST, and membranes were incubated for 1 h at room temperature. Membranes were washed three times in TBST, incubated with secondary antibody conjugated to horseradish peroxidase (anti-mouse, 1:2000, Amersham Biosciences; anti-goat, 1:5000, Santa Cruz Biotechnology). Blots were visualized by use of ECL (Pierce). Purification of soluble neurexin-1 β fusion protein and the control FC-IgG protein was carried out as described in Ushkaryov et al. (Ushkaryov et al., 1994). For treatment using the purified proteins, neurons were transfected with the appropriate construct. Transfection medium was then replaced with Neurobasal medium containing either vehicle only (HBSS), neurexin-1 β fusion protein, or FC-IgG (for each well of neurons, approximately 8 μ l of each was added to 500 μ l of Neurobasal medium).

2.2.5 Imaging and Analysis

Images were acquired on a Zeiss Axiovert M200 motorized microscope by using a monochrome 14-bit Zeiss AxioCamHR charge-coupled device camera. In some experiments, exposure times

were individually adjusted to yield an optimum brightness of immunofluorescent clusters without saturation. In other experiments, images were acquired with equal exposure and scaled to the same extent, without saturation. For analysis of cluster density (number), images were analyzed in Northern Eclipse (Empix Imaging, Missauga, Canada), by using custom-written software routines as previously described (Prange et al., 2004a). Briefly, images were processed at a constant threshold level (of 32,000 pixel values) to create a binary image and dendrites of the cell of interest were outlined by using fluorescence signal. Only clusters with average pixel values 2 times greater than corresponding background pixel values were used for analysis. The number of stained clusters was measured as a function of dendritic length. For analysis of changes in NL2 localization, puncta from the NL2 channel were manually outlined, and the intensity of each punctum was subtracted from the dendritic background intensity value, and multiplied by punctum area to obtain an integrated intensity. In the case of cells transfected with PSD-95 GFP, each punctum on the NL2 channel was then scored for colocalization with either PSD-95 GFP or VGAT puncta. Mean intensity of NL2 puncta colocalizing with PSD-95 GFP was then compared to that of NL2 puncta colocalizing with VGAT, and a ratio obtained. In the case of untransfected controls, the same process was conducted for VGluT1-positive or negative NL2 puncta. The ratios for PSD-95 GFP-transfected cells were then compared to those for untransfected controls. For statistical analyses, Mann-Whitney U or Wilcoxin signed ranks tests were used.

2.2.6 Electrophysiology

Recording of miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) were performed at least 2 days post-transfection. Hippocampal neurons on coverslips were transferred to a recording chamber that was continuously perfused with extracellular solution (ECS, pH 7.4; 320-330 mOsm) containing (in mM): 140 NaCl, 5.4 KCl, 1 MgCl₂, 1.3 CaCl₂, 25 HEPES, 33 glucose, 0.0005 tetrodotoxin (Alomone Labs). Transfected cells were identified from their GFP signal under a fluorescence upright microscope. Patch pipettes were pulled from borosilicate glass capillaries (Sutter Instrument) and filled with an intracellular solution (pH 7.2; 300-310 mOsm), composed of (in mM): 115 Cs gluconate, 17.5 CsCl, 10 HEPES, 2 MgCl₂, 10 EGTA, 4 ATP, 0.1 GTP, and 0.1% Lucifer Yellow (Sigma). An Axopatch 200B amplifier (Axon Instruments) was used for the recording. The access resistance

was monitored throughout each experiment. Recordings with a series resistance variation of more than 10% were rejected. No electronic compensation for series resistance was used. Whole-cell patch-clamp recordings were performed in voltage-clamp mode and the membrane potential was maintained at either the reversal potential for GABA_A receptor-mediated mIPSCs (-60 mV) to isolate mEPSCs, or at the reversal potential for ionotropic glutamate receptor-mediated mEPSCs (+10 mV) to isolate mIPSCs. The recorded spontaneous mIPSCs and mEPSCs were blocked completely by the GABA_A receptor antagonist bicuculine (Sigma) and by the ionotropic glutamate receptor antagonist cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma), respectively (data not shown). Recordings were low-pass filtered at 2 kHz, sampled at 10 kHz, and stored in a PC using Clampex 8.2 (Axon). The t-test was used for statistical analysis.

2.3 Results

2.3.1 Neuroligins Drive Excitatory and Inhibitory Presynaptic Contact Formation

Previous work showed that NL1 drives excitatory synapse formation (Scheiffele et al., 2000). However, recently we have revealed the surprising finding that NL1 overexpression can also induce inhibitory presynaptic contact formation (Prange et al., 2004a). These results suggest that NL1 may be involved *in vivo* in establishing both excitatory and inhibitory synapses. NL2 and NL3 are two other known members of the NL family expressed in the brain (Ichtchenko et al., 1996). To explore whether the effect on inhibitory synapses is unique to NL1, we analyzed the effects of hemagglutinin (HA)-tagged NL2 (HA-NL2) and NL3 (HA-NL3) on both excitatory and inhibitory synapse formation. For this analysis, days *in vitro* (DIV) 5 hippocampal neurons were transfected with HA-NL2 and HA-NL3; fixed at DIV 8 and stained for either the vesicular gamma aminobutyric acid (GABA) transporter (VGAT), a marker for inhibitory synapses; or the vesicular glutamate transporter (VGluT1), a marker for excitatory synapses. Remarkably, HA-NL2 and HA-NL3 overexpression significantly enhanced the average number of contacting presynaptic boutons; both excitatory (1.6 ± 0.1 -fold for HA-NL2 and 1.5 ± 0.2 -fold for HA-NL3) and inhibitory (3.0 ± 0.9 -fold for HA-NL2 and 2.7 ± 0.4 -fold for HA-NL3), when compared to GFP-transfected cells (Fig. 2.1). Other changes observed include enhanced number of dendritic filopodia (data not shown). These results demonstrate that various members of the NL family exert similar effects on establishing new synaptic contacts, regardless of type.

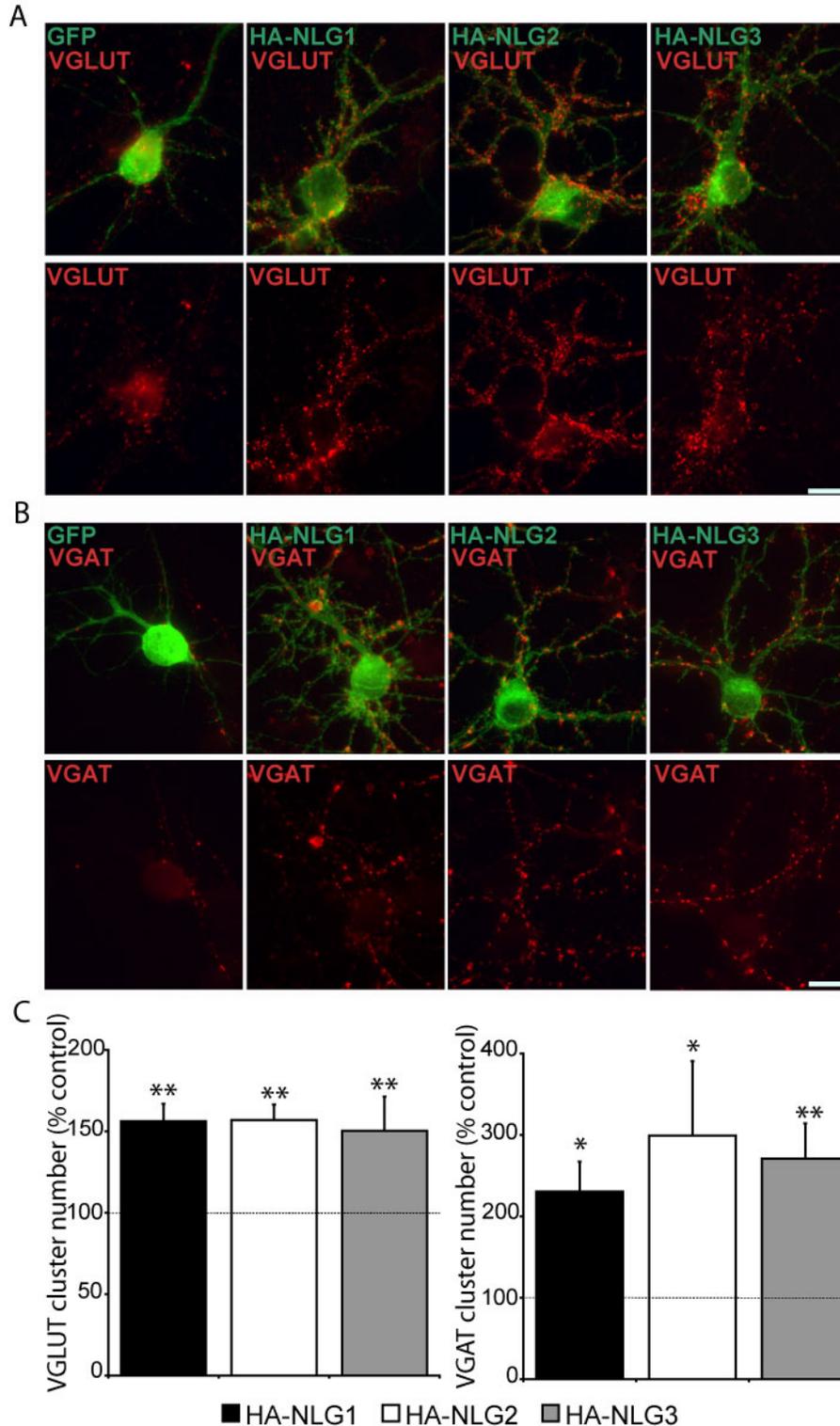


Fig. 2.1. Members of the NL family drive excitatory and inhibitory synapse formation.

DIV 5 hippocampal neurons were transfected with either GFP, HA-NL1, HA-NL2, or HA-NL3, and then fixed at DIV 8. (A,B) Cells were then stained for either (A) VGLuT1 or (B) VGAT. The number of VGLuT1-positive terminals contacting dendrites of neurons transfected with either HA-NL construct was enhanced as compared to GFP-transfected cells (GFP, n = 14; NL1 and NL2, n = 10; NL3, n = 16). An increase in VGAT positive puncta was also observed (GFP, n = 12; NL1-3, n = 10). (C) Quantification of changes in number of VGLuT1- and VGAT-positive terminals contacting transfected cells. Dashed lines indicate 100% (GFP control) levels. **, p < 0.01; *, p < 0.05. Scale bars: 10 μ m.

2.3.2 NL-Induced Inhibitory Synapse Formation is Mediated by Neurexin-1 β

The differential recruitment of proteins to their respective synaptic compartments is likely to be mediated by heterotypic trans-synaptic signaling. NLs have been shown to associate with neurexin-1 β and this interaction leads to the recruitment of elements required for the structural reorganization of presynaptic compartments (Ichtchenko et al., 1996; Irie et al., 1997; Butz et al., 1998; Scheiffele et al., 2000). Work performed by Dean et al. (Dean et al., 2003) demonstrated that synapse formation involves direct interaction between NL1 and neurexin-1 β and that the effects of NL1 on excitatory synapses are blocked using an FC fusion protein containing the extracellular domain of neurexin-1 β (Scheiffele et al., 2000; Dean et al., 2003). However, it remains unclear how NLs influence the maturation of GABA presynaptic terminals. Previous studies showed that β -neurexins are also expressed by inhibitory neurons (Ullrich et al., 1995). Moreover, recent work by Graf et al. (Graf et al., 2004) showed that expression of neurexin-1 β in non-neuronal cells can drive clustering of NLs and several other excitatory and inhibitory postsynaptic proteins in neurons. To examine whether NL interaction with neurexin-1 β is required for inhibitory synapse formation *in vivo*, DIV 6 neurons were transfected with either HA-NL1 or HA-NL2 and then incubated in a medium containing either vehicle solution, 30 μ g/ml FC-IgG (control), or 30 μ g/ml of a soluble form of neurexin-1 β lacking splice site 4 fused to FC-IgG (NXN-FC; Fig. 2.2).

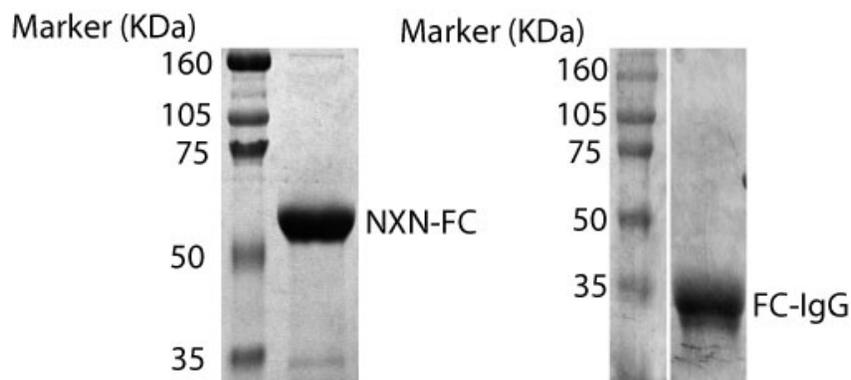


Fig. 2.2. Purification of a soluble fragment of neurexin-1 β . Constructs expressing FC-IgG and neurexin-1 β -FC (NXN-FC) fusion proteins were transfected into COS cells and secreted fusion proteins were purified from cell medium as described in Materials in Methods. Purified proteins were subjected to SDS-PAGE and stained with Coomassie Blue.

Two days post-transfection, neurons were fixed and analyzed for induction of inhibitory synapses. Remarkably, inhibitory synapse formation mediated by either NL1 or NL2 was dramatically diminished upon incubation with NXN-FC (Fig. 2.3 and 2.4). This was manifested by a decrease in the number of VGAT-positive puncta contacting dendrites of cells transfected with either construct as compared to vehicle-treated controls (Fig. 2.3A and B; $34\pm7\%$ of control for NL1 and $39\pm9\%$ of control for NL2). Moreover, no significant effect of FC-IgG treatment was observed (Fig. 2.3B and 2.4).

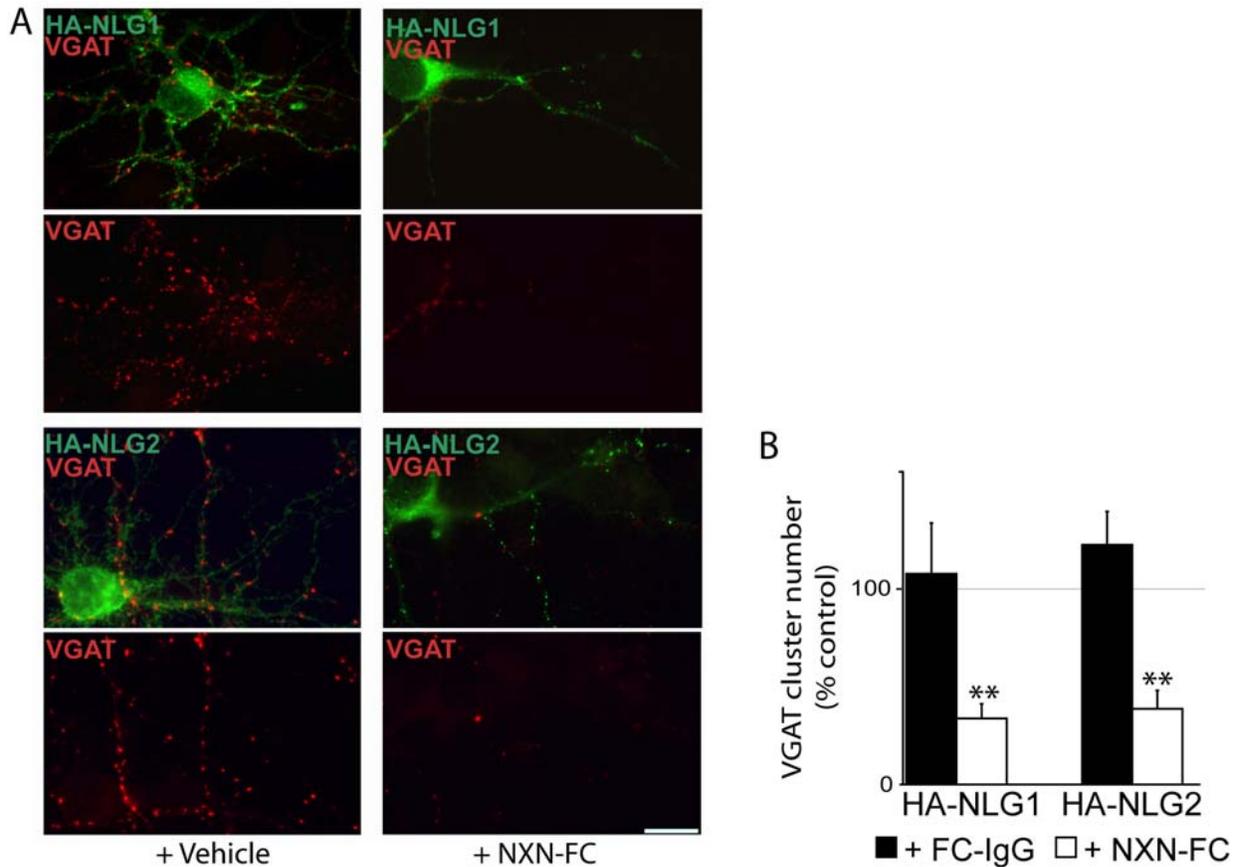


Fig. 2.3. NL induced inhibitory synapse formation involves neurexin-1 β . DIV 6 hippocampal neurons were transfected with either HA-NL1 or HA-NL2, and incubated with medium containing either 30 μ g/ml FC-IgG, or 30 μ g/ml FC-neurexin-1 β peptide (NXN-FC), or HBSS (vehicle) only. Cells were fixed two days following treatment, and then immunostained with HA and VGAT antibodies. (A,B) Addition of NXN-FC results in a decrease in the number of VGAT-positive terminals contacting dendrites of HA-NL1 (vehicle, n = 10; FC-IgG, n = 8; NXN-FC, n = 11) or HA-NL2 (vehicle, n = 10; FC-IgG, n = 13; NXN-FC, n = 10) transfected cells. (B) Quantification of changes in number of VGAT-positive terminals contacting transfected cells presented as % of control (vehicle; dotted line). $P < 0.01$. Scale bars: 10 μ m.

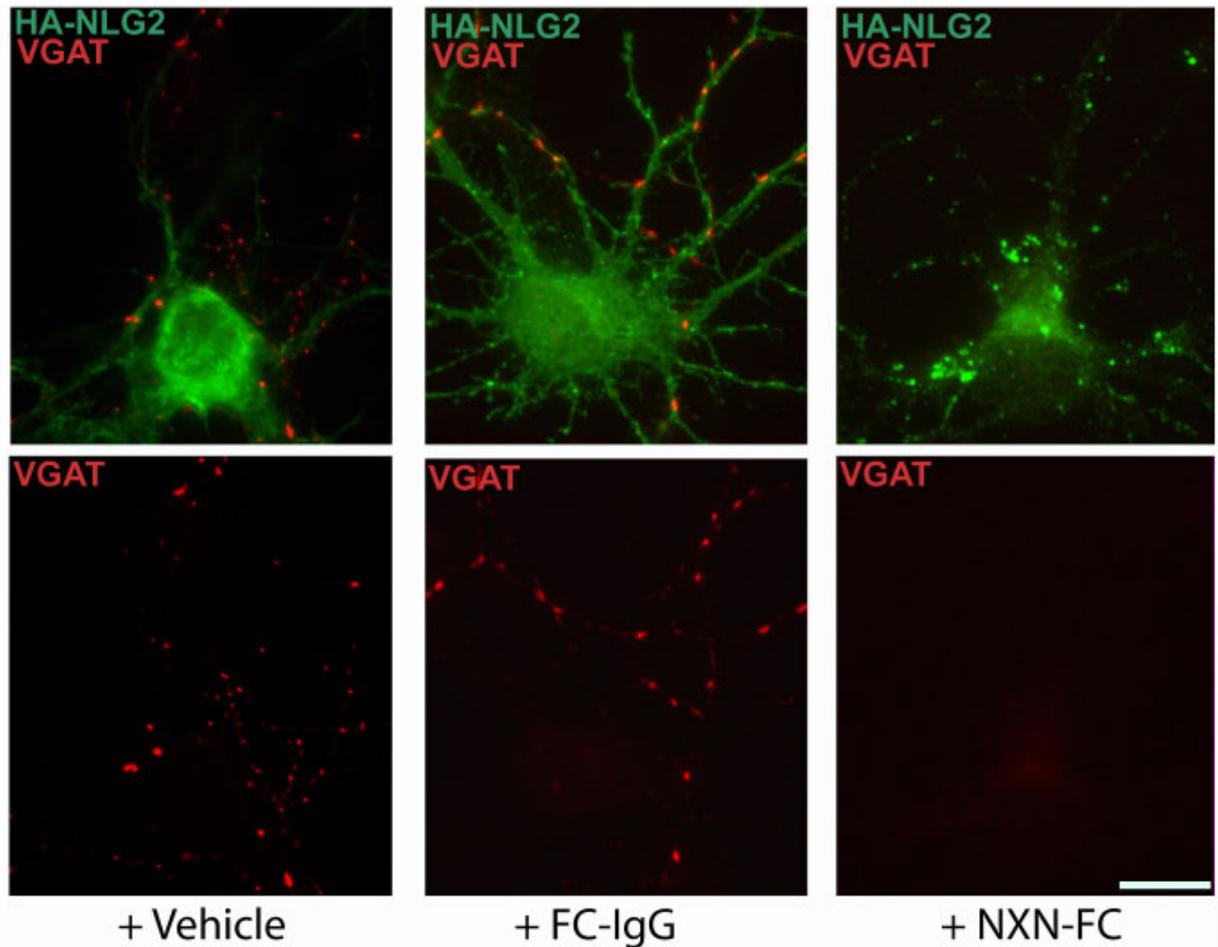


Fig. 2.4. A soluble form of neurexin blocks NL-induced inhibitory contacts. DIV 6 hippocampal neurons were transfected with HA-NL2 and incubated with medium containing either 30 $\mu\text{g/ml}$ FC-IgG, 30 $\mu\text{g/ml}$ NXN-FC, or HBSS (vehicle) only. Cells were fixed two days following treatment, and then immunostained with HA and VGAT antibodies. Addition of NXN-FC results in a decrease in the number of VGAT-positive terminals contacting dendrites of HA-NL2 transfected cells. Scale bars: 10 μm .

These results show that a neurexin-1 β -dependent interaction regulates NL-induced inhibitory synapse formation. Significantly, treatment of GFP-transfected cells with NXN-FC resulted in a decrease in the number of inhibitory synapses ($51\pm 9\%$ of control) when compared to GFP cells treated with vehicle only (Fig. 2.5). This is further evidence that neurexin-1 β is involved in the formation of inhibitory synapses *in vivo*.

Treatment with NXN-FC also induced aggregation of HA-NL1 and HA-NL2 (Fig. 2.4). This phenomenon is consistent with that observed in the case of the integrin transmembrane cell-

adhesion receptors, where ligand binding induces aggregation of the receptor (Miyamoto et al., 1995). Interestingly, NL clusters were present at sites positive for PSD-95 but lacking synaptophysin staining, indicating non-synaptic localization (Fig. 2.6A and B). Similar co-clustering of NLs and PSD-95 by neurexin-1 β was recently reported (Graf et al., 2004). The aggregation of NLs and PSD-95 upon treatment with NXN-FC is intriguing since NL expression alone was not sufficient to enhance PSD-95 clustering (Fig. 2.7). Further work is required to clarify these differences.

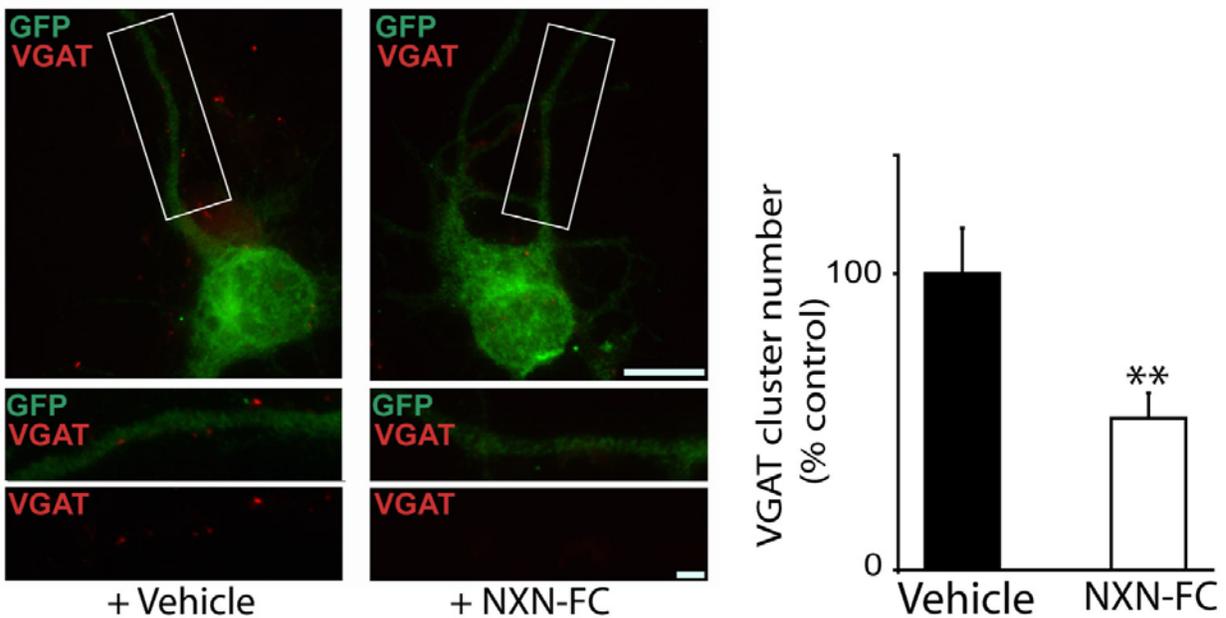


Fig. 2.5. Changes in number of VGAT-positive terminals contacting cells transfected with GFP. Enlarged boxed areas are shown below. Each group has been normalized to vehicle only treatment (vehicle, n = 10; NXN-FC, n = 10). $p < 0.01$. Scale bars: Full view images = 10 μm , enlarged panels = 1 μm .

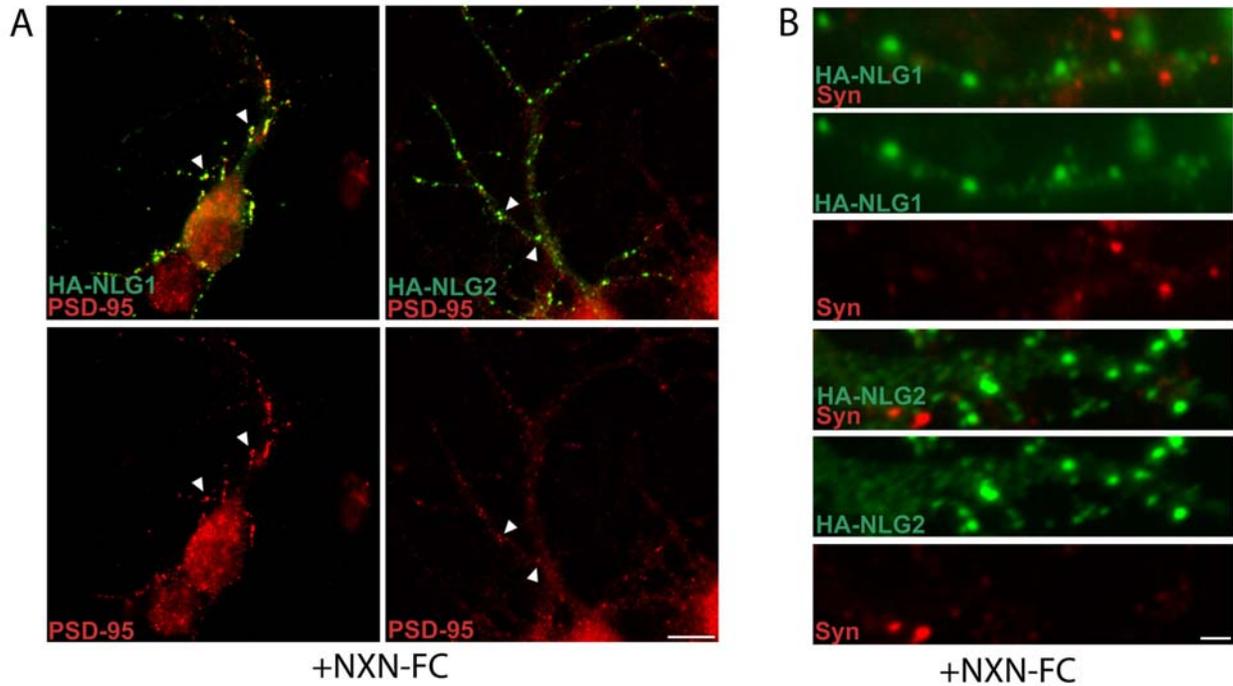


Fig. 2.6. Soluble neurexin-1 β induces clustering of both HA-NL1 and HA-NL2 at sites positive for PSD-95. (A) Treatment of neurons with 30 μ g/ml NXN-FC. PSD-95 clusters are indicated with arrowheads. (B) Lack of synaptophysin (Syn) staining at sites containing HA-NL1 or HA-NL2 after treatment with NXN-FC. Scale bars: A = 10 μ m, B = 1 μ m.

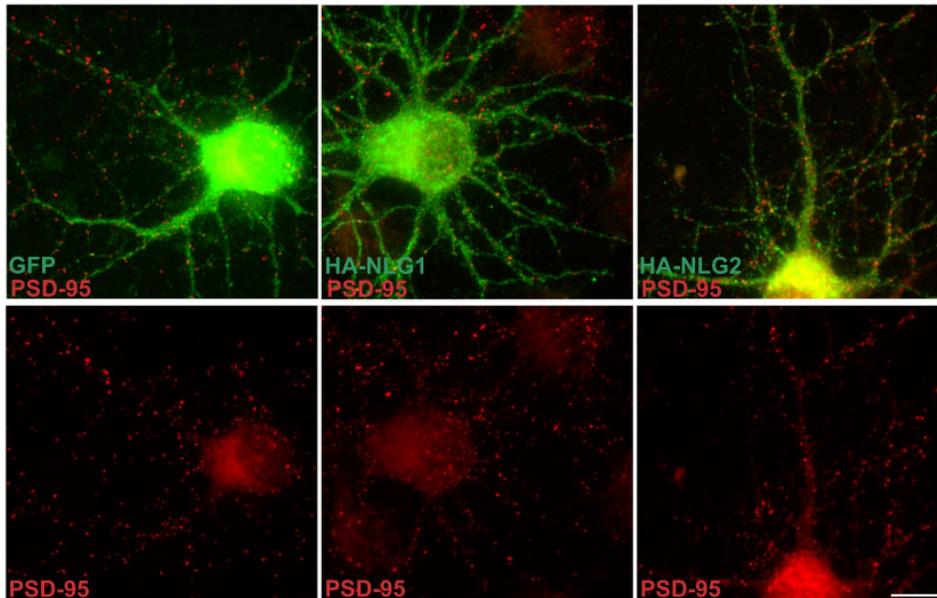


Fig. 2.7. Expression of HA-NL1 or HA-NL2 does not alter clustering of PSD-95. Neurons were transfected at DIV 5 and stained at DIV 8 with antibodies against HA (green) or PSD-95 (red). Scale bars: 10 μ m.

2.3.3 Functional Effects of Neurexin-1 β on Excitatory and Inhibitory Synapses

To examine functional correlates of the immunocytochemical changes induced by application of NXN-FC fusion protein, an electrophysiological approach was taken (Fig. 2.8). DIV 8-9 hippocampal neurons were transfected with either GFP alone or HA-NL1, and incubated with either NXN-FC or FC-IgG for 2-3 days. Changes in miniature excitatory (mEPSCs) and inhibitory (mIPSCs) postsynaptic currents were compared by using whole-cell voltage-clamp recordings (Fig. 2.8A). Ectopic expression of HA-NL1 in cells treated with control peptide (FC-IgG) significantly increases the frequency of mEPSCs and mIPSCs compared to GFP transfected cells (Fig. 2.8B and C; upper panels). Strikingly, the effects on both mEPSCs and mIPSCs were blocked by treatment with NXN-FC. In addition, NXN-FC treatment reduced basal frequency of mEPSCs and mIPSCs in cells transfected with GFP alone. These results parallel our immunocytochemical data, and support a novel role for β -neurexins in the induction of inhibitory synapses. Importantly, treatment of HA-NL1 transfected cells with NXN-FC increases the ratio of excitatory to inhibitory (E/I) synaptic currents (~ 4 fold), suggesting that β -neurexins play a more critical role in inhibitory synapse formation.

It should also be noted that neither HA-NL1 expression nor NXN-FC treatment had a significant effect on mEPSC amplitude, and the effect on mIPSC amplitude was only moderate (Fig. 2.8B and C; lower panels). The lack of effect on the amplitude of excitatory currents, which mainly reflects postsynaptic changes, is consistent with the lack of change in clustering of postsynaptic proteins such as PSD-95 (Fig. 2.7). These findings indicate that NL1 is mainly involved in presynaptic rather than postsynaptic maturation.

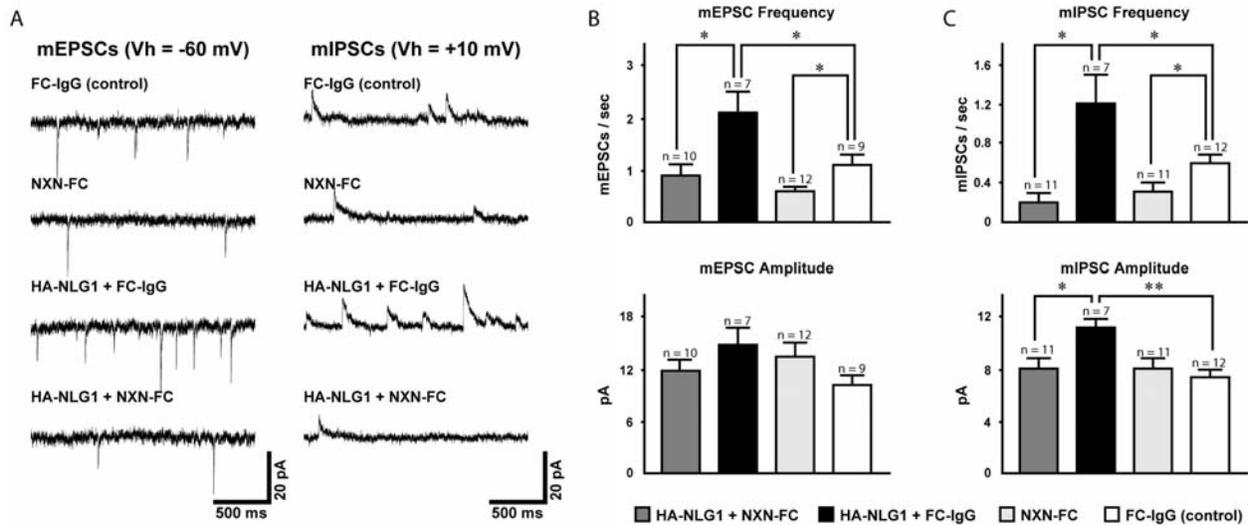


Fig. 2.8. Blocking neurexin-1 β function diminishes NL1 mediated excitatory and inhibitory synaptic function. Electrophysiological recordings from cultured rat hippocampal neurons co-expressing HA-NL1 and GFP, or GFP alone, and incubated with medium containing either 30 μ g/ml FC-IgG or 30 μ g/ml neurexin-1 β -FC peptide (NXN-FC). Spontaneous mEPSCs and mIPSCs were recorded in voltage-clamp mode at holding potentials of -60 mV and +10 mV, respectively. (A) Representative traces of mEPSCs (left) and mIPSCs (right) recorded from these neurons. (B,C) Both the mEPSC and mIPSC frequencies were enhanced in neurons expressing HA-NL1 when compared to neurons transfected with GFP alone. In contrast, treatment of HA-NL1 expressing cells with NXN-FC resulted in a significant reduction in both mEPSC and mIPSC frequency when compared to FC-IgG treated controls. Moreover, NXN-FC treatment reduced both basal mEPSCs and mIPSCs frequency in neurons expressing GFP alone. *, $p < 0.05$; **, $p < 0.05$.

2.3.4 Localization of Endogenous NLs to Excitatory and Inhibitory Synapses

The effect of various NLs on both excitatory and inhibitory synaptic contacts suggested that members of the NL family are endogenously localized at both excitatory and inhibitory synapses. Using NL1-specific antibodies, Song et al. (Song et al., 1999) showed that NL1 is present at excitatory synapses. In this study we analyzed the distribution of NL1 and NL2 at both excitatory and inhibitory synapses using antibodies specific to these proteins (Fig. 2.9, 2.10 and 2.11). Immunostaining analysis showed that both NL1 and NL2 are weakly clustered in young (DIV 7) neurons (Fig. 2.9A and 2.10). However, in more mature (DIV 14) neurons, NL1 was enriched at excitatory synapses, but was also weakly detected at inhibitory synapses (Fig. 2.10B and C, left panels). In contrast, NL2 was enriched at inhibitory synapses, but was weakly detected at excitatory synapses (Fig. 2.10B and C, right panels). Similar observations on the localization of NL2 at inhibitory synapses have recently been reported (Graf et al., 2004; Varoqueaux et al.,

2004). The differential enrichment of specific members of the NL family to either VGAT- or VGlut1-positive contacts supports an *in vivo* role for these proteins in building both excitatory and inhibitory synapses.

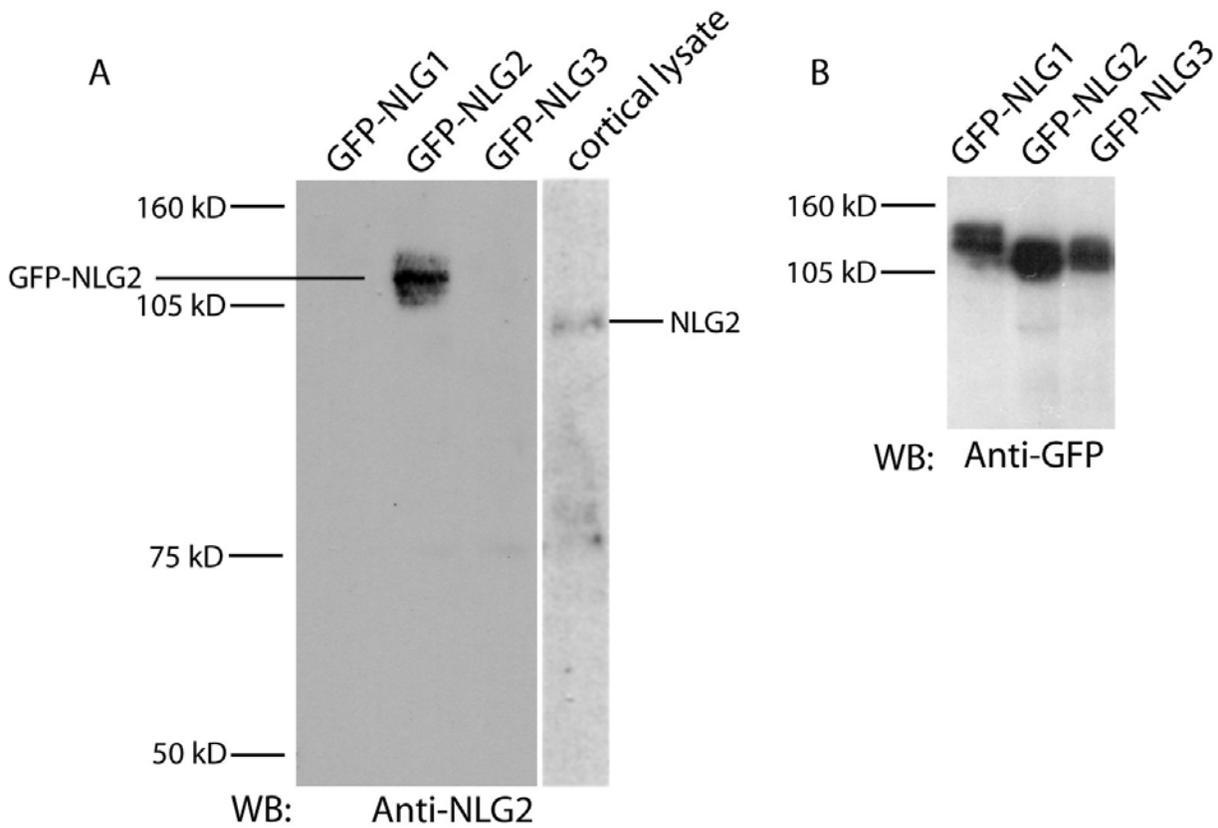


Fig. 2.9. Specificity of NL2 antibody. (A) COS cells overexpressing GFP-tagged NL1, NL2, or NL3 were analyzed by Western blot analysis, using NL2 specific antibody (anti-NL2). A band of the predicted size of NL2 was seen in lysate obtained from DIV 14 cortical neurons. (B) Expression of generated GFP fusion proteins was confirmed by GFP specific antibody (anti-GFP).

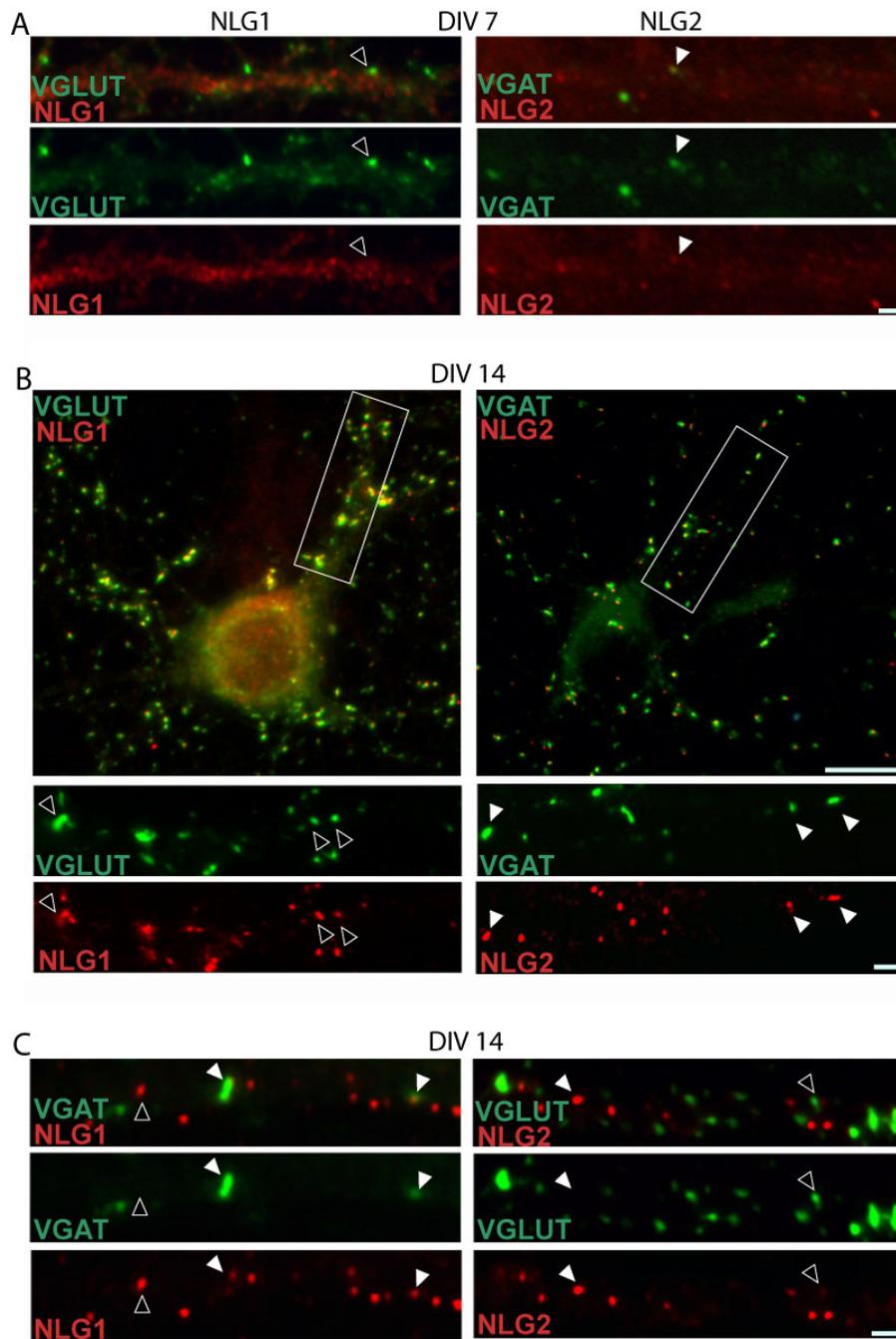


Fig. 2.10. Endogenous localization of NLs to excitatory and inhibitory synapses. Hippocampal neurons were immunostained for endogenous NL1 or NL2, and either VGluT1 or VGAT. (A) NL1 and NL2 are weakly clustered in young (DIV 7) neurons. (B) In older (DIV 14) neurons, both NL1 and NL2 are strongly clustered at synaptic sites. NL1 clusters are enriched at VGluT1-positive sites (empty arrowheads), whereas NL2 clusters are mainly found at sites apposed to VGAT-positive terminals (white arrowheads). Enlarged boxed areas are shown below. (C) NL1 is weakly detected at VGAT-positive contact sites (white arrowheads). In contrast, NL2 is weakly clustered at VGluT1-positive contacts (empty arrowheads). Scale bars: Full view images = 10 μ m; enlarged panels = 1 μ m.

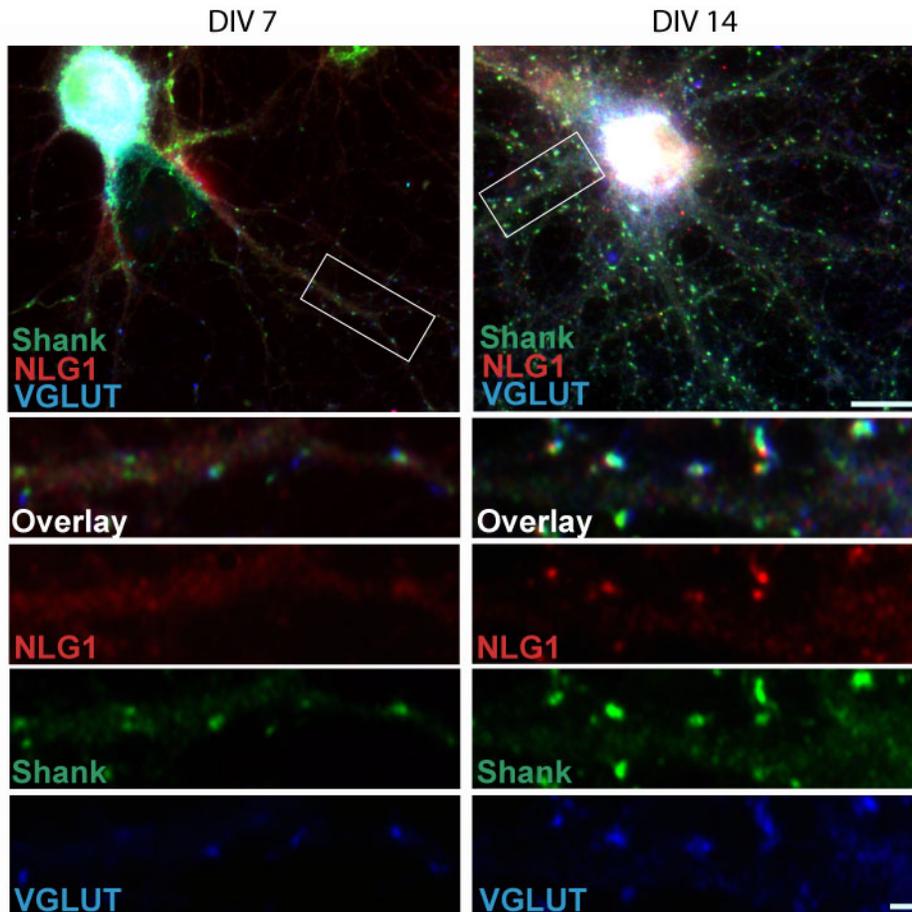


Fig. 2.11. Localization of endogenous NL1 with excitatory postsynaptic proteins in developing neurons. Cells were fixed at DIV 7 and DIV 14 for analysis of endogenous NL1 localization with respect to other postsynaptic and presynaptic proteins. Enlarged boxed areas are shown below. At DIV 7, Shank, a scaffolding postsynaptic protein is found in distinct clusters; synaptic and non-synaptic as assessed by staining for VGLuT1, an excitatory presynaptic marker. Similar distribution was observed for PSD-95 and GKAP (not shown). At DIV 7, NL1 poorly colocalizes with Shank. At DIV 14, NL1 puncta colocalize with Shank and VGLuT1. Scale bars: Full view images = 10 μ m, enlarged panels = 1 μ m.

2.3.5 Manipulation of PSD-95 Levels Regulates Clustering of NLs and Shifts the Distribution of NL2 from Inhibitory to Excitatory Synapses

We have previously shown that enhanced expression of PSD-95 accelerates maturation of excitatory synapses and that this process involves recruitment of NL1 to clusters containing PSD-95 (Prange et al., 2004a). We have also shown that PSD-95 overexpression results in a decrease in the total number of inhibitory synapses ($53 \pm 5\%$) and an overall increase in the E/I synaptic ratio (Prange et al., 2004a). These effects are most likely due to sequestration of synaptogenic factors to specifically build excitatory synapses. Here we explored whether PSD-95

manipulates the balance between excitatory and inhibitory synapses by sequestering various members of the NL family to excitatory synapses. We first evaluated whether PSD-95 can enhance clustering of overexpressed NL2 and NL3. For this analysis, neurons were transfected at DIV 6 with GFP tagged PSD-95 (PSD-95 GFP) and either HA-NL2 or HA-NL3 and then fixed at DIV 9. Similar to HA-NL1 (Prange et al., 2004a), PSD-95 GFP induced clustering of both HA-NL2 and HA-NL3 (Fig. 2.12). These results demonstrate that PSD-95 can accelerate clustering of members of the NL family.

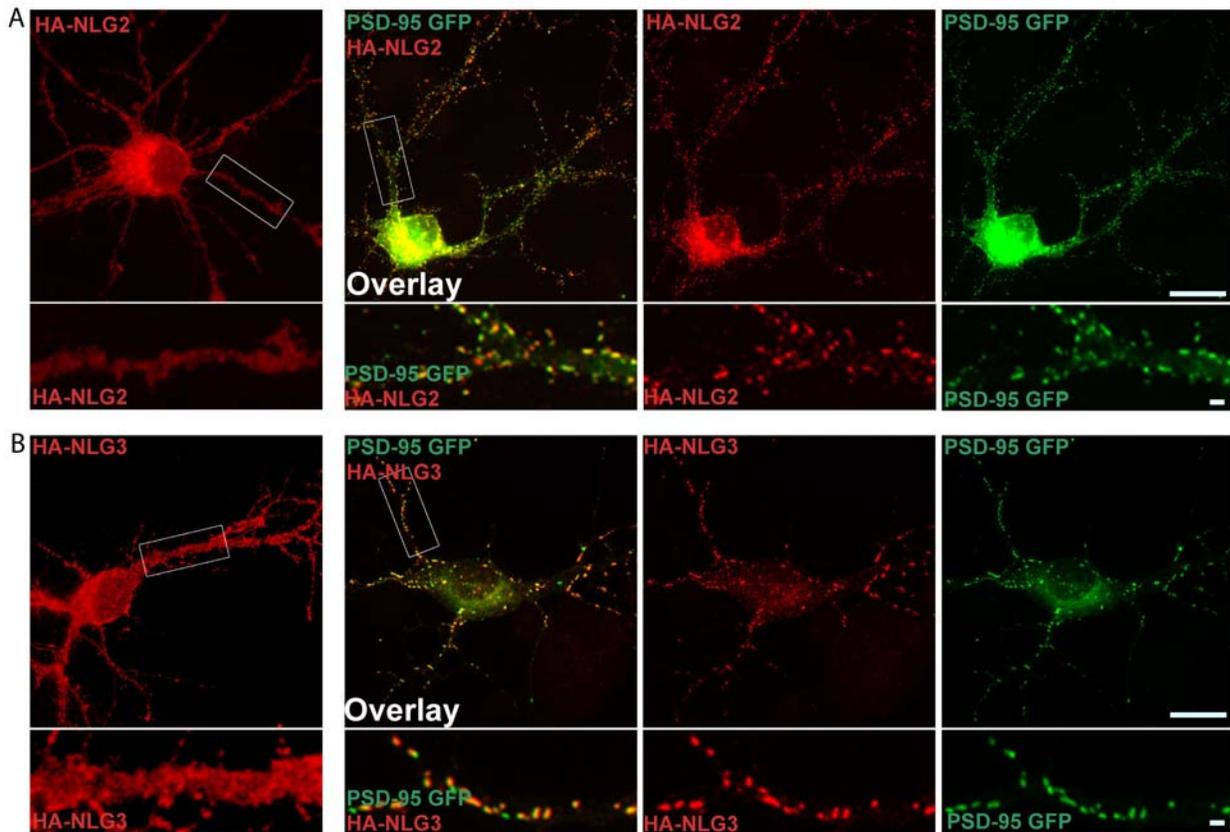


Fig. 2.12. Regulation of NL2 and NL3 clustering by PSD-95. Hippocampal cells were transfected with HA-NL2 or HA-NL3, either alone or with PSD-95 GFP. Cells were fixed and immunostained for HA and GFP. (A) HA-NL2 expression alone (left panel) results in a diffuse staining pattern. When PSD-95 GFP is coexpressed (right panels), clustering of HA-NL2 is strongly enhanced. (B) Similar effects were observed in the case of HA-NL3. Enlarged boxed areas in A and B are shown below. Scale bars: Full view images = 10 μ m, enlarged panels = 1 μ m.

The presence of NL2 at inhibitory contacts suggested that the decreased number of inhibitory synapses previously observed upon overexpression of PSD-95 may have resulted from

manipulation of NL2 accumulation at inhibitory sites, whereby it was sequestered to excitatory synapses. To assess this, hippocampal neurons were transfected with PSD-95 GFP, then fixed and immunostained with antibodies specific to VGAT and NL2 (at DIV 10). Strikingly, the relative amounts of NL2 at excitatory synapses in cells overexpressing PSD-95 GFP was significantly enhanced when compared to controls (Fig. 2.11B and C, right panels and Fig. 2.13A and B). The ratio of intensity of staining of NL2 at VGluT1-positive (excitatory) to VGluT1-negative (inhibitory) sites in untransfected cells was 0.30 ± 0.03 . In contrast, the ratio of intensity of staining of NL2 at excitatory (PSD-95 GFP-positive) to inhibitory (VGAT-positive) sites in PSD-95 GFP-overexpressing cells was 1.7 ± 0.2 . The enhanced accumulation of NL2 at excitatory synapses correlates with reduced number of inhibitory contacts in neurons expressing PSD-95 GFP. This strongly suggests that enhanced levels of PSD-95 manipulate the E/I synapse ratio by depleting NL2 from inhibitory synapses and directing it to excitatory postsynaptic sites. Fig. 2.14 depicts a model that illustrates this phenomenon.

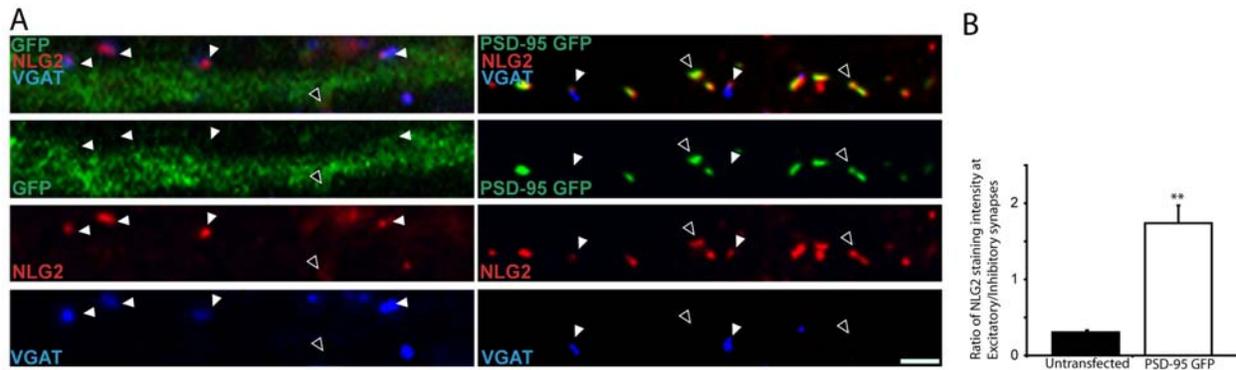


Fig. 2.13. Enhanced PSD-95 expression alters the distribution of endogenous NL2. (A) DIV 6 hippocampal neurons were transfected with PSD-95 GFP and fixed at DIV 10. Cells were then immunostained for GFP, NL2, and VGAT. As compared to GFP-transfected neurons, PSD-95 GFP overexpression shifts endogenous NL2 from inhibitory (VGAT-positive) to excitatory (PSD-95-positive) synapses. VGAT-positive sites are indicated by white arrowheads, and VGAT-negative sites by empty arrowheads (B) Quantification of the redistribution of NL2 from inhibitory to excitatory synapses (see Results and Discussion for a description of the analysis). Untransfected, $n = 10$; PSD-95 GFP, $n = 8$. $p < 0.005$. Scale bar: $1 \mu\text{m}$.

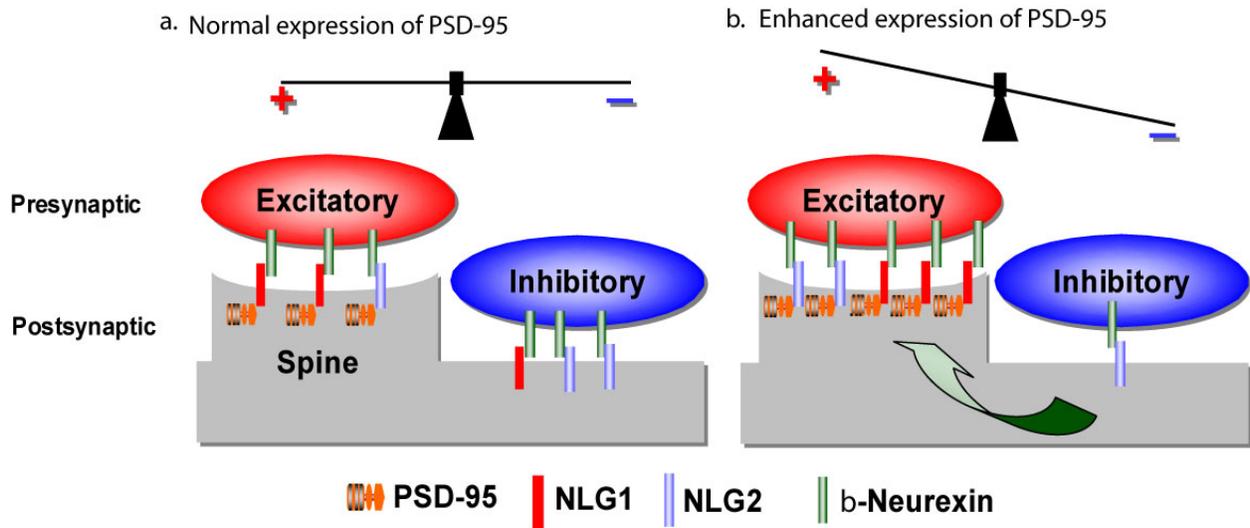


Fig. 2.14. Model illustrating the effects of altered balance between NLs and PSD-95 on E/I ratio. Normally, NL1 is enriched at excitatory synapses, while NL2 is found concentrated at inhibitory synapses. NLs signaling through β -neurexin can induce excitatory and inhibitory presynaptic contacts. Association with postsynaptic scaffolding proteins such as PSD-95 controls the type of synapses formed. An increase in PSD-95 levels shifts NL1 and NL2 from inhibitory to excitatory postsynaptic sites. The resulting effect is a shift in E/I ratio.

2.4 Discussion

Several lines of evidence indicate that appropriate stoichiometry of PSD-95 and NLs controls the number and type of synapses formed (Fig. 6C). First, when transfected alone, NLs increase the number of both excitatory and inhibitory presynaptic terminals, however, as previously shown by our lab, co-expression of NL1 with PSD-95 restricts NL-induced presynaptic effects to excitatory synaptic contacts (Prange et al., 2004a). Second, clustering of both endogenous and transfected NLs at postsynaptic excitatory sites was enhanced by PSD-95. Third, overexpression of PSD-95 resulted in a shift in the distribution of NL2 from inhibitory sites to excitatory synapses and this shift correlates with the previously observed decrease in the number and activity of inhibitory synapses in neurons overexpressing PSD-95 (Prange et al., 2004a). Taken together, these observations indicate that the relative amount of endogenous PSD-95 controls the E/I ratio through modulation of the localization and/or retention of NLs.

It is striking that PSD-95 can manipulate the localization of multiple members of the NL family and that it can redistribute NL2 from inhibitory to excitatory synapses. However, it remains unclear how endogenous NL2 preferentially clusters at inhibitory synapses. In addition to PDZ-dependent interactions, the C-terminal domain of each of these proteins may associate with other specific elements that cooperate or compete with PSD-95 for targeting members of the NL family to excitatory or inhibitory synapses. Further work is required to determine whether this process involves competition between PDZ-containing proteins and molecules such as gephyrin or GABA receptor interacting proteins to regulate NL2 accumulation at inhibitory synapses (Levi et al., 2004; Luscher and Keller, 2004).

By manipulating the localization of cell adhesion molecules involved in building excitatory and inhibitory synapses, PSD-95 can alter the E/I synapse ratio and hence neuronal excitability. These findings are critical in light of the new observation that the balance in E/I ratio is affected in many psychiatric disorders, including autism and mental retardation (Rubenstein and Merzenich, 2003). The implication of NL genes, as well as PSD-95, in autism suggest that an imbalance in E/I ratio may result in the manifestation of abnormalities in patients affected with this disease (Risch et al., 1999; Jamain et al., 2003; Chih et al., 2004; Laumonnier et al., 2004). In addition to the NLs examined in this study, a fourth member (NL4) has recently been shown

to be affected in autism (Jamain et al., 2003). It will be important to determine the endogenous localization of the additional NL proteins and their role in the process of synaptic development. The involvement of neurexin-1 β in NL mediated inhibitory presynaptic contacts is also intriguing and provides a novel mechanism for the development of inhibitory synapses.

Of particular interest, physiological and pathological paradigms have recently been shown to alter the levels of PSD-95. For example, PSD-95 association with the PSD is dynamic and is regulated by synaptic activity and palmitate cycling on PSD-95 (El-Husseini Ael et al., 2002). Synaptic activity also upregulates PSD-95 expression through a neuregulin mediated pathway (Bao et al., 2004). In contrast, administration of cocaine, a drug known to cause hyperexcitability, results in down regulation of PSD-95 in the striatum, a region mainly composed of inhibitory neurons (Yao et al., 2004). Moreover, mutation of FMRP, a gene associated with fragile X mental retardation, results in a loss of regulation of PSD-95 expression (Todd et al., 2003). Thus, changes in PSD-95 levels under these conditions may also induce profound effects on the localization of NLs at excitatory and inhibitory synapses and thus a change in the E/I ratio.

It appears then that localization of NLs rather than their intrinsic characteristics determine which members are involved in formation of a particular type of synapse. The following question therefore arises: How does sequestering particular NLs to either excitatory or inhibitory postsynaptic sites result in induction of a particular synapse type with respect to the presynaptic side? It may be the case that NLs are the cue for recruitment of presynaptic machinery which is common to both types of synapses. Additional postsynaptic cues would then be required for specification of synaptic type. Alternatively, NLs may only be required for synaptic stabilization. Indeed, we observed that both NL1 and NL2 are only weakly clustered in young neurons, at an age when PSD-95 and presynaptic clusters have already been well established. Thus, the action of NLs may come into play later in development, with different members stabilizing synapse formation at their respective synapse type.

We conclude that members of the NL family exert similar effects on both excitatory and inhibitory synapses. These results reveal a novel role for NLs in building inhibitory synapses through interaction with β -neurexin. Another critical finding is that synaptogenic activity of NLs

is dictated by interactions with scaffolding proteins and these interactions regulate the E/I synaptic ratio.

2.5 References

- Bao J, Lin H, Ouyang Y, Lei D, Osman A, Kim TW, Mei L, Dai P, Ohlemiller KK, Ambron RT (2004) Activity-dependent transcription regulation of PSD-95 by neuregulin-1 and Eos. *Nat Neurosci* 7:1250-1258.
- Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 35:567-576.
- Brose N (1999) Synaptic cell adhesion proteins and synaptogenesis in the mammalian central nervous system. *Naturwissenschaften* 86:516-524.
- Butz S, Okamoto M, Sudhof TC (1998) A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain. *Cell* 94:773-782.
- Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Brecht DS, Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* 408:936-943.
- Chih B, Afridi SK, Clark L, Scheiffele P (2004) Disorder-associated mutations lead to functional inactivation of neuroligins. *Hum Mol Genet* 13:1471-1477.
- Craig AM, Boudin H (2001) Molecular heterogeneity of central synapses: afferent and target regulation. *Nat Neurosci* 4:569-578.
- Craven SE, El-Husseini AE, Brecht DS (1999) Synaptic targeting of the postsynaptic density protein PSD-95 mediated by lipid and protein motifs. *Neuron* 22:497-509.
- Dean C, Scholl FG, Choih J, DeMaria S, Berger J, Isacoff E, Scheiffele P (2003) Neurexin mediates the assembly of presynaptic terminals. *Nat Neurosci* 6:708-716.
- El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Brecht DS (2000a) PSD-95 involvement in maturation of excitatory synapses. *Science* 290:1364-1368.
- El-Husseini AE, Craven SE, Chetkovich DM, Firestein BL, Schnell E, Aoki C, Brecht DS (2000b) Dual palmitoylation of PSD-95 mediates its vesiculotubular sorting, postsynaptic targeting, and ion channel clustering. *J Cell Biol* 148:159-172.
- El-Husseini AE, Schnell E, Dakoji S, Sweeney N, Zhou Q, Prange O, Gauthier-Campbell C, Aguilera-Moreno A, Nicoll RA, Brecht DS (2002) Synaptic strength regulated by palmitate cycling on PSD-95. *Cell* 108:849-863.
- Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM (2004) Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 119:1013-1026.
- Ichtchenko K, Nguyen T, Sudhof TC (1996) Structures, alternative splicing, and neurexin binding of multiple neuroligins. *J Biol Chem* 271:2676-2682.
- Ichtchenko K, Hata Y, Nguyen T, Ullrich B, Missler M, Moomaw C, Sudhof TC (1995) Neuroligin 1: a splice site-specific ligand for beta-neurexins. *Cell* 81:435-443.
- Irie M, Hata Y, Takeuchi M, Ichtchenko K, Toyoda A, Hirao K, Takai Y, Rosahl TW, Sudhof TC (1997) Binding of neuroligins to PSD-95. *Science* 277:1511-1515.
- Jamain S, Quach H, Betancur C, Rastam M, Colineaux C, Gillberg IC, Soderstrom H, Giros B, Leboyer M, Gillberg C, Bourgeron T (2003) Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat Genet* 34:27-29.

- Kim E, Sheng M (2004) PDZ domain proteins of synapses. *Nat Rev Neurosci* 5:771-781.
- Laumonnier F, Bonnet-Brilhault F, Gomot M, Blanc R, David A, Moizard MP, Raynaud M, Ronce N, Lemonnier E, Calvas P, Laudier B, Chelly J, Fryns JP, Ropers HH, Hamel BC, Andres C, Barthelemy C, Moraine C, Briault S (2004) X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *Am J Hum Genet* 74:552-557.
- Lee SH, Sheng M (2000) Development of neuron-neuron synapses. *Curr Opin Neurobiol* 10:125-131.
- Levi S, Logan SM, Tovar KR, Craig AM (2004) Gephyrin is critical for glycine receptor clustering but not for the formation of functional GABAergic synapses in hippocampal neurons. *J Neurosci* 24:207-217.
- Luscher B, Keller CA (2004) Regulation of GABAA receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. *Pharmacol Ther* 102:195-221.
- Missler M, Sudhof TC (1998) Neurexins: three genes and 1001 products. *Trends Genet* 14:20-26.
- Miyamoto S, Akiyama SK, Yamada KM (1995) Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science* 267:883-885.
- Prange O, Wong TP, Gerrow K, Wang YT, El-Husseini A (2004a) A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. *Proc Natl Acad Sci U S A* 101:13915-13920.
- Prange O, Wong TP, Gerrow K, Wang YT, El-Husseini A (2004b) A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. *PNAS* 101:13915-13920.
- Rao A, Harms KJ, Craig AM (2000) Neuroligation: building synapses around the neurexin-neuroligin link. *Nat Neurosci* 3:747-749.
- Risch N et al. (1999) A genomic screen of autism: evidence for a multilocus etiology. *Am J Hum Genet* 65:493-507.
- Rubenstein JL, Merzenich MM (2003) Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav* 2:255-267.
- Scheiffele P, Fan J, Choih J, Fetter R, Serafini T (2000) Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101:657-669.
- Song JY, Ichtchenko K, Sudhof TC, Brose N (1999) Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc Natl Acad Sci U S A* 96:1100-1105.
- Todd PK, Mack KJ, Malter JS (2003) The fragile X mental retardation protein is required for type-I metabotropic glutamate receptor-dependent translation of PSD-95. *Proc Natl Acad Sci U S A* 100:14374-14378.
- Ullrich B, Ushkaryov YA, Sudhof TC (1995) Cartography of neurexins: more than 1000 isoforms generated by alternative splicing and expressed in distinct subsets of neurons. *Neuron* 14:497-507.
- Ushkaryov YA, Hata Y, Ichtchenko K, Moomaw C, Afendis S, Slaughter CA, Sudhof TC (1994) Conserved domain structure of beta-neurexins. Unusual cleaved signal sequences in receptor-like neuronal cell-surface proteins. *J Biol Chem* 269:11987-11992.
- Varoqueaux F, Jamain S, Brose N (2004) Neuroligin 2 is exclusively localized to inhibitory synapses. *Eur J Cell Biol* 83:449-456.
- Washbourne P, Dityatev A, Scheiffele P, Biederer T, Weiner JA, Christopherson KS, El-Husseini A (2004) Cell adhesion molecules in synapse formation. *J Neurosci* 24:9244-9249.

Yao WD, Gainetdinov RR, Arbuckle MI, Sotnikova TD, Cyr M, Beaulieu JM, Torres GE, Grant SG, Caron MG (2004) Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioral plasticity. *Neuron* 41:625-638.

Ziv NE (2001) Recruitment of synaptic molecules during synaptogenesis. *Neuroscientist* 7:365-370.

Ziv NE, Smith SJ (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* 17:91-102.

3. Postsynaptic Scaffolding Molecules Modulate the Localization of Neuroligins²

3.1 Introduction

Synapse formation is thought to be a highly ordered process, beginning with the recognition of appropriate targets and formation of incipient contacts, and culminating with the recruitment of pre- and postsynaptic proteins to well-delineated microdomains, to form nodes of communication at points of cell-cell contact. Presynaptic compartments are characterized by the presence of hundreds of neurotransmitter-filled synaptic vesicles and by electron dense active zones, whereas postsynaptic compartments consist of a dense matrix of neurotransmitter receptors, scaffolding proteins and signaling molecules (reviewed in (McAllister, 2007; Jin and Garner, 2008).

In the mature mammalian central nervous system, glutamatergic and GABAergic synapses constitute the major classes of excitatory and inhibitory synaptic contacts, respectively. Processing of neural information is thought to occur by the integration of excitatory and inhibitory synaptic inputs onto a given neuron (Schummers et al., 2002). The relative proportion of excitatory and inhibitory synaptic inputs received by a neuron, or excitatory/inhibitory (E/I) balance, determines its overall level of excitability, and thus its function within a given neural circuit. In recent years, a number of neuropsychiatric diseases, including autism spectrum disorders and some forms of mental retardation, have been attributed to shifts in E/I balance (reviewed in (Rubenstein and Merzenich, 2003). This underscores the importance of establishing and maintaining proper E/I balance, and suggests that cellular processes involved in determining whether a nascent synapse will develop into an excitatory or inhibitory contact may be critical for proper brain function. Despite this, the molecular mechanisms that confer synaptic identity remain elusive.

The molecular mechanisms underlying the proper development and function of excitatory synapses have been well studied. A number of cell adhesion and scaffolding molecules have come to the forefront as key players in the development of excitatory synapses (Washbourne et

² A version of this chapter has been published. **Levinson JN**, Li R, Kang R, Moukhles H, El-Husseini A, Bamji SX. 2009. Postsynaptic scaffolding molecules modulate the localization of neuroligins. *Neuroscience* **165**:782-93.

al., 2004; Gerrow and El-Husseini, 2006). The neuroligin family of postsynaptic cell adhesion molecules, for instance, has been shown to play important roles in synapse development. Overexpression of neuroligin family members in cultured hippocampal neurons can trigger the formation of glutamatergic and GABAergic presynaptic terminals on contacting axons, as well as increase spine number and enhance the clustering of postsynaptic proteins such as PSD-95 and the NMDA receptor subunit, NR1 (Prange et al., 2004; Chih et al., 2005; Levinson et al., 2005). These changes are associated with enhanced frequency and amplitude of miniature EPSCs and IPSCs (Graf et al., 2004; Prange et al., 2004; Levinson et al., 2005). Conversely, knockdown of neuroligins in cultured neurons reduces the number of excitatory and inhibitory contacts, and specifically reduces inhibitory currents, shifting E/I balance towards greater excitation (Chih et al., 2005).

Previous work has shown that neuroligin 1 (NL1), neuroligin 3 (NL3) and neuroligin 4 (NL4) are localized mainly at glutamatergic synapses, while neuroligin 2 (NL2) is enriched at GABAergic synapses (Song et al., 1999; Varoqueaux et al., 2004; Levinson et al., 2005; Varoqueaux et al., 2006). This stark contrast in the synaptic localization of different neuroligins suggests that the ways in which these proteins exert their effects on synapse development may rely on differential sorting of various neuroligin isoforms. Therefore, understanding mechanisms that control trafficking of these proteins to synaptic sites may be crucial for elucidating the cellular processes involved in maintaining E/I balance.

Neuroligins have been shown to mediate their effects on excitatory and inhibitory synapse development through trans-synaptic interaction with neurexins, a family of primarily presynaptic cell adhesion molecules (Ichtchenko et al., 1995; Dean et al., 2003; Graf et al., 2004; Levinson et al., 2005; Nam and Chen, 2005; Budreck and Scheiffele, 2007). By altering trans-synaptic interactions with neurexins, splice sites in the extracellular domains of NL1 and NL2 have been shown to play critical roles in determining the localization of these proteins (Chih et al., 2006). However, the targeting of neuroligins to excitatory synapses is also affected by association with PSD-95, a postsynaptic scaffolding molecule enriched at excitatory contacts (Prange et al., 2004). While highly overexpressed NL1 can induce the maturation of both excitatory and inhibitory presynaptic terminals, PSD-95, which binds the C-terminal PDZ-binding domain of neuroligins, restricts the localization and effects of NL1 to excitatory synapses, strongly

supporting a role for the intracellular region of neuroligins in modulating their synaptic localization (Irie et al., 1997; Prange et al., 2004). In addition, overexpression of PSD-95 can shift the localization of NL2 from inhibitory to excitatory contacts (Graf et al., 2004; Levinson et al., 2005), and decreasing endogenous levels of PSD-95 with siRNA shifts NL1 from excitatory to inhibitory synapses (Gerrow et al., 2006). These results have led to a model which proposes that the synapse-specific localization patterns of different neuroligins are modulated by their interactions with postsynaptic scaffolding proteins that are specific to a particular synapse type. These interactions between postsynaptic cell adhesion molecules and scaffolding proteins would then partially dictate the balance between excitatory and inhibitory synapse development and/or function.

Despite the progress that has been made with respect to understanding postsynaptic factors influencing the targeting of NL1 to excitatory synapses, it is unknown what postsynaptic factors are involved in the localization of NL2 and NL3 to inhibitory and excitatory synapses, respectively. Substantial sequence divergence exists in the C-termini of neuroligin proteins, with NL1, NL3 and NL4 differing the most from NL2, consistent with their subcellular localization patterns. Thus, these sequence variations may lead to differential interactions with specific excitatory or inhibitory postsynaptic scaffolding molecules, underlying the differences in the targeting of various family members. Gephyrin, a cytoplasmic multidomain protein which in neurons is found almost exclusively at inhibitory contact sites along the dendrite, is thought to link inhibitory neurotransmitter receptors to the cytoskeleton, as well as to certain signal transduction pathways involving *cdc42* (reviewed in (Kneussel and Loeblich, 2007). Furthermore, gephyrin is important for clustering of both glycine and GABA_A receptors (Kirsch et al., 1993; Essrich et al., 1998; Feng et al., 1998; Kneussel et al., 1999; Levi et al., 2004). Thus, gephyrin presents a good candidate scaffolding protein for regulating the targeting of neuroligins to inhibitory synapses.

In this study, we have examined factors involved in the targeting of NL2 and NL3 to synapses. We show that siRNA-mediated knockdown of gephyrin leads to a shift of endogenous NL2 from inhibitory to excitatory synaptic contacts, indicating a role for gephyrin in the proper targeting of NL2 to inhibitory synapses. Consistent with this, deletion analysis demonstrates a role for the C-terminus in modulating the clustering of NL2 at inhibitory synapses. Finally, to further clarify

factors involved in the synaptic localization of NL3, we generated an antibody specifically against this neuroligin family member. Characterization of the endogenous distribution of NL3 demonstrated a primarily excitatory synaptic localization pattern. Using this antibody, we show that knockdown of PSD-95 partially shifts NL2 and NL3 from excitatory to inhibitory synapses, analogous but opposite to what happens in the case of gephyrin and NL2. Our data show that association between NL2 and gephyrin is required for modulating the localization of NL2 at inhibitory synapses, while functional interaction with PSD-95 partially controls the localization of NL2 and NL3 at excitatory contacts. These data further strengthen the idea that proper synaptic targeting of neuroligins is partially controlled by interactions with postsynaptic scaffolding proteins.

3.2 Methods and Materials

3.2.1 cDNA Cloning and Mutagenesis

Full-length rat HA-NL2 was as previously described (Levinson et al., 2005). NL2 Δ PRR (NL2 lacking amino acids 798-826) was constructed by PCR of full-length NL2 using oligonucleotides with BglIII and HindIII restriction sites (forward, GGGCCCAGATCTcggggaggagggggtccc; reverse,

GAGTCCAAGCTTCTATACCCGAGTGGTGGAGTGGGGATGGGGTAGCCCCAGGCCAC TGGGCAGCAGGGTC), and subsequently subcloning the resultant fragment into full-length HA-NL2. NL2 Δ PDZb was constructed as above, with the exception that the following reverse primer was used: GGGCCCAAGCTTCTAGGAGTGGGGATGGGG. NL2 Δ 716-782 was constructed in two steps. First, PCR of full-length NL2 was done using the following primers: forward, ggactcAAGCTTctcttggccccgggggccc; reverse, GGGCCCGTCGACCTATACCCGAGTGGTGGAGTGGGGATGGGGTAG. The resulting fragment, corresponding to amino acids 783-836 of NL2, was then subcloned into an HA-NL2 construct lacking the C-terminal 120 amino acids, using HindIII and SalI restriction sites. Next, a stop codon just after amino acid 715 was removed using the Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Briefly, the entire plasmid was amplified by PCR using oligonucleotides containing a deletion for TAG (GGCGGCTTAGCCCAAAGCTTCTCTTGGC), the methylated template strand was destroyed enzymatically with DpnI, the purified construct was transformed into bacteria, and verified by direct sequencing. All constructs have been verified by sequencing. GFP-NL1 was made by subcloning a PCR fragment of full length NL1 lacking the signal sequence into pEGFP-C1, downstream of GFP, and subsequently inserting the NL1 signal sequence (amino acids 1-46) upstream of GFP. GFP-NL2 and GFP-NL3 were as previously described (Levinson et al., 2005). The generation of PSD-95 fused to GFP was previously described (El-Husseini et al., 2000b). CFP-gephyrin was a gift from Dr. Ann Marie Craig.

For siRNA experiments, we annealed HPLC/PAGE-purified siRNA oligonucleotides designed to specifically interfere with gephyrin and PSD-95 expression levels in hippocampal neurons. The sense sequence of siRNA for rat gephyrin was TAGGAGACAACCCAGATGACTATCTCA

(Jacob et al., 2005). The oligonucleotide, TAGGAGACAACCCAGAAGAGTTTCTCA, was used to generate a control gephyrin siRNA construct containing three point mutations (underlined) relative to the gephyrin siRNA sequence. PSD-95 siRNA consisted of the sequence, GCCTTCGACAGAGCCACGA (Nakagawa et al., 2004). Control PSD-95 siRNA consisted of a scrambled version of the PSD-95 siRNA sequence. All oligonucleotide sequences were designed to generate sticky end overhangs corresponding to BglII and HinDIII. Subsequent annealing products were inserted into pSuper (Oligoengine, Seattle, WA) which was digested with BglII and HinDIII.

For antibody production experiments, rat NL3 was PCR amplified using the primers, GGGCCCGGATCCgtaaggacaaacggcgcca and GGGCCCGTCGACGTCGGGCAATGCTGTGAG. The resulting PCR product was inserted into the BamHI and Sall sites of pGEX-6P3.

3.2.2 Cell Culture and Transfections

Dissociated primary neuronal cultures were prepared from hippocampi of embryonic day 18/19 Wistar rats. Cells were dissociated by papain digestion followed by brief mechanical trituration, and plated on poly-lysine (Sigma, St. Louis, MO)-treated coverslips at a density of about 1.5×10^5 per 18 mm glass coverslip (Deckglaser, Freiburg, Germany) into Minimal Essential Medium (GIBCO) supplemented with 10% fetal bovine serum (HyClone, Waltham, MA), glucose (Sigma, St. Louis, MO), sodium pyruvate, GlutaMAX, and penicillin/streptomycin (GIBCO-Invitrogen, Burlington, ON). After 1-2 hours, the medium was replaced with NeuroBasal medium (GIBCO-Invitrogen, Burlington, ON) supplemented with B-27, GlutaMAX, penicillin and streptomycin (GIBCO-Invitrogen, Burlington, ON), as previously described (Brewer et al., 1993). Every 3-4 days, half of the volume of maintenance medium was taken out and replaced with fresh medium. Cultured neurons were transfected by calcium-phosphate technique, using the CalPhos Mammalian Transfection Kit (Clontech, Mountain View, CA), as previously described (Jiang et al., 2004). For all siRNA experiments other than those used to assess the knockdown efficiency of siRNA constructs, control siRNA refers to the gephyrin point mutation control construct.

COS-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO-Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (HyClone, Waltham, MA), sodium pyruvate and penicillin/streptomycin (GIBCO-Invitrogen, Burlington, ON). COS-7 cells were transfected with Lipofectamine 2000 reagent (Invitrogen, Burlington, ON), as per the manufacturer's recommendations, and harvested 24 h later.

3.2.3 Antibody Production and Purification

Antibodies were raised in rabbit using a GST-fused C-terminal fragment of NL3, corresponding to amino acids 732-791. This GST-fusion protein was purified by affinity chromatography, and injected into rabbits. Rabbit sera were tested for the presence of anti-NL3 antibodies by Western blot analysis of COS-7 cell lysates. Anti-NL3 antibodies were subsequently affinity purified from sera using the GST-fused NL3 antigen used for injection.

3.2.4 Immunocytochemistry

Coverslips were fixed in 4% PFA with 4% sucrose for 2 min., followed by -20°C methanol for 6 min., and permeabilized with 0.3% Triton-X100 in phosphate-buffered saline. The following primary antibody solutions were used: HA (mouse, 1:1000, BABCO; rat, 1:1000, Roche, Mississauga, ON), GFP (chicken, 1:1000, Abcam, Cambridge, MA), VGluT1 (rabbit, 1:1000, Synaptic Systems, Goettingen, Germany), VGAT (rabbit, 1:1000, Synaptic Systems, Goettingen, Germany), NL2 (goat, 1:50, Santa Cruz, Santa Cruz, CA; and gift from Dr. Anne Marie Craig, Graf et al., 2006), gephyrin (mouse, 1:300, Synaptic Systems, Goettingen, Germany), PSD-95 (mouse, 1:500, ABR, Rockford, IL), NL3 (homemade; see above). Secondary antibodies were generated in goat and conjugated with Alexa488 or Alexa568 (1:1000, Molecular Probes, Burlington, ON), or in donkey and conjugated with AMCA (1:100) or Cy3 (1:500) (Jackson ImmunoResearch, West Grove, PA), and used as previously described (Prange et al., 2004). All antibody reactions were performed in blocking solution containing 2% normal goat and/or donkey serum for 1 h at room temperature or overnight at 4°C. Coverslips were then mounted on slides (Frost Plus; Fisher, Ottawa, ON) with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL).

3.2.5 Western Blot Analysis

COS-7 cells were harvested in lysis buffer containing 25 mM Tris, 150 mM NaCl, 3 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), 0.8% Triton X-100, 0.2% SDS, and 1 protease inhibitor mixture tablet/10 ml (Roche Applied Science, Mississauga, ON). For analysis of total brain lysates, whole brains from adult or postnatal day 16-19 Wistar rats were quickly removed. Brain tissue was homogenized in TEEN buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl) supplemented with 10 mM ATP, 10 mM MgCl₂, 2.5 mM Na₃Va₄, 1 mM phenylmethylsulfonyl fluoride (Sigma), and 1 protease inhibitor mixture tablet/10 ml (Roche Applied Science, Mississauga, ON).

Samples were boiled for 10 min. in loading buffer (62.5 mM Tris-HCl, 2% SDS, 1% β-mercaptoethanol, 7.5% glycerol, 15 μM bromophenol blue), and the proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, England). Nonspecific binding was blocked by incubating membranes with 3% BSA or 10% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h. After three washes in TBST, primary antibodies (GFP, rabbit, 1:1000, Synaptic Systems, Goettingen, Germany; β-actin, mouse, 1:10000, Sigma, St. Louis, MO; NL3, rabbit, homemade, see below) were diluted in 3% bovine serum albumin or 5% milk in TBST, and membranes were incubated for 1 h at room temperature. Membranes were washed three times in TBST, incubated with secondary antibody conjugated to horseradish peroxidase (anti-rabbit or anti-mouse, 1:3000, Bio-Rad, Mississauga, ON). Blots were visualized by use of SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

3.2.6 Imaging and Analysis

Images were acquired on an inverted Zeiss Axiovert M200 motorized microscope, using a monochrome 14-bit Zeiss AxioCamHR charge-coupled device camera. In some experiments, exposure times were individually adjusted to yield an optimum brightness of immunofluorescent clusters without saturation. In other experiments, images were acquired with equal exposure and scaled to the same extent, without saturation. Images were analyzed in Northern Eclipse (Empix Imaging, Mississauga, ON) using custom written software routines, as previously described (Prange et al., 2004). Briefly, images were processed at a constant threshold level (32,000 pixel

values) to create a binary image, and dendrites of the cell of interest were outlined by using fluorescence signal. Only clusters with minimum average pixel values 2-3 times greater than corresponding background pixel values were used for analysis. The density of stained clusters was measured as a function of number per dendritic length. For intensity analysis, average background intensity was subtracted from the average intensity of individual puncta, and multiplied by puncta area to obtain integrated intensity. For colocalization analysis, background-subtracted immunofluorescence clusters for all imaging channels were correlated for overlapping signal. Colocalization was scored if clusters in two channels were overlapping by at least 1 pixel. For all statistical analyses, two-tailed Student's T-test was performed.

3.3 Results

3.3.1 Distribution of Endogenous NL2 in Cultured Hippocampal Neurons

Previous work using immunoelectron microscopy and immunohistochemistry has demonstrated that NL1 and NL3 are enriched at excitatory synaptic contacts, while NL2 is primarily localized at inhibitory synapses (Song et al., 1999; Varoqueaux et al., 2004; Levinson et al., 2005; Varoqueaux et al., 2006; Budreck and Scheiffele, 2007). To confirm its localization at inhibitory synapses and to more thoroughly quantify the distribution of NL2 for further analyses, cells were fixed, permeabilized, and immunostained for NL2 in conjunction with various synaptic markers. This staining method identifies both cell surface and intracellular pools of NL2. We first examined the localization of NL2 with VGAT or VGluT1, integral synaptic vesicle membrane proteins that label inhibitory and excitatory presynaptic terminals, respectively. NL2 exhibited a punctate pattern along dendritic processes, as previously shown (Fig. 3.1A, B; (Levinson et al., 2005). Quantitative analysis demonstrates that $74\pm 3\%$ of NL2 clusters colocalize with VGAT, with only $21\pm 2\%$ of NL2 clusters colocalizing with VGluT1 (Fig. 3.1C). This analysis largely recapitulated previous findings, validating our antibodies and our method of analysis. As VGluT1 and VGAT label mobile synaptic vesicles in addition to those found at stable synaptic contacts, these proteins alone are not accurate synaptic markers. We therefore quantified the localization of NL2 at sites where pre- and postsynaptic markers were colocalized. Excitatory synapses were defined as sites positive for clusters of VGluT1 and PSD-95, whereas sites where VGAT and gephyrin were colocalized were considered sites of inhibitory contact. According to these criteria, $68\pm 3\%$ of NL2 clusters are localized to inhibitory synapses, while only $15\pm 2\%$ are localized to excitatory synapses (Fig. 3.1C). These results confirm previous findings indicating that the majority of NL2 is associated with inhibitory synapses, and extend these observations to provide more defined quantifications of its localization at different synapse types. Once again, it is important to note that the immunostaining method used in these experiments identifies both cell-surface and intracellular pools of NL2, and thus colocalization of NL2 with pre- and postsynaptic markers does not necessarily indicate the incorporation of NL2 at the synaptic membrane. These data also reveal a subset of non-synaptic NL2 clusters at this developmental stage. These non-synaptic clusters may represent transport packets containing NL2, which are subsequently incorporated into the plasma membrane at postsynaptic sites, as has been shown in

the case of preassembled protein complexes containing PSD-95, GKAP and Shank (Gerrow et al., 2006).

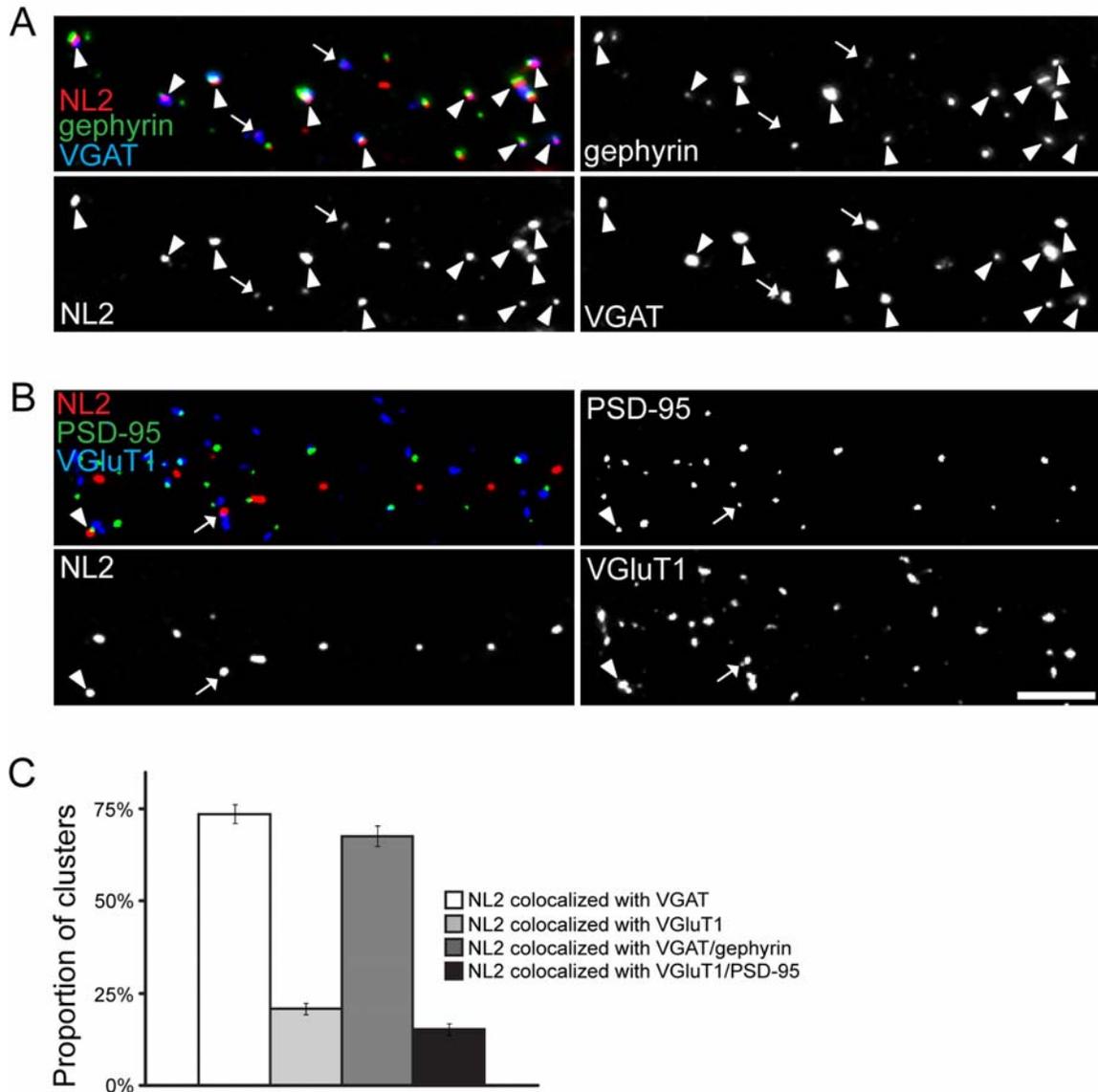


Fig. 3.1. Localization of endogenous NL2 at excitatory and inhibitory synapses. (A, B) Images of 14 DIV cultured hippocampal neurons immunolabeled with anti-NL2 in conjunction with antibodies against markers specific for excitatory or inhibitory synapses. The majority of NL2 clusters are associated with the inhibitory synaptic markers, gephyrin and VGAT (A, C), whereas only a minority of NL2 colocalizes with the excitatory markers, PSD-95 and VGluT1 (B, C). Arrowheads indicate triple colocalization between NL2 and the synaptic markers, while arrows denote colocalization between NL2 and either VGAT (A) or VGluT1 (B). (C) Quantification of the proportion of NL2 colocalized with synaptic markers. Error bars represent SEM. N=10-15 cells per condition. Scale bar, 5 μ m.

3.4.2 Knockdown of Gephyrin or PSD-95 Alters the Proportion of NL2 at Inhibitory and Excitatory Synapses

Postsynaptic compartments are composed of a network of cell surface and scaffolding molecules that regulate synapse formation and efficacy by recruiting proteins to specific synaptic subtypes. For example, at excitatory postsynaptic compartments, PSD-95 plays a large role in recruiting AMPA receptor subunits via the adaptor protein, stargazin 1 (Schnell et al., 2002; Bats et al., 2007). PSD-95 has also been shown to play a large role in localizing NL1 to excitatory synapses (Prange et al., 2004). Similarly, at inhibitory postsynaptic compartments, the scaffolding protein, gephyrin, regulates the recruitment of GABA_A and glycine receptors (Essrich et al., 1998; Kneussel et al., 1999; Hanus et al., 2004; Levi et al., 2004; Jacob et al., 2005; Tretter et al., 2008). As gephyrin is primarily localized at GABAergic synapses (Craig et al., 1996), we postulated that gephyrin may be involved in clustering cell adhesion molecules at inhibitory synapses, and specifically tested whether gephyrin acts to restrict NL2 to inhibitory postsynaptic sites.

The role of gephyrin in the recruitment of NL2 to inhibitory synapses was assessed by examining the subcellular localization of NL2 clusters in gephyrin knockdown cells. Specifically, a previously published siRNA sequence was used to attenuate gephyrin levels (Jacob et al., 2005). The efficacy of this siRNA was assayed in two ways. First, gephyrin siRNA attenuated CFP-gephyrin levels, but not PSD-95-GFP levels, in COS-7 cells (Fig. 3.2A). Second, there was a 58% decrease in the density of dendritic gephyrin clusters in hippocampal neurons transfected with gephyrin siRNA, demonstrating the efficacy of this siRNA sequence in neurons (Fig. 3.3)

To determine the role of gephyrin in localizing NL2 to synapses, cells expressing gephyrin siRNA were fixed, permeabilized, and immunostained with NL2 in combination with either VGAT or VGluT1. Consistent with previous reports (Yu et al., 2007), knockdown of gephyrin resulted in a reduction in the density of VGAT clusters contacting dendrites of transfected cells (35±9% of control; Fig. 3.4A, C). No changes in the density of VGluT1 or NL2 clusters were observed in these cells (Fig. 3.4B, D, E). However, a 31% reduction was observed in the proportion of NL2 clusters apposed to VGAT (Fig. 3.4A, F), with a concomitant 26% increase in the proportion of NL2 clusters apposed to VGluT1 (Fig. 3.4B, F). These data indicate a shift in the localization of NL2 from inhibitory to excitatory synaptic contacts upon knockdown of

gephyrin, suggesting that gephyrin modulates the synaptic localization of NL2 at inhibitory synapses.

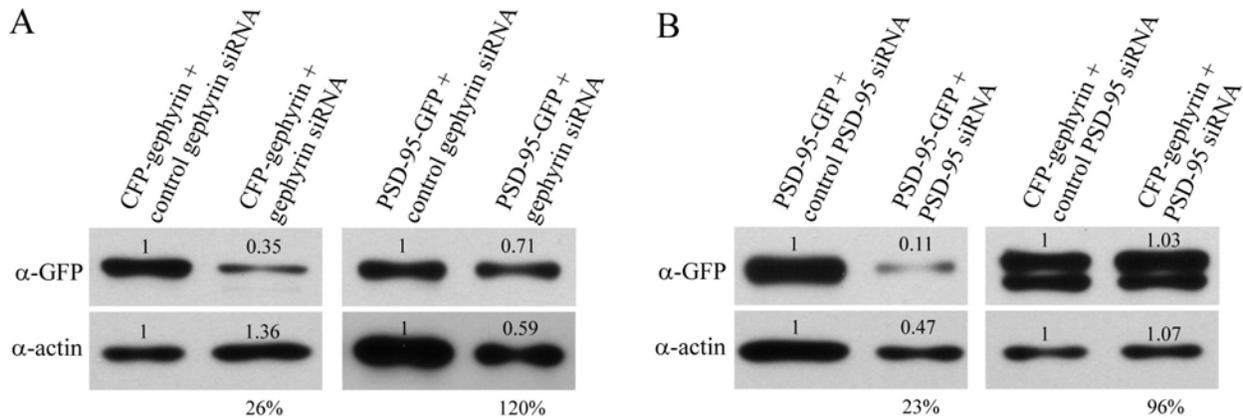


Fig. 3.2. Knockdown of gephyrin and PSD-95 in COS-7 cells. COS-7 cells were transfected with either CFP-gephyrin or PSD-95-GFP in combination with gephyrin (A) or PSD-95 (B) siRNA constructs, or their corresponding control siRNAs. Western blots were probed with either anti-GFP or anti-β-actin. Numbers above each band represent the intensity relative to its control band (in arbitrary integrated density units). Numbers at the bottom represent band intensities relative to the corresponding controls, after normalization with actin.

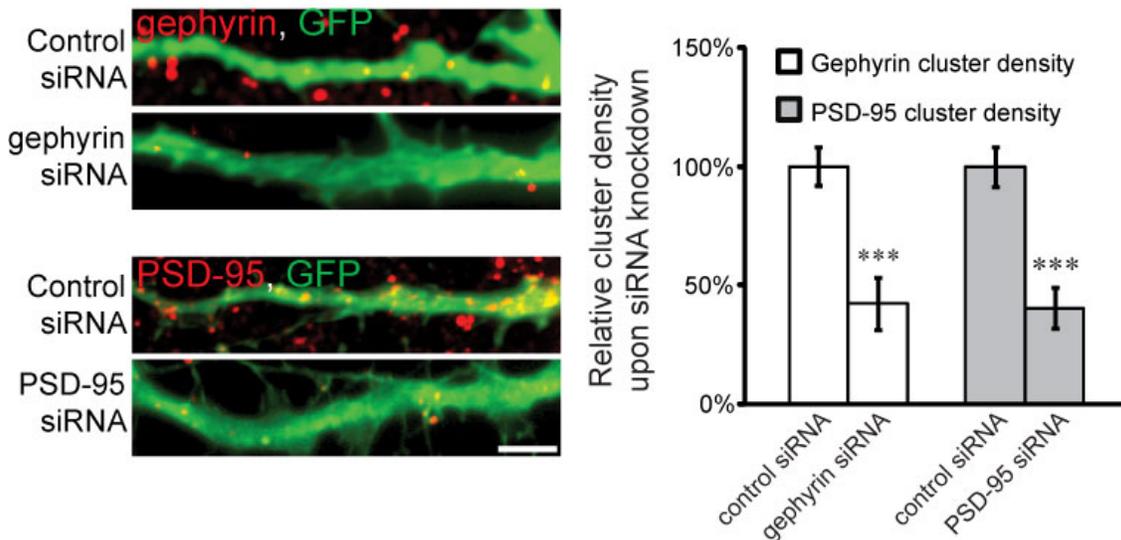


Fig. 3.3. Knockdown of gephyrin or PSD-95 in cultured hippocampal neurons. 5-6 DIV hippocampal neurons were co-transfected with GFP plus either gephyrin or PSD-95 siRNA, and fixed at 11-12 DIV. Cells were immunolabeled using antibodies against either gephyrin or PSD-95, and quantification of gephyrin and PSD-95 knockdown in cultured hippocampal neurons, as assessed by a reduction in cluster density for the targeted protein, was performed. $p < 0.001$. Scale bar, 5 μm .

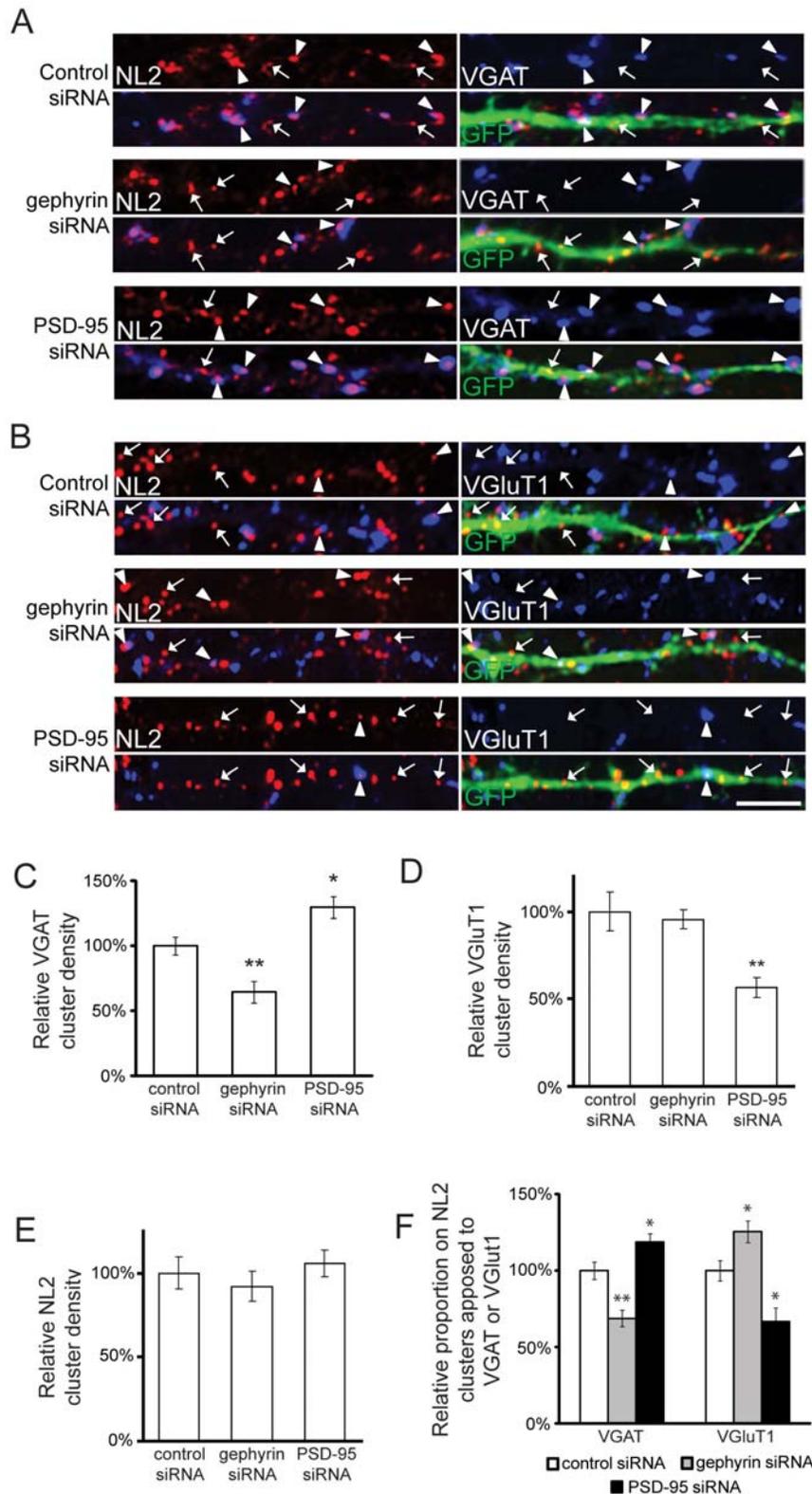


Fig. 3.4. Knockdown of gephyrin or PSD-95 alters the distribution of NL2 at inhibitory and excitatory synapses.

5-6 DIV hippocampal neurons were co-transfected with GFP plus either gephyrin or PSD-95 siRNA, and fixed at 11-12 DIV. Cells were immunolabeled using antibodies against either NL2 and VGAT (A), or NL2 and VGlut1 (B). Localization of NL2 clusters relative to VGAT (A) or VGlut1 (B) was assessed in neurons transfected with either gephyrin or PSD-95 siRNA. Arrowheads indicate colocalization of NL2 with either VGAT or VGlut1 on transfected neurons. Arrows denote NL2 clusters which do not colocalize with either VGAT or VGlut1. (C) Quantification of VGAT cluster density upon knockdown of either gephyrin or PSD-95, relative to control neurons. (D) Quantification of VGlut1 cluster density upon knockdown of either gephyrin or PSD-95, relative to control neurons. (E) Quantification of NL2 cluster density upon knockdown of either gephyrin or PSD-95, relative to control neurons. (F) Quantification of the localization of NL2 relative to synaptic markers upon knockdown of either gephyrin or PSD-95 knockdown. The proportion of NL2 clusters associated with either VGAT or VGlut1 was measured in neurons

transfected with either gephyrin or PSD-95 siRNA, and compared to control neurons. N=17-18 cells per condition from at least 3 independent experiments. Error bars represent SEM. *, $p < 0.05$; **, $p < 0.01$. Scale bar, 5 μm .

As a small pool of NL2 can also be found at excitatory synapses (Fig. 3.1B, C), and overexpression of PSD-95 has been shown to shift NL2 from inhibitory to excitatory synapses (Graf et al., 2004; Levinson et al., 2005), we also addressed the role of PSD-95 in the targeting of NL2 to excitatory postsynaptic sites. Specifically, we used a previously characterized PSD-95 siRNA construct (Nakagawa et al., 2004) to knock down PSD-95 protein levels in neurons. This PSD-95 siRNA sequence attenuated expression of PSD-95-GFP, but not CFP-gephyrin, in COS-7 cells (Fig. 3.2B). Furthermore, transfection of this construct in hippocampal neurons resulted in a 59% reduction in the density of PSD-95 clusters relative to control neurons (Fig. 3.3), confirming that this siRNA sequence efficiently reduces PSD-95 levels. Consistent with previous studies (Prange et al., 2004; Gerrow et al., 2006), knockdown of PSD-95 causes a significant reduction in the density of VGluT1 puncta ($57\pm 6\%$ of control cells; Fig. 3.4B, D), with a corresponding increase in the density of VGAT clusters ($130\pm 8\%$ of control cells; Fig. 3.4A, C). The density of NL2 clusters was not significantly different in cells expressing PSD-95 siRNA (Fig. 3.4A, B, E). However, the proportion of NL2 clusters apposed to VGluT1 puncta was significantly decreased ($67\pm 9\%$ of control cells; Fig. 3.4B, F), while the proportion of NL2 clusters associated with VGAT was significantly increased ($119\pm 6\%$ of control cells; Fig. 3.4A, F). This data indicates that knockdown of PSD-95 shifts the localization of NL2 from excitatory to inhibitory synaptic contacts. Together, these data suggest that scaffolding molecules such as PSD-95 and gephyrin modulate the targeting of NL2 to specific synaptic subtypes.

3.3.3 The C-terminal Domain of NL2 Modulates its Recruitment to Inhibitory Synapses

Previous work has shown the importance of C-terminal regions in mediating the postsynaptic clustering of NL1 (Dresbach et al., 2004; Prange et al., 2004). As gephyrin is involved in localizing NL2 to inhibitory synapses, we hypothesized that the C-terminus of NL2 plays a similar role in modulating the recruitment of NL2 to inhibitory synapses. Various candidate regions exist within the intracellular tail of NL2, including a C-terminal PDZ-binding domain, a proline-rich region which corresponds to an area of particularly high sequence divergence between NL2 and other neuroligin family members, and the region between the transmembrane domain and the proline-rich region (Fig. 3.5).

To assess the importance of these regions in NL2 targeting, we generated a series of C-terminal deletion mutant constructs (Fig. 3.5). Surface expression levels of the deletion constructs were similar to that of wild-type HA-NL2 (data not shown). When expressed at high levels, NL2 aberrantly localizes throughout the transfected neuron, and has a strong effect on presynaptic terminal maturation (Graf et al., 2004; Prange et al., 2004; Chih et al., 2005; Levinson et al., 2005). To avoid issues associated with high levels of NL2 overexpression, constructs were transfected using the calcium phosphate method, with small amounts of DNA. Using this method, transfected HA-tagged wild-type NL2 was found to cluster at inhibitory synaptic sites, as defined by colocalization with a postsynaptic gephyrin cluster apposed to a presynaptic VGAT cluster (Fig. 3.6A). Moreover, when transfected using this method, wild-type and mutant NL2 constructs did not significantly alter the density of inhibitory synapses compared to cells transfected with GFP (Fig. 3.6A, B).

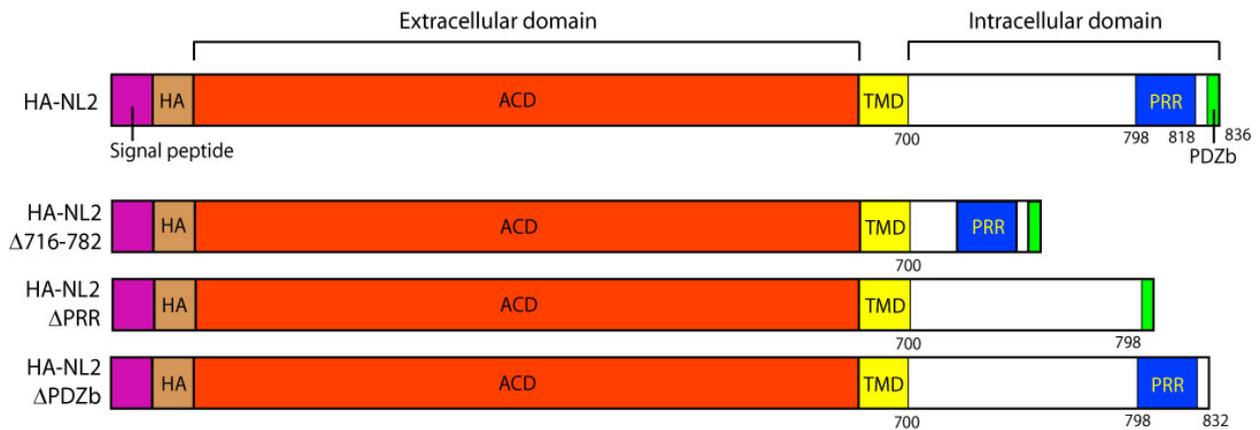


Fig. 3.5. Schematic representation of C-terminal NL2 deletion constructs. ACD, acetylcholinesterase-like domain; TMD, transmembrane domain; PRR, proline-rich region; PDZb, PDZ-binding motif.

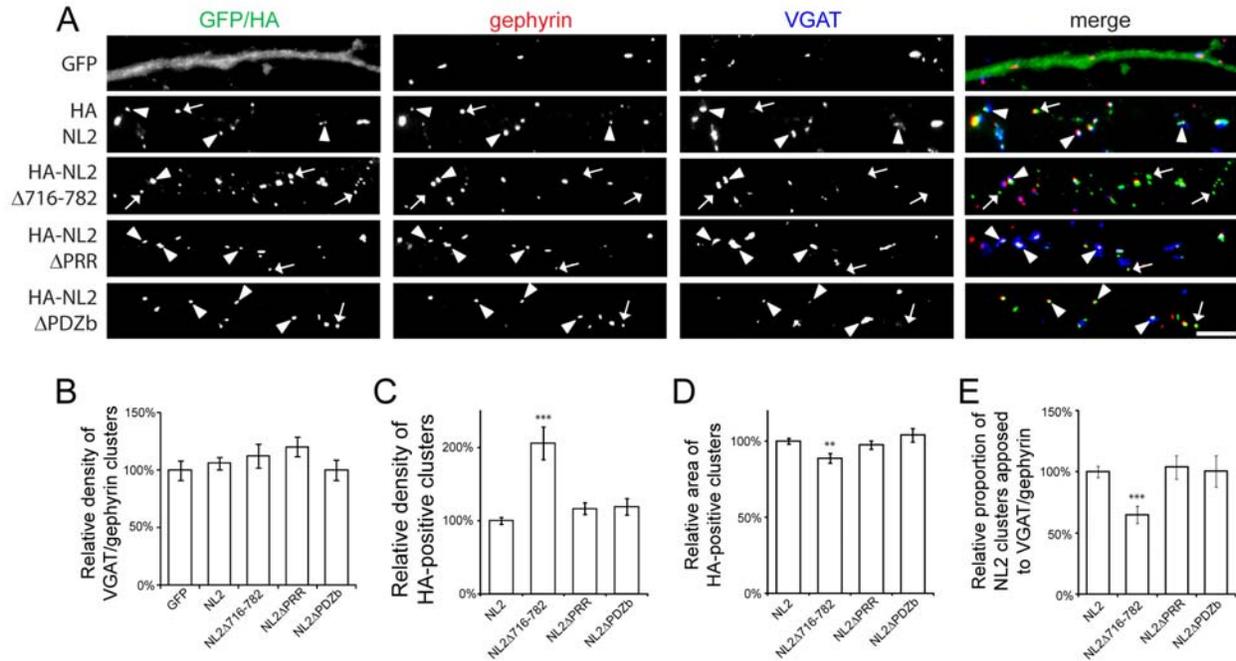


Fig. 3.6. The C-terminal domain of NL2 modulates its localization at inhibitory synapses. (A) 7-8 DIV cultured hippocampal neurons were transfected with each of the NL2 deletion constructs, and immunolabeled using antibodies against HA, VGAT and gephyrin at 10-11 DIV. Arrowheads indicate HA-NL2-positive clusters that colocalize with both gephyrin and VGAT, while HA-NL2 positive clusters that do not colocalize with both VGAT and gephyrin are marked by arrows. (B) Transfection of the various NL2 C-terminal deletion mutants does not result in increased inhibitory synapse density, relative to GFP controls. (C, D) Deletion of amino acids 716-782 increases the density (C) and decreases the area (D) of HA-positive clusters. (E) Deletion of amino acids 716-782 reduces the proportion of NL2 clusters at inhibitory synapses. N=17-48 cells per condition from at least 3 independent experiments. Error bars represent SEM. **, $p < 0.01$; ***, $p < 0.001$. Scale bar, 5 μm .

Deletion of the NL2 proline-rich region (NL2 Δ PRR) or PDZ-binding domain (NL2 Δ PDZb) had no effect on the density of NL2 clusters, compared to wild-type HA-NL2 (Fig. 3.6A, C). However, deletion of the region between amino acids 716 and 782 resulted in a 106% increase in the density of HA-positive clusters (Fig. 3.6A, C), as well as an 11% decrease in HA-positive cluster area (Fig. 3.6A, D), relative to wild-type HA-NL2. Interestingly, this region between the transmembrane domain and the proline-rich region of NL2 overlaps with a region required for the synaptic clustering of NL1, as well as a region required for the polarized dendritic localization of NL1 (Dresbach et al., 2004; Rosales et al., 2005). To address which C-terminal domains of NL2 are important for its targeting to inhibitory synapses, cells were transfected with specific C-terminal domain deletion constructs, and the proportion of HA-positive clusters found at sites containing a VGAT cluster apposed to a gephyrin cluster was quantified. Neither deletion

of the NL2 proline-rich region, nor the PDZ-binding domain had an effect on this measure. However, deletion of the region between amino acids 716 and 782 resulted in a 35% reduction in the proportion of HA-positive clusters colocalizing with gephyrin/VGAT (Fig. 3.6A, E). Together, these data suggest that region 716-782 is required for discrete clustering of NL2 at inhibitory contacts.

3.3.4 NL3 is Expressed in the Adult Brain and is Localized Mainly at Excitatory Synapses in Cultured Hippocampal Neurons

NL3 has previously been identified in the rodent brain and has been shown to enhance the formation of both excitatory and inhibitory synapses in cultured neurons (Ichtchenko et al., 1996; Gilbert et al., 2001; Chih et al., 2005; Levinson et al., 2005; Varoqueaux et al., 2006; Budreck and Scheiffele, 2007). To further characterize NL3, we generated and purified a polyclonal rabbit antibody against an intracellular epitope of NL3 with minimal homology to the other neuroligin family members. To test the specificity of this antibody, Western blot analysis of COS-7 cells transfected with GFP-tagged NL1, NL2, or NL3 was carried out. Immunolabeling with our anti-NL3 antibody revealed a single band exclusively in COS-7 cells expressing GFP-NL3 (Fig. 3.7A). Staining with anti-GFP antibody confirmed that GFP-tagged NL1 and NL2 were being properly expressed (Fig. 3.7A). Pre-adsorption with the peptide used to generate anti-NL3 abolished detection of the band corresponding to NL3, further demonstrating the specificity of this antibody (Fig. 3.7A, bottom). The NL3 antibody also recognized a single band of approximately 100 kD in whole-brain homogenates from adult rats, consistent with previous findings (Fig. 3.7B). To further examine the synaptic localization of NL3, 14 DIV cultured hippocampal neurons were immunolabeled for NL3, VGluT1 and VGAT. NL3 was expressed in a punctate pattern and was shown to localize predominantly with the excitatory presynaptic marker, VGluT1 (Fig. 3.8A). $64\pm 2\%$ of NL3 clusters were apposed to VGluT1 clusters, while only $20\pm 2\%$ were apposed to VGAT-positive sites (Fig. 3.8B, D).

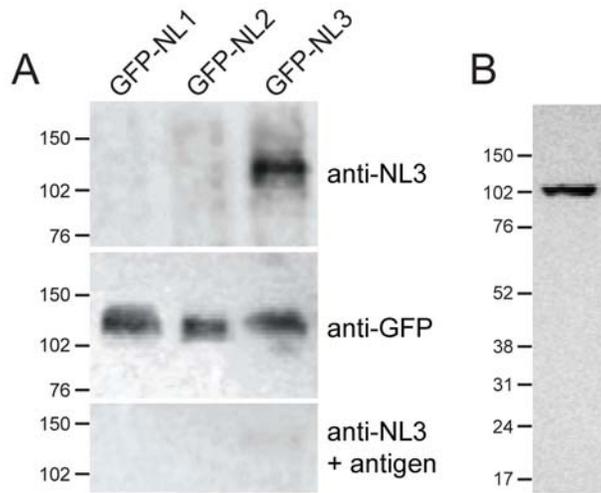


Fig. 3.7. NL3 is expressed in rat brain. (A) Western blot analysis of lysates from COS-7 cells expressing GFP-tagged versions of NL1, NL2 or NL3. The NL3 antibody recognized a single band in lysates expressing GFP-NL3, but not in lysates expressing GFP-NL1 or GFP-NL2. Protein expression was verified using anti-GFP. Pre-adsorption with the peptide used to generate the anti-NL3 antibody abolished the presence of the GFP-NL3 band. (B) The anti-NL3 antibody recognized a single band at ~100 kD from adult rat whole brain lysates.

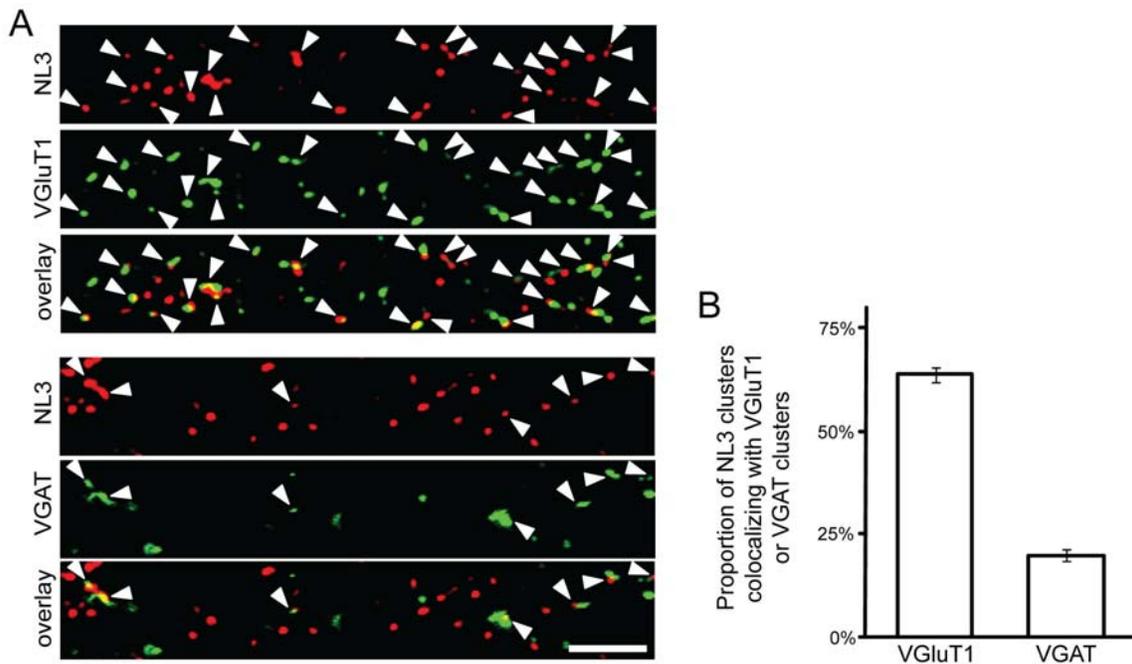


Fig. 3.8. NL3 is found mainly at excitatory synapses in cultured hippocampal neurons. (A) Confocal images of 14 DIV cultured hippocampal neurons immunolabeled with anti-NL3 in combination with anti-VGluT1 or anti-VGAT. Arrowheads indicate NL3 clusters that colocalize with VGluT1 or VGAT. (B) Quantification of colocalization between NL3 and either VGluT1 or VGAT. N=16-18 cells per immunostaining set. Error bars represent SEM. Scale bar, 5 μ m.

3.3.5 Knockdown of PSD-95 or Gephyrin Alters the Proportion of NL3 at Excitatory and Inhibitory Synapses

As PSD-95 is involved in the targeting of NL1 to excitatory synaptic sites, we examined whether PSD-95 plays a similar role in localizing NL3. Neurons transfected with PSD-95 siRNA exhibited no significant change in the density of NL3 clusters (Fig. 3.9A-C), similar to NL2 (Fig. 3.4). However, the proportion of NL3 clusters apposed to VGluT1 puncta was decreased by approximately 30% (Fig. 3.9A, D), with a concomitant 46% increase in the proportion of NL3 clusters apposed to VGAT puncta (Fig. 3.9B, D). This suggests that PSD-95 knockdown partially shifts NL3 from excitatory to inhibitory contacts, analogous to what has been shown for NL1 (Gerrow et al., 2006) and to what our data show for NL2 (Fig. 3.4).

Although NL3 is primarily localized to excitatory sites, a smaller proportion of NL3 clusters can be found apposed to inhibitory presynaptic terminals (Fig. 3.8). As such, we assessed whether gephyrin is involved in the targeting of this small pool of NL3 to inhibitory contacts. As with PSD-95 knockdown, knockdown of gephyrin had no effect on the density of NL3 clusters (Fig. 3.9A-C). The proportion of NL3 clusters apposed to VGAT puncta, however, was decreased by approximately 34% (Fig. 3.9B, D). No corresponding increase in the colocalization of NL3 with VGluT1 was observed (Fig. 3.9A, D), which may suggest that the NL3 lost from inhibitory sites is not recruited to excitatory synaptic sites. Alternatively, due to the high proportion of NL3 found at excitatory synapses under control conditions, further accumulation of relatively small amounts of protein, resulting from the loss of NL3 from inhibitory contacts, may not be resolvable by the detection means used in this study.

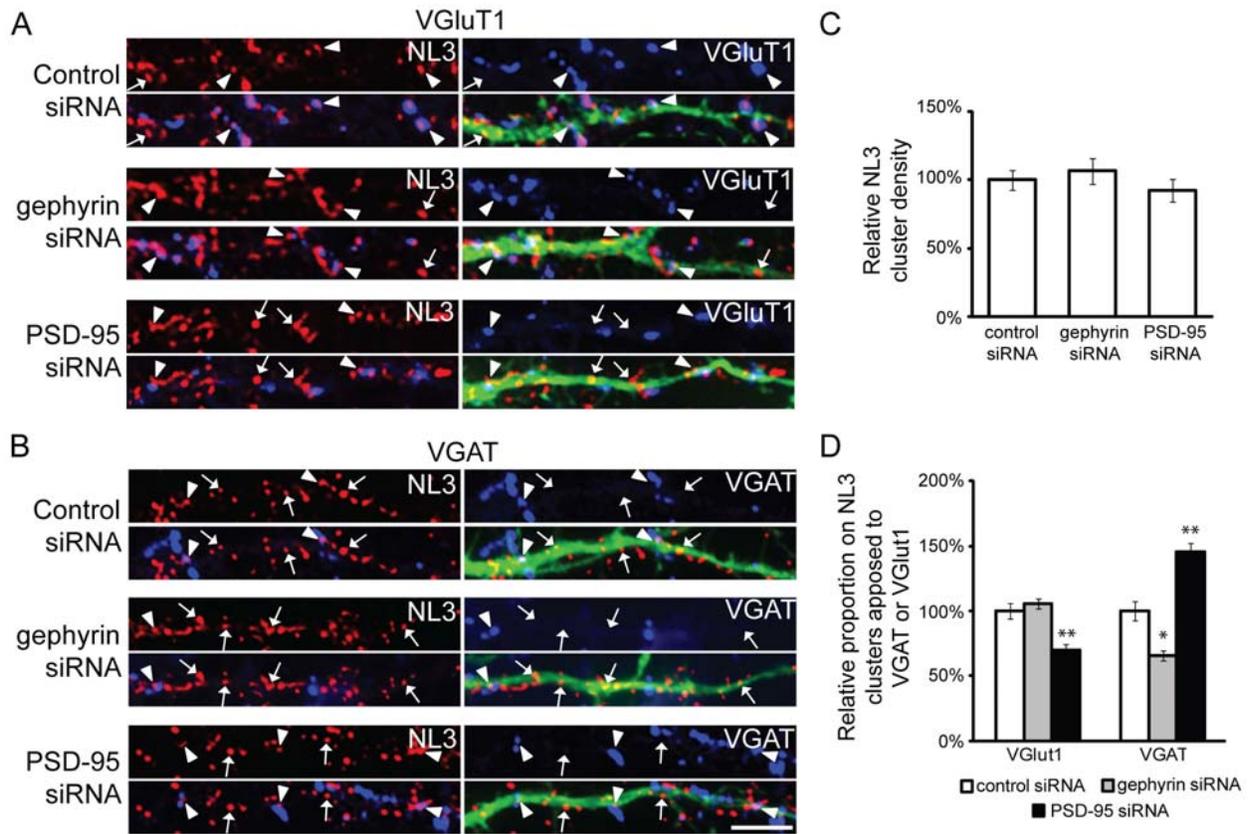


Fig. 3.9. Knockdown of PSD-95 or gephyrin alters the synaptic localization of NL3 in cultured neurons. 5-6 DIV hippocampal neurons were co-transfected with GFP plus either gephyrin or PSD-95 siRNA and fixed at 11-12 DIV. Cells were immunolabeled with anti-NL2 in combination with either anti-VGlut1 (A) or anti-VGAT (B). Arrowheads indicate NL3 clusters apposed to the presynaptic marker being examined. Arrows denote NL3 clusters which do not colocalize with the presynaptic marker being examined. (C) Quantification of the relative density of NL3 clusters upon knockdown of either gephyrin or PSD-95. No significant differences were detected. N=30-37 cells per condition from 3 independent experiments. (D) Quantification of the proportion of NL3 apposed to VGlut1 or VGAT in neurons transfected with either gephyrin or PSD-95 siRNA. N=15-20 cells per condition from 3 independent experiments. Error bars represent SEM. *, $p < 0.05$; **, $p < 0.01$. Scale bar, 5 μm .

3.4 Discussion

The neuroligin family of transmembrane proteins is both sufficient and necessary for the formation of synapses in cultured hippocampal neurons. Indeed, expression of neuroligins is sufficient to induce the formation of presynaptic terminals in contacting neurons (Scheiffele et al., 2000; Graf et al., 2004), while knockdown of neuroligin protein levels decreases synapse density, and alters the ratio of excitatory to inhibitory synaptic activity, in favour of more excitation (Chih et al., 2005). Interestingly, overexpression of PSD-95, which enhances excitatory synapse maturation (El-Husseini et al., 2000a), increases the accumulation of neuroligins at this synapse type, and knockdown of PSD-95, which is associated with a shift of NL1 from excitatory to inhibitory synapses (Gerrow et al., 2006), increases the number of inhibitory contacts at the expense of excitatory contacts. Given the importance of neuroligins in promoting the formation and maturation of specific synaptic subtypes and in dictating the balance of excitatory and inhibitory inputs, it is crucial to understand the mechanism(s) by which these proteins are localized to excitatory and inhibitory synapses. Evidence indicates that the C-terminal regions of neuroligins are involved in this process. For instance, Dresbach and colleagues demonstrate that the region of the NL1 C-terminal tail located between the transmembrane and PDZ-binding domains is important for the clustering of NL1 at postsynaptic compartments (Dresbach et al., 2004). Furthermore, while highly overexpressed NL1 does not cluster appropriately and promotes the formation of both excitatory and inhibitory presynaptic terminals, overexpression of its postsynaptic binding partner, PSD-95, in these cells enhances the clustering of NL1 at synapses, and specifically enhances the formation of excitatory synapses (Prange et al., 2004). Moreover, overexpression of PSD-95 has been shown to shift the localization of NL2 from inhibitory to excitatory synapses (Graf et al., 2004; Levinson et al., 2005).

Our data provide additional evidence that the C-terminal domains of neuroligins are important for the synaptic localization of these proteins, and specifically demonstrate that intracellular mechanisms can modulate the localization of NL2 and NL3 at inhibitory and excitatory synapses (Fig. 3.10). Indeed, siRNA-mediated knockdown of gephyrin shifts the localization of NL2 from inhibitory to excitatory synapses, while knockdown of PSD-95 has the opposite effect, shifting NL2 and NL3 from excitatory to inhibitory contacts. Moreover, our findings indicate that the C-

terminal region of NL2 between amino acids 716 and 782 plays an important role in clustering NL2 at inhibitory synapses. This region may facilitate the synaptic clustering of NL2 independently of gephyrin, however it is also possible that gephyrin interacts with this region, either directly or indirectly, to localize NL2 to inhibitory synapses. Indeed, as mentioned above, a similar region in the C-terminus of NL1 is necessary for the clustering of this protein at synapses (Dresbach et al., 2004). Further work is required to determine whether this intracellular region is involved in targeting different family members to a specific type of synapse, or whether these regions are simply required for the ability of neuroligins to cluster in more general terms.

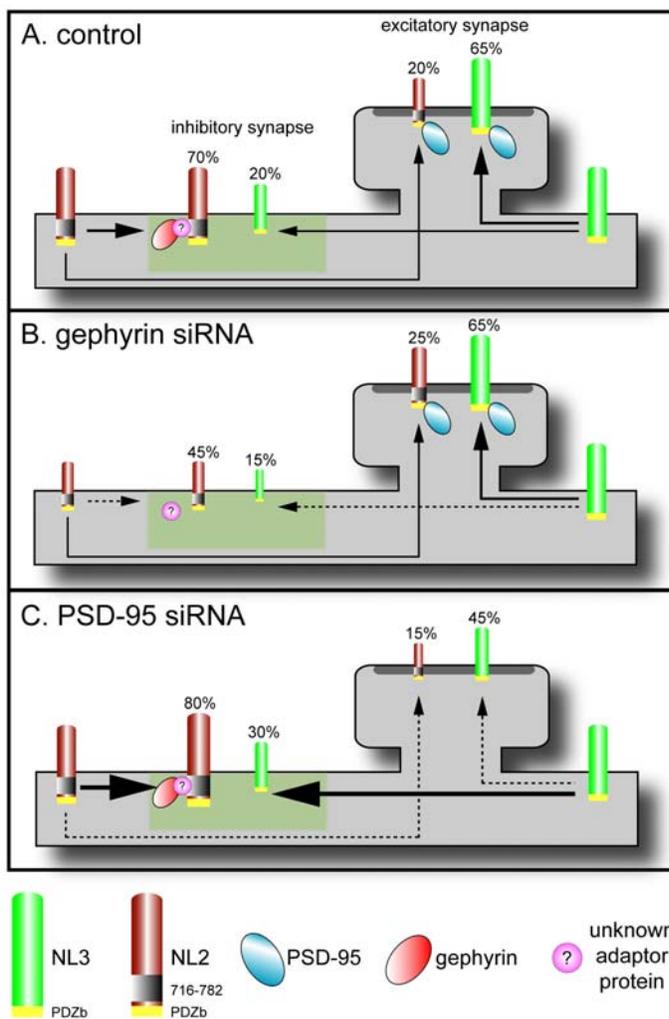


Fig. 3.10. Model illustrating the involvement of gephyrin and PSD-95 in the synaptic localization of NL2 and NL3. (A) In control cells, the majority of NL2 is localized at inhibitory synapses, while only a subset is localized at excitatory synapses. Conversely, the majority of NL3 is localized at excitatory contacts, with only a subset is localized at inhibitory synapses. We postulate that these neuroligins are recruited to inhibitory synapses, in part, through interaction with gephyrin, and to excitatory synapses through interactions with PSD-95. (B) Attenuation of gephyrin protein levels reduces the proportion of NL2 and NL3 at inhibitory synapses, and increases the proportion of NL2 at excitatory synapses, presumably due to the increased availability of NL2 for binding PSD-95. (C) Attenuation of PSD-95 protein levels reduces the proportion of NL2 and NL3 at excitatory synapses, with concomitant increases at inhibitory sites. The proportions of neuroligin localization at inhibitory and excitatory synapses are indicated in the model, and represent approximations of our data.

Knockdown of gephyrin or PSD-95 produced a rather modest effect on neuroligin localization at synapses in our experiments (Figs. 3.4 and 3.9). One possible reason for this is that additional

mechanisms likely participate in this process. For example, alternative splice sites within the extracellular domains of neuroligins have been shown to determine the recruitment of these proteins to excitatory versus inhibitory synapses (Chih et al., 2006), as well as their binding to specific neurexin isoforms (Boucard et al., 2005). Indeed, manipulation of the extracellular domains of NL1 and NL2 produced ~3-fold differences in the synaptic localization patterns of these proteins (Chih et al., 2006). In addition, different splice isoforms of neurexins have differential effects on the clustering of NL1/3/4 versus NL2, as well as on induction of excitatory versus inhibitory postsynaptic differentiation (Chih et al., 2006; Graf et al., 2006). Furthermore, other scaffolding proteins found at inhibitory synapses could aid in the targeting of NL2 to these sites. One candidate is S-SCAM, a scaffolding protein with a structural organization similar to that of PSD-95 that has been shown to interact with NL2 and β -dystroglycan at inhibitory synapses (Hirao et al., 1998; Sumita et al., 2006). Further evidence of the involvement of additional factors in the localization of NL2 comes from a recent study by O'Sullivan et al. (2009), where NL2 clusters were found apposed to inhibitory nerve terminals in gephyrin knockout mice. While it is possible that compensatory mechanisms may underlie the retention of NL2 at inhibitory synapses in these knockout animals, these results suggest the involvement of gephyrin-independent mechanisms. Thus, it is clear from ours and previous data that intracellular mechanisms are involved in the modulation of neuroligin localization, rather than playing a major role in this process. However, the effects observed in our study may be underestimates of the actual contributions of the intracellular domains of neuroligins to their synaptic localization patterns. First, in the case of our siRNA experiments, the moderate effects of gephyrin or PSD-95 knockdown may simply be due to incomplete knockdown of these proteins (Fig. 3.3). Second, for the NL2 C-terminal deletion experiments, the presence of wild-type, endogenous NL2 may mask the extent of the effects of the NL2 Δ 716-782 mutant, through the ability of neuroligins to form functional oligomers (Comoletti et al., 2003; Dean et al., 2003). Thus, it is likely that both extra- and intracellular mechanisms act together to determine the specific synaptic targeting of neuroligins.

Neuroligins have been shown to interact with PSD-95 through the C-terminal type I PDZ-binding domain that is preserved in all family members. Indeed, PDZ-dependent interaction between PSD-95 and neuroligins has been demonstrated biochemically and via yeast two-hybrid

(Irie et al., 1997), and the effects of PSD-95 on the clustering of NL1 are abolished when the third PDZ domain of PSD-95 is deleted (Prange et al., 2004). These observations raise the possibility of competition between different scaffolding molecules for binding to neuroligins via distinct or overlapping regions within their intracellular tails. For example, gephyrin, or an adaptor protein which mediates interaction of gephyrin with NL2, may have a higher affinity for NL2 than does PSD-95, perhaps due to the presence of C-terminal sequences unique to NL2. Under normal conditions, the majority of NL2 would then be localized at inhibitory contacts, while only a small proportion would be found at excitatory sites. With reduced gephyrin levels however, more NL2 would be available to bind PSD-95 via the PDZ-binding domain of NL2, resulting in a higher level of NL2 localized at excitatory contacts. Conversely, PSD-95 upregulation might enable it to out-compete gephyrin, resulting in the recruitment of a larger pool of NL2 to excitatory synapses.

While previous *in vitro* work suggests that targeting of neuroligins to specific synaptic sites may be relevant to the development of synapses and the initial establishment of E/I balance, *in vivo* work suggests that neuroligins may in fact participate more in the maturation and function of synapses (Varoqueaux et al., 2006). Triple knockout of NL1-3 in mice results in reduced spontaneous inhibitory and excitatory activity in the respiratory brainstem, resulting in an increased E/I ratio. However, these changes in synaptic activity were not accompanied by changes in the total number of synapses in this region. It is possible, then, that specificity of neuroligin targeting has more of an impact on synaptic function in already-developed synapses.

3.5 References

- Bats C, Groc L, Choquet D (2007) The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. *Neuron* 53:719-734.
- Boucard AA, Chubykin AA, Comoletti D, Taylor P, Sudhof TC (2005) A splice code for trans-synaptic cell adhesion mediated by binding of neuroligin 1 to alpha- and beta-neurexins. *Neuron* 48:229-236.
- Brewer GJ, Torricelli JR, Eवेge EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 35:567-576.
- Budreck EC, Scheiffele P (2007) Neuroligin-3 is a neuronal adhesion protein at GABAergic and glutamatergic synapses. *Eur J Neurosci* 26:1738-1748.
- Chih B, Engelman H, Scheiffele P (2005) Control of excitatory and inhibitory synapse formation by neuroligins. *Science* 307:1324-1328.
- Chih B, Gollan L, Scheiffele P (2006) Alternative splicing controls selective trans-synaptic interactions of the neuroligin-neurexin complex. *Neuron* 51:171-178.
- Comoletti D, Flynn R, Jennings LL, Chubykin A, Matsumura T, Hasegawa H, Sudhof TC, Taylor P (2003) Characterization of the interaction of a recombinant soluble neuroligin-1 with neurexin-1beta. *J Biol Chem* 278:50497-50505.
- Craig AM, Banker G, Chang W, McGrath ME, Serpinskaya AS (1996) Clustering of gephyrin at GABAergic but not glutamatergic synapses in cultured rat hippocampal neurons. *J Neurosci* 16:3166-3177.
- Dean C, Scholl FG, Choih J, DeMaria S, Berger J, Isacoff E, Scheiffele P (2003) Neurexin mediates the assembly of presynaptic terminals. *Nat Neurosci* 6:708-716.
- Dresbach T, Neeb A, Meyer G, Gundelfinger ED, Brose N (2004) Synaptic targeting of neuroligin is independent of neurexin and SAP90/PSD95 binding. *Mol Cell Neurosci* 27:227-235.
- El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Brecht DS (2000a) PSD-95 involvement in maturation of excitatory synapses. *Science* 290:1364-1368.
- El-Husseini AE, Craven SE, Chetkovich DM, Firestein BL, Schnell E, Aoki C, Brecht DS (2000b) Dual palmitoylation of PSD-95 mediates its vesiculotubular sorting, postsynaptic targeting, and ion channel clustering. *J Cell Biol* 148:159-172.
- Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin. *Nat Neurosci* 1:563-571.
- Feng G, Tintrop H, Kirsch J, Nichol MC, Kuhse J, Betz H, Sanes JR (1998) Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. *Science* 282:1321-1324.
- Gerrow K, El-Husseini A (2006) Cell adhesion molecules at the synapse. *Front Biosci* 11:2400-2419.
- Gerrow K, Romorini S, Nabi SM, Colicos MA, Sala C, El-Husseini A (2006) A preformed complex of postsynaptic proteins is involved in excitatory synapse development. *Neuron* 49:547-562.
- Gilbert M, Smith J, Roskams AJ, Auld VJ (2001) Neuroligin 3 is a vertebrate gliotactin expressed in the olfactory ensheathing glia, a growth-promoting class of macroglia. *Glia* 34:151-164.
- Graf ER, Kang Y, Hauner AM, Craig AM (2006) Structure function and splice site analysis of the synaptogenic activity of the neurexin-1 beta LNS domain. *J Neurosci* 26:4256-4265.

- Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM (2004) Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 119:1013-1026.
- Hanus C, Vannier C, Triller A (2004) Intracellular association of glycine receptor with gephyrin increases its plasma membrane accumulation rate. *J Neurosci* 24:1119-1128.
- Hirao K, Hata Y, Ide N, Takeuchi M, Irie M, Yao I, Deguchi M, Toyoda A, Sudhof TC, Takai Y (1998) A novel multiple PDZ domain-containing molecule interacting with N-methyl-D-aspartate receptors and neuronal cell adhesion proteins. *J Biol Chem* 273:21105-21110.
- Ichtchenko K, Nguyen T, Sudhof TC (1996) Structures, alternative splicing, and neurexin binding of multiple neuroligins. *J Biol Chem* 271:2676-2682.
- Ichtchenko K, Hata Y, Nguyen T, Ullrich B, Missler M, Moomaw C, Sudhof TC (1995) Neuroligin 1: a splice site-specific ligand for beta-neurexins. *Cell* 81:435-443.
- Irie M, Hata Y, Takeuchi M, Ichtchenko K, Toyoda A, Hirao K, Takai Y, Rosahl TW, Sudhof TC (1997) Binding of neuroligins to PSD-95. *Science* 277:1511-1515.
- Jacob TC, Bogdanov YD, Magnus C, Saliba RS, Kittler JT, Haydon PG, Moss SJ (2005) Gephyrin regulates the cell surface dynamics of synaptic GABA_A receptors. *J Neurosci* 25:10469-10478.
- Jiang M, Deng L, Chen G (2004) High Ca²⁺-phosphate transfection efficiency enables single neuron gene analysis. *Gene Ther* 11:1303-1311.
- Jin Y, Garner CC (2008) Molecular mechanisms of presynaptic differentiation. *Annu Rev Cell Dev Biol* 24:237-262.
- Kirsch J, Malosio ML, Wolters I, Betz H (1993) Distribution of gephyrin transcripts in the adult and developing rat brain. *Eur J Neurosci* 5:1109-1117.
- Kneussel M, Loebrich S (2007) Trafficking and synaptic anchoring of ionotropic inhibitory neurotransmitter receptors. *Biol Cell* 99:297-309.
- Kneussel M, Brandstatter JH, Laube B, Stahl S, Muller U, Betz H (1999) Loss of postsynaptic GABA(A) receptor clustering in gephyrin-deficient mice. *J Neurosci* 19:9289-9297.
- Levi S, Logan SM, Tovar KR, Craig AM (2004) Gephyrin is critical for glycine receptor clustering but not for the formation of functional GABAergic synapses in hippocampal neurons. *J Neurosci* 24:207-217.
- Levinson JN, Chery N, Huang K, Wong TP, Gerrow K, Kang R, Prange O, Wang YT, El-Husseini A (2005) Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. *J Biol Chem* 280:17312-17319.
- McAllister AK (2007) Dynamic aspects of CNS synapse formation. *Annu Rev Neurosci* 30:425-450.
- Nakagawa T, Futai K, Lashuel HA, Lo I, Okamoto K, Walz T, Hayashi Y, Sheng M (2004) Quaternary structure, protein dynamics, and synaptic function of SAP97 controlled by L27 domain interactions. *Neuron* 44:453-467.
- Nam CI, Chen L (2005) Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. *Proc Natl Acad Sci U S A* 102:6137-6142.
- Prange O, Wong TP, Gerrow K, Wang YT, El-Husseini A (2004) A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. *Proc Natl Acad Sci U S A* 101:13915-13920.
- Rosales CR, Osborne KD, Zuccarino GV, Scheiffele P, Silverman MA (2005) A cytoplasmic motif targets neuroligin-1 exclusively to dendrites of cultured hippocampal neurons. *Eur J Neurosci* 22:2381-2386.

- Rubenstein JL, Merzenich MM (2003) Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav* 2:255-267.
- Scheiffele P, Fan J, Choih J, Fetter R, Serafini T (2000) Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101:657-669.
- Schnell E, Sizemore M, Karimzadegan S, Chen L, Brecht DS, Nicoll RA (2002) Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. *Proc Natl Acad Sci U S A* 99:13902-13907.
- Schummers J, Marino J, Sur M (2002) Synaptic integration by V1 neurons depends on location within the orientation map. *Neuron* 36:969-978.
- Song JY, Ichtchenko K, Sudhof TC, Brose N (1999) Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc Natl Acad Sci U S A* 96:1100-1105.
- Sumita K, Sato Y, Iida J, Kawata A, Hamano M, Hirabayashi S, Ohno K, Peles E, Hata Y (2006) Synaptic scaffolding molecule (S-SCAM) membrane-associated guanylate kinase with inverted organization (MAGI)-2 is associated with cell adhesion molecules at inhibitory synapses in rat hippocampal neurons. *J Neurochem* 100:154-166.
- Tretter V, Jacob TC, Mukherjee J, Fritschy JM, Pangalos MN, Moss SJ (2008) The clustering of GABA(A) receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor alpha 2 subunits to gephyrin. *J Neurosci* 28:1356-1365.
- Varoqueaux F, Jamain S, Brose N (2004) Neuroligin 2 is exclusively localized to inhibitory synapses. *Eur J Cell Biol* 83:449-456.
- Varoqueaux F, Aramuni G, Rawson RL, Mohrmann R, Missler M, Gottmann K, Zhang W, Sudhof TC, Brose N (2006) Neuroligins determine synapse maturation and function. *Neuron* 51:741-754.
- Washbourne P, Dityatev A, Scheiffele P, Biederer T, Weiner JA, Christopherson KS, El-Husseini A (2004) Cell adhesion molecules in synapse formation. *J Neurosci* 24:9244-9249.

4. General Discussion

4.1 Summary of Findings

The objectives of this work were to determine:

- (1) What are the roles of different neuroligin family members in the development of different synapse types?
- (2) What roles do postsynaptic mechanisms play in differentially regulating the localization, and therefore effects, of various neuroligin proteins?

Previous studies have shown that members of the neuroligin family of postsynaptic CAMs are synaptogenic, in that their expression in non-neuronal cells is sufficient to induce the formation of presynaptic terminals on contacting neuronal axons. Furthermore, the effects of neuroligins on synapse formation were shown to be mediated through neurexins, a family of primarily presynaptic CAMs that have been shown to bind to neuroligins (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Scheiffele et al., 2000; Dean et al., 2003). In addition, expression of NL1 in cultured neurons has been shown to induce the formation of both glutamatergic and GABAergic presynaptic terminals (Prange et al., 2004). The work presented in this thesis addresses the importance of NL2 and NL3 in the development of GABAergic synapses, and assesses the role of neurexins in this process. I show that, like NL1, expression of NL2 or NL3 induce glutamatergic and GABAergic presynaptic differentiation, indicating that additional neuroligin family members play a role in inhibitory synapse formation. Moreover, the effects of neuroligins on the formation of GABAergic terminals are abrogated upon treatment with a soluble form of neurexin-1 β . This suggests that neurexins generally mediate the effects of multiple members of the neuroligin family on both glutamatergic and GABAergic synapse development. Consistent with other reports, I show that, while the majority of NL1 is localized to excitatory synaptic sites, NL2 is primarily found at inhibitory synapses. Interestingly, overexpression of PSD-95 in neurons causes a shift of endogenous NL2 from GABAergic to glutamatergic synapses, suggesting that interactions between PSD-95 and neuroligins may participate in the regulation of neuroligin localization, and ultimately in the modulation of synaptic balance.

Due to the findings that (1) co-expression of PSD-95 with NL1 in hippocampal neurons restricts the effects of NL1 on presynaptic terminal maturation to excitatory synapses (Prange et al., 2004) and (2) overexpression of PSD-95 shifts NL2 from inhibitory to excitatory synaptic sites, I investigated the importance of postsynaptic scaffolding molecules in the targeting of neuroligins to specific synapse types. My findings demonstrate that siRNA-mediated knockdown of PSD-95 leads to redistribution of NL2 and NL3 from glutamatergic to GABAergic synapses. Conversely, knockdown of the inhibitory postsynaptic scaffolding protein, gephyrin, results in a shift of NL2 from GABAergic to glutamatergic contacts. Additionally, we find that deletion of a region of the NL2 intracellular domain located between the transmembrane domain and the proline-rich domain leads to reduced targeting of NL2 to GABAergic synapses, indicating that this region is required for the normal clustering of NL2 to inhibitory contact sites. Together, these data suggest that, via the intracellular C-terminal tail of neuroligins, postsynaptic scaffolding proteins specific to different synapse types modulate the synaptic targeting of neuroligins. Thus, this mechanism may partially underlie the synapse specificity of different neuroligin family members, and could therefore play a role in the control of E/I balance.

4.2 Role of Neuroligin-Neurexin Interaction in Synapse Development and Specificity

4.2.1 Initiation of Synapse Formation vs. Synapse Maturation vs. Synaptic Plasticity. Which One is it?

Neuroligins and neurexins were originally considered as candidate organizers of synaptic contacts as they met the following criteria for such molecules: (1) They are both CAMs expressed at the surface of neurons, (2) they are present in multiple isoforms, with different neurons expressing different variants, making them well suited to encoding specificity codes (Ichtchenko et al., 1996; Missler and Sudhof, 1998), (3) NL1 and neurexin 1 β bind each other and mediate cell adhesion in native membranes (Ichtchenko et al., 1995; Nguyen and Sudhof, 1997), (4) neurexins are receptors for α -latrotoxin, which triggers massive neurotransmitter release, suggesting a presynaptic localization for these proteins (Ushkaryov et al., 1992), (5) NL1 is found postsynaptically at excitatory contacts (Song et al., 1999) and (6) neurexins and neuroligins bind to proteins involved in pre- and postsynaptic organization, including the scaffolding proteins, CASK and PSD-95, respectively (Hata et al., 1996; Irie et al., 1997).

The first solid functional evidence for a role for trans-synaptic interaction between neuroligins and neurexins in synapse development came from a pivotal study by Scheiffele and colleagues showing that neuroligins expressed in non-neuronal cells induces the formation of presynaptic terminals on contacting axons (Scheiffele et al., 2000). Furthermore, a follow-up study showed that clustering of β -neurexin by NL1 is sufficient to trigger the recruitment of presynaptic vesicles induced by NL1 (Dean et al., 2003). These results suggested that neuroligin-neurexin interaction may have a synaptogenic role, perhaps being a cue for initial contact formation between pre- and postsynaptic elements. In addition to the work presented in this thesis, a number of other recent findings support the concept that neuroligins and neurexins are involved in early stages of synapse formation. Additional overexpression studies confirm that increased levels of NL1, NL2 or NL3 in cultured hippocampal neurons leads to enhanced presynaptic terminal formation (Prange et al., 2004; Chih et al., 2005; Sara et al., 2005). Furthermore, overexpression of NL1 was shown to promote postsynaptic differentiation, as evidenced by higher spine densities, as well as increased clustering of several postsynaptic proteins, including homer, NR1 and PSD-95 (Chih et al., 2005; Sara et al., 2005). I also present data showing that

synapses induced by neuroligins are functionally active, as evidenced by increased frequency of mEPSCs and mIPSCs upon expression of either NL1 or NL2. Conversely, siRNA-mediated suppression of neuroligins leads to decreased densities of both excitatory and inhibitory presynaptic terminals, as well as decreased mIPSC frequency, again indicative of a role in synapse formation (Chih et al., 2005). Demonstrating a functional significance for extracellular binding between neurexins and neuroligins, neurexins presented on the surface of heterologous cells or beads also induce local accumulation of postsynaptic scaffolding proteins and neurotransmitter receptors, via aggregation of neuroligins on the surface of dendrites (Graf et al., 2004; Nam and Chen, 2005; Chih et al., 2006; Graf et al., 2006). Thus, a great deal of *in vitro* evidence exists to suggest that neuroligins and neurexins may play important roles in the formation of synaptic contacts.

However, a study looking at genetic knockout of NL1-3 in mice has raised the possibility that, *in vivo*, trans-synaptic cell adhesion mediated by neuroligins and neurexins may be more important for later stages of synapse maturation and/or synapse activity, rather than initial contact formation (Varoqueaux et al., 2006). Due to respiratory failure, *Nlgn1;Nlgn2;Nlgn3* triple knockout mice die within a day or so following birth. Surprisingly, overall synapse morphology and density remained unaltered in hippocampal and neocortical, as well as brainstem regions of the brains of these mice. This was puzzling, in light of the abundance of previous data supporting an important role for neuroligins in synapse formation. More detailed examination of brainstem regions involved in respiration, however, revealed that, along with modest increases in the ratio of VGluT to VGAT, large decreases in spontaneous GABAergic/glycinergic and glutamatergic currents occurred. Furthermore, failure rates of evoked GABAergic/glycinergic transmission were dramatically increased relative to control mice. While reduced levels of several synaptic vesicle and postsynaptic proteins in triple knockout mice support a role for neuroligins in recruitment of critical synaptic elements, the specific effect of neuroligin loss on synaptic activity and not on overall synapse number suggests that these proteins are not essential for induction of synapse formation in intact brains, and instead are more important for establishing and/or maintaining normal levels of synaptic transmission at more advanced stages of synapse/brain development.

How then do we reconcile the wealth of *in vitro* data hinting at a role for neuroligins in synapse formation and control of E/I balance early in synapse development with knockout data that suggests that neuroligins are dispensable for initial steps of synapse formation in live animals? The most straightforward explanation is that redundant synaptogenic factors can functionally compensate for loss of neuroligins in knockout mice. Although compensation does not occur in the case of neuroligin knockdown (Chih et al., 2005), the possibility of compensatory mechanisms coming into play *in vivo* cannot be ruled out. Such a situation occurs, for instance, in the case of PSD-95 and PSD-93 double knockout. Simultaneous knockdown of PSD-95 and PSD-93 in cultured hippocampal neurons has been found to result in a more dramatic reduction in AMPAR-mediated synaptic transmission than double knockout of these proteins in mice (Elias et al., 2006). In these double knockout mice, expression of another PSD-MAGUK, SAP-102, was shown to be upregulated and siRNA knockdown of SAP-102 in a PSD-95/PSD-93 double knockout background further reduced AMPAR-mediated synaptic transmission to a level more closely resembling that seen in double knockdown neurons. Thus, compensation can occur more readily upon gene knockout than in the case of acute knockdown in single dissociated cells. Another possibility to explain the discrepancies between cell culture and *in vivo* data with respect to the role of neuroligin-neurexin signaling in synapse development is that neuroligins and neurexins behave aberrantly when expressed at excessively high levels. In this scenario, neuroligins and neurexins would normally act to recruit molecules important for synaptic maturation and stabilization, such as neurotransmitter receptors, scaffolding molecules and their associated proteins. However, given the complex nature of pre- and postsynaptic protein networks, as well as the ability of neuroligins and neurexins to mediate cell-cell adhesion (Nguyen and Sudhof, 1997), expression of these proteins at levels beyond a certain threshold could lead to initiation of axodendritic contact and ultimately trigger the formation of ectopic contact sites. Such a phenomenon may occur in *in vitro* experiments conducted in cell culture that rely on overexpression of neuroligins and associated proteins.

In addition to the data from triple neuroligin knockout mice, evidence that neuroligins can cluster neurotransmitter receptors at postsynaptic sites also implicates neuroligins in the recruitment of proteins required for synapse maturation and stabilization. For example, overexpression of NL1 in neurons leads to enhanced accumulation of NR1 (Chih et al., 2005), and in non-neuronal cells

expressing gephyrin, collybistin and GABA_AR subunits, transfection with NL2 results in clustering of GABA_ARs at the plasma membrane (Poulopoulos et al., 2009). Also, glutamate application results in the recruitment of AMPARs to β -neurexin-induced dendritic clusters containing PSD-95 and NMDARs, supporting a role for neuroligin-neurexin interaction in synapse maturation (Nam and Chen, 2005). In addition, various lines of evidence support the notion that other CAMs play more important roles than neuroligin and neurexin in early stages of contact formation. The affinity for interaction between soluble neuroligin 1 and neurexin-1 β is low relative to other trans-synaptic adhesive interaction partners, such as Eph and ephrins (Gale et al., 1996; Comoletti et al., 2003), which may therefore be better suited for aligning synaptic compartments. Moreover, other CAMs, such as cadherins and members of the Ig superfamily are thought to mediate initiation of contact between pre- and postsynaptic membranes (Shapiro and Colman, 1999; Benson et al., 2001; Yamagata et al., 2003).

4.2.2 Which Came First: The Chicken or the Egg? Presynaptic vs. Postsynaptic Mechanisms of Neuroligin-based Synapse Development

Whether neuroligins and neurexins are involved in early events that trigger synapse formation and convey synaptic identity, or whether they play a role in promoting the maturation and stabilization of more established synaptic contacts, one unanswered question that persists in the field is whether clustering of neuroligins in postsynaptic compartments leads to clustering of presynaptically localized neurexins, or whether the reverse situation occurs. Complicating the answer to this question, each of these scenarios is supported by a number of studies. Evidence stemming from two separate reports indicates that postsynaptic development may be regulated by presynaptically-induced clustering of neuroligins, through differential recruitment of postsynaptic scaffolding proteins, ultimately leading to synapse-specific clustering of neurotransmitter receptors. In particular, one set of studies shows that neurexin-mediated clustering of NL2 in neurons results in co-aggregation of both PSD-95 and gephyrin. On the other hand, clustering of NL1, NL3, or NL4 induces aggregation of PSD-95 but not gephyrin (Graf et al., 2004; Graf et al., 2006). On a similar note, work carried out by Nam et al. (2005) shows that β -neurexin expressed in non-neuronal cells induces formation of PSD-95 and NMDAR co-clusters in contacting dendrites (Nam and Chen, 2005). These clusters were disrupted by expression of a dominant-negative version of NL1, which also impaired excitatory

neurotransmission in transfected neurons, indicating that postsynaptic assembly of glutamatergic synapses may involve neurexin-mediated clustering of NL1 and its associated postsynaptic proteins (Nam and Chen, 2005). Further supporting a role for clustering of neuroligins in the downstream assembly of postsynaptic components, overexpression of NL1 in neurons induces clustering of a number of postsynaptic proteins found at excitatory synapses, including PSD-95, homer and NMDA receptors (Chih et al., 2005). Other studies have shown that splice sites within the extracellular regions of both neuroligins and neurexins determine which subsets of neuroligins and other key proteins cluster at a particular postsynaptic site, again suggesting presynaptic control of postsynaptic development (Table 4.1; (Chih et al., 2006; Graf et al., 2006)). Finally, a recent study showed that NL2 activates collybistin, enabling it to form submembraneous microaggregates with gephyrin that can subsequently recruit GABA_ARs to the membranes of COS7 cells, and that NL2 is required for the postsynaptic recruitment of gephyrin to perisomatic synaptic sites, in support of NL2 being a regulator of postsynaptic rather than presynaptic assembly (Poulopoulos et al., 2009).

Isoform	Binding Partners	Effects on protein clustering	References
NL1 B(+)	β -NXN SS4(-)	Clusters mainly at glutamatergic contacts Mainly induces clustering of VGluT1	Boucard et al., 2005; Chih et al., 2006
NL1 B(-)	α - and β -NXN SS4(+/-)	Clusters mainly at GABAergic contacts Induces clustering of VGluT1 and VGAT equally	Boucard et al., 2005; Chih et al., 2006
NL2 A(+)	α - and β -NXN SS4(+/-)	Clusters mainly at GABAergic contacts	Chih et al., 2006
NL2 A(-)	α - and β -NXN SS4(+/-)	Clusters mainly at glutamatergic contacts	Chih et al., 2006
NXN1 β SS4(-)	NL1B(+/-)	Clusters both PSD-95 and gephyrin	Boucard et al., 2005; Chih et al., 2006; Graf et al., 2006
NXN1 β SS4(+)	NL1B(-), NL2	Clusters gephyrin only	Boucard et al., 2005; Chih et al., 2006; Graf et al., 2006
NXN1 α SS4(-)	NL1B(-)	Clusters gephyrin only	Boucard et al., 2005; Chih et al., 2006; Graf et al., 2006

Table 4.1. Neuroligin and neurexin splice isoforms and their synaptic binding partners and clustering effects.

However, these results conflict with reports that overexpression of neuroligins does not significantly alter clustering of any postsynaptic proteins examined, including PSD-95, as well as AMPA and NMDA receptor subunits (Prange et al., 2004; Levinson et al., 2005; Sara et al., 2005). These data also contrast with a number of other studies that support the idea that neuroligins are instructive to presynaptic differentiation. These reports lead to models whereby clustering of scaffolding molecules recruits neuroligins to postsynaptic sites, which in turn leads to trans-synaptic clustering of neurexins, ultimately triggering further steps of presynaptic development. For instance, NL1 expressed in neurons or presented on the surface of beads induces clustering of neurexin at nascent presynaptic sites (Dean et al., 2003). In addition, when PSD-95 and NL1 are coexpressed in hippocampal neurons, the effects of NL1 on inhibitory synapses is abolished, with a corresponding increase in NL1 accumulation at excitatory contacts, suggesting that postsynaptic, rather than presynaptic, factors regulate the clustering of NL1 (Prange et al., 2004). Accordingly, electrophysiological recordings of PSD-95 transfected cells show an overall increase in the ratio of excitatory to inhibitory (E/I) synaptic currents, associated

with increased postsynaptic clustering and activity of glutamate receptors, enhanced spine formation, and induction of presynaptic maturation (El-Husseini et al., 2000; Prange et al., 2004). Conversely, RNA knockdown of PSD-95 reduces the E/I synapse ratio (Prange et al., 2004; Gerrow et al., 2006). The results presented in this thesis also support the idea that clustering of neuroligins and presynaptic development are at least partially determined by postsynaptic mechanisms involving scaffolding proteins, as evidenced by the observations that overexpression of neuroligins enhances the formation of presynaptic terminals, and knockdown of PSD-95 or gephyrin leads to shifts in the localization of neuroligin clustering.

Introducing further complexity into the story, overexpression of the postsynaptic scaffolding molecule, S-SCAM, results in enhanced clustering of NL1, which in turn leads to enhanced accumulation of PSD-95 at synapses (Iida et al., 2004). This result presents the possibility that additional postsynaptic scaffolding proteins may regulate the synaptic clustering of neuroligins. Furthermore, oligomerization of neuroligins has been shown to occur, and is thought to be important for their normal functioning in synapse development (Comoletti et al., 2003; Dean et al., 2003). Thus, neuroligin clustering, whether pre- or postsynaptically induced, may be facilitated by its own inherent ability to self-assemble. Aside from introducing another point from which neuroligin clustering can be controlled, oligomerization of neuroligins also presents complications with respect to the interpretation of certain data relating to pre- vs. postsynaptic mechanisms for neuroligin targeting. For example, I have presented data in this thesis suggesting that, while the region between amino acids 716 and 782 within the C-terminal tail of NL2 is required for its proper localization at excitatory synapses, deletion of other regions within the intracellular portion do not result in mistargeted protein. However, the possibility that oligomerization of NL2 protein masks the loss of key regions within the intracellular tail cannot be ruled out in this case. Considering the simplest situation possible, where a homodimer is formed between two NL2 molecules, three possibilities exist: Dimers containing two copies of wild-type protein, those containing two copies of mutant protein and those with one copy of each. In the latter case, through “piggy backing” onto wild-type protein, mutant protein may be localized properly to synapses, even if it inherently lacks this ability.

It therefore remains unclear whether the initial trigger for neuroligin-neurexin-based synapse development is presynaptically-induced clustering of these CAMs at the cell surface (presynaptic → postsynaptic), or accumulation of scaffolding proteins at postsynaptic sites (postsynaptic → presynaptic). Some of the observed differences may simply be due to the developmental stages examined or differences in expression levels of manipulated proteins. For example, the lack of enhanced clustering of PSD-95 seen in Fig. 2.7 and other work (Prange et al., 2004) may be due to excessively high levels of overexpression of neuroligins, which has indeed previously been shown to have a dominant-negative effect, causing the dispersal of postsynaptic proteins (Graf et al., 2004). However, a complete explanation is likely to be far more complex. Such apparently inconsistent results can be found when examining other aspects of synapse development as well. For instance, the finding that preformed protein complexes containing PSD-95, GKAP and Shank can recruit presynaptic terminals to nascent synaptic sites is at odds with other work showing that formation of active presynaptic sites often precedes rapid recruitment of excitatory postsynaptic molecules (Bresler et al., 2001; Bresler et al., 2004; Gerrow et al., 2006), indicating that, at least in some situations, presynaptic maturation can occur independently of signaling from the postsynaptic side. However, the fact that both pre- and postsynaptic mechanisms were captured in the former study, as well as the fact that neurons used in this study were at an earlier developmental stage, suggests that the observed differences could be accounted for by a gradual shift in the mechanism used for synapse formation at different ages. Thus, similar age-dependent shifts may underlie the differences seen in the case of neuroligin-neurexin-induced synapse formation. Alternatively, a simple unidirectional mechanism for synapse development may not be sufficient to explain synapse formation in this context. It is conceivable - perhaps even likely given the vast complexity of biological systems - that synapse formation is a dynamic, ever-shifting process, where signals are continually flowing in both directions between pre- and postsynaptic compartments, as a means of fine-tuning the process. Such a scenario is consistent with data from both this thesis and previous work (Chih et al., 2006), which show only partial contributions of pre- and postsynaptic control mechanisms in the localization of neuroligins. Despite these discrepancies, the reported observations suggest that appropriate clustering of neuroligins and their binding partners is critical for the development of fully functional synapses.

4.2.3 Involvement of Other Cell Adhesion and Scaffolding Molecules

As the field of neuroligin-neurexin research has been explored over the last fifteen years, a great deal of research efforts have been focused on the roles of other cell adhesion systems and scaffolding molecules in synapse development (some of these systems have been discussed in detail in Chapter 1 of this thesis). It is becoming clear that both of these classes of protein are likely key factors in every step of synapse development and function, and that each step requires the contribution of, and possibly cooperation or competition between, multiple CAMs and scaffolding proteins.

NGL-2, for example, presents an interesting case in this respect. NGL-2 regulates excitatory, but not inhibitory, synapse formation, and like neuroligins, is recruited specifically to excitatory synapses through binding to PSD-95 (Kim et al., 2006). While PSD-95 is known to bind NL1 through its third PDZ domain, NGL-2 binds to the first and second PDZ domains of PSD-95 (Irie et al., 1997; Kim et al., 2006). As such, NGL-2 may cooperate with NL1 to regulate excitatory synapse formation, and possibly E/I ratio. One interesting difference between the neuroligin and NGL-2 adhesion systems, however, is that, while NGL-2 has the ability to induce presynaptic differentiation in contact axons, its known binding partner, netrin-G2, is unable to cluster NGL-2 in a reciprocal manner (Kim et al., 2006). It is therefore a possibility that the NGL-2-netrin-G2 complex fundamentally differs from the NL1-neurexin complex, or that the NGL-2 adhesion system operates through multiple presynaptic binding partners (Lin et al., 2003). However, it should be noted that it is uncertain whether neurexins are in fact the only presynaptic receptor for neuroligins, or even whether these molecules interact trans-synaptically *in vivo*.

It is also unclear how SynCAM, another adhesion molecule with synaptogenic characteristics, fits into the model of CAM-based synaptic development. Artificial synapse induction by NL1 and SynCAM is identical, in that expression of either molecule in heterologous cells leads to the formation of presynaptic terminals on contacting axons that can undergo both spontaneous and evoked neurotransmitter release (Sara et al., 2005). However, when expressed in pure neuronal cultures, only expression of SynCAM increased synaptic activity, whereas only NL1 expression increased the number of morphologically-defined synapses. Aside from highlighting the limits of artificial co-culture assays for analysis of synapse development, these results also reflect an

important difference in the physiological functions of these molecules. One possible conclusion from this data is that NL1 and SynCAM may have complementary roles in synapse development, where, for example, NL1 is required for the formation of synaptic contacts, while SynCAM is important for establishing a fully functional synapse. The nature of the relationship between these molecules may differ significantly *in vivo*, perhaps playing more compensatory roles. Further genetic experiments will be required to resolve this issue.

Another interesting possibility comes from the idea that postsynaptic scaffolding proteins may compete for binding to a particular CAM. For instance, my data shows that, while NL2 is normally found primarily at GABAergic synaptic sites, overexpression of PSD-95 leads to a shift of endogenous NL2 to excitatory contacts. Conversely, my data also indicates that knockdown of PSD-95 causes a shift of neuroligins from glutamatergic to GABAergic synapses, and that knockdown of gephyrin results in the movement of NL2 from inhibitory to excitatory synapses. These results support the notion of competition between different postsynaptic scaffolding molecules for binding to CAMs. In such a model, when relative PSD-95 levels are high enough (i.e. upon overexpression of PSD-95 or knockdown of gephyrin), PSD-95 is able to “hijack” neuroligin molecules that would otherwise localize at inhibitory synapses, and bring them over to excitatory synapses. In the inverse situation, high enough relative levels of scaffolding proteins that are able to recruit neuroligins to GABAergic contacts (possibly gephyrin; i.e. upon knockdown of PSD-95) would enable these scaffolding proteins to get a foothold on “excitatory” neuroligins and shift them to inhibitory synapses (Fig. 3.10 and 4.1). Binding of PSD-95 to all neuroligins via their C-terminal PDZ-binding domain is consistent with such a scenario. Moreover, a gephyrin-binding motif has recently been identified in all neuroligin family members; this 15-residue region is sufficient for binding of these proteins to gephyrin in a collybistin-dependent manner (Poulopoulos et al., 2009). Thus, the inherent ability of both PSD-95 and gephyrin to bind all neuroligins strengthens the idea of competitive control of neuroligin targeting.

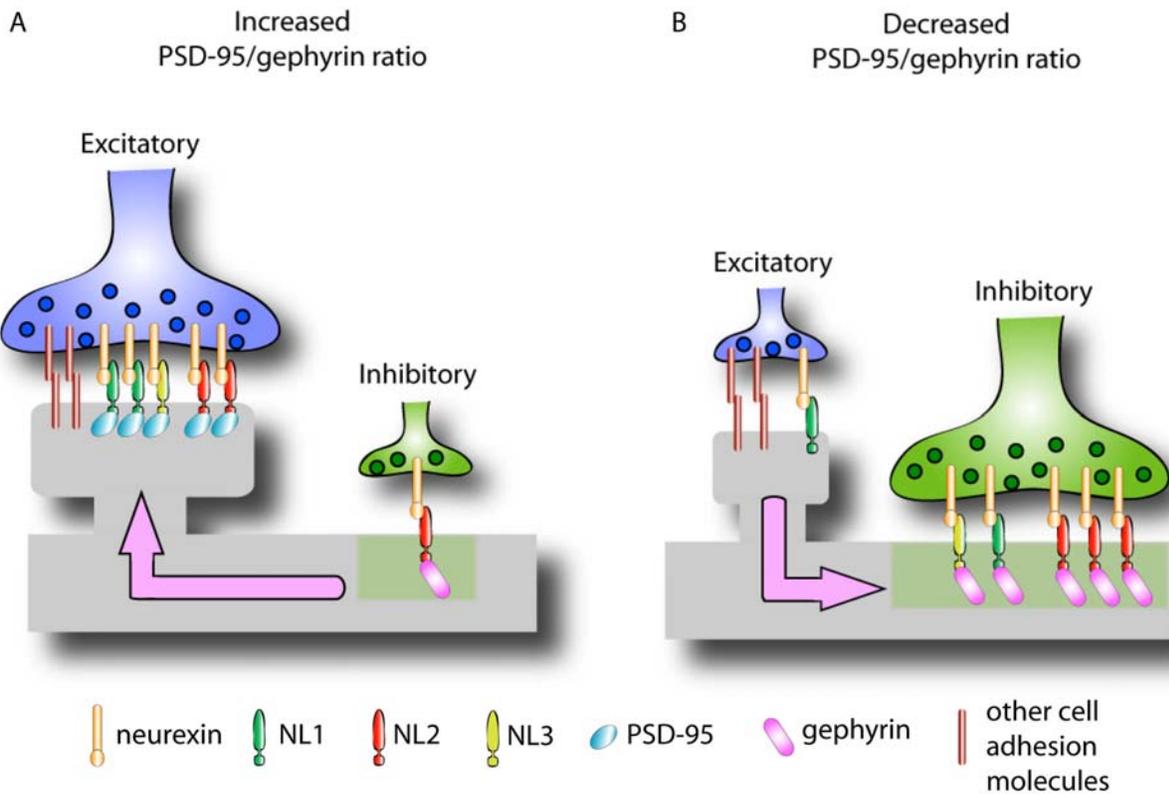


Fig. 4.1. PSD-95 and gephyrin regulate the targeting of neuroligins. NL1, NL2 and NL3 induce excitatory and inhibitory synapses. Neuroligins associate with PSD-95, which is exclusively localized at excitatory sites, or with the inhibitory synaptic scaffolding protein, gephyrin. Interaction with PSD-95 enhances accumulation of neuroligins at excitatory synapses, while interaction with gephyrin promotes neuroligin accumulation at inhibitory contacts. (A) Increases in PSD-95 level relative to gephyrin results in a shift of neuroligins from inhibitory to excitatory synapses. (B) Conversely, decreases in the amount of PSD-95 relative to gephyrin lead to a shift of neuroligins from excitatory to inhibitory synapses. Additional cell adhesion molecules present at both synapse types may contribute to synapse development. Adapted with permission from (Levinson and El-Husseini, 2005).

4.3 Implications in Disease

Several physiological and pathological paradigms alter the levels of PSD-95. For example, PSD-95 association with the PSD is dynamic and is regulated by synaptic activity and palmitate cycling on PSD-95 (El-Husseini Ael et al., 2002). Synaptic activity also upregulates PSD-95 expression through a neuregulin mediated pathway (Bao et al., 2004). In contrast, administration of cocaine, a drug known to cause hyperexcitability, results in down regulation of PSD-95 in the striatum, a region mainly composed of inhibitory neurons (Yao et al., 2004). Moreover, mutation of FMRP, a gene associated with fragile X mental retardation, results in a loss of regulation of PSD-95 expression (Todd et al., 2003).

As discussed in Chapter 1, neuroligins and their binding partners have been linked to a handful of cases of ASD (Mariner et al., 1986; Konstantareas and Homatidis, 1999; Risch et al., 1999; Thomas et al., 1999; Auranen et al., 2002; Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2004). In addition, changes in the E/I synapse balance have been proposed to be affected in many neurodevelopmental psychiatric disorders, including autism and some forms of mental retardation (Rubenstein and Merzenich, 2003). In particular, it is thought that autism is associated with enhanced E/I neurotransmission due to either increased excitation or reduced inhibition, and that this enhanced excitability leads to disruption of memory formation and abnormal social behaviour associated with this disorder. It is therefore tempting to consider the possibility that gaining a better understanding of the roles of CAMs and scaffolding proteins may help uncover the mysteries associated with these debilitating diseases, and perhaps, one day, lead to therapeutic applications for this knowledge in the treatment of these disorders. A potential defect in E/I ratio in autism and related disorders is emphasized by the recent discovery that frame shift mutations in the NL3 and NL4 genes, which result in early protein truncation and misfolding, are associated with autism (Jamain et al., 2003; Chih et al., 2004; Comoletti et al., 2004; Laumonnier et al., 2004). Indeed, a number of NL3 and NL4 mutant mouse models have been generated recently in an attempt to develop mouse models of autism. However, differing results have been obtained, ranging from abnormal social interactions resembling those seen in patients with autism, to a near lack of discernable behavioural phenotypes (Tabuchi et al., 2007; Chadman et al., 2008; Jamain et al., 2008; Radyushkin et al., 2009). Transgenic mouse models expressing NL1 or NL2 have also recently been generated to examine the effects of synaptic

imbalance and altered neuronal excitability on animal behaviour (Hines et al., 2008). In mice overexpressing NL2, but not NL1, changes in anxiety were observed along with impaired social interactions, suggestive of an autism spectrum phenotype. In addition, chromosomal rearrangements in regions that harbor the NL1, NL2 and PSD-95 genes have also been implicated in autism (Konstantareas and Homatidis, 1999; Auranen et al., 2002; Zoghbi, 2003). The potential involvement of neuroligin genes as well as PSD-95 in autism therefore provides a possible molecular and synaptic basis for this imbalance in E/I ratio, which manifests itself as abnormalities in patients affected with neurodevelopmental psychiatric disorders.

In the adult brain, formation of new synaptic contacts is far less common, and thus CAMs and scaffolding proteins may be involved in controlling synaptic activity rather than synapse number. Alterations in the amounts of these proteins may therefore result in weakening or strengthening of either excitatory or inhibitory synaptic activity and in turn modulate the E/I balance, consistent with what was observed in the case of NL1-3 triple knockout mice (Varoqueaux et al., 2006).

4.4 Conclusions and Future Directions

In this thesis, I have presented the results of my research relating to the study of the neuroligin family of CAMs, and their role in synapse development. First, my work has implicated multiple neuroligins in the development of both glutamatergic and GABAergic synapses, and has uncovered neurexin as a likely key trans-synaptic mediator of the effects of neuroligins on both synapse types. Second, I have shown that, in addition to extracellular factors playing a role in neuroligin-mediated synaptic differentiation, interactions between neuroligins and scaffolding molecules within postsynaptic compartments are important regulators of this process.

Despite the heavy focus on the study of neuroligins in the last decade, however, there remain a number of unanswered fundamental questions in this field. As alluded to above, such unresolved issues include those relating to the precise mechanisms of action of neuroligins. The trans-synaptic interactions between neuroligins and neurexins, as well as the interactions between neuroligins and a small subset of postsynaptic scaffolding proteins, have been examined in detail over the years; however, our understanding of the role of neuroligins in synaptic development, activity and plasticity, and possibly in other as of yet unidentified cellular functions, may be enhanced by shifting focus onto a more exhaustive identification and analysis of the full complement of molecules that directly or indirectly interact with neuroligin family members. Such an approach may reveal new angles from which to approach the study of neuroligins, and eventually nail down the molecular mechanisms surrounding these molecules at synapses.

Related to this, but on a broader scale, it remains unclear what functional roles neuroligins in fact play in the life of a synapse in an intact brain. Are neuroligins involved in early stages of contact initiation? If so, do they confer the identity of specific synaptic subtypes at these initial steps? Are neuroligins instead involved in more advanced stages of synapse development, such as contact stabilization and/or maturation, perhaps via the sustained recruitment and retention of proteins required for synaptic organization and function? It may then be at these more intermediate stages that synaptic identity is given a solid foothold. Or are neuroligins primarily involved in synaptic activity and plasticity in live animals? Experiments looking more directly at the effects of neuroligins on the synaptic recruitment and/or modulation of neurotransmitter receptors *in vivo* may help answer some of these questions. In addition, due to the emerging

complexity of synapses and the likely interplay between synaptic cell adhesion systems, more detailed investigations of cross-talk between these adhesion systems may provide insight into these issues, and could help shed light on functional compensation that may be obscuring our understanding of the effects of these molecules *in vivo*. Also, although neuroligins are unlikely to be a major underlying basis of neuropsychiatric disorders such as ASD diseases, elucidating the contribution this protein family makes to synaptic development *in vivo* may help us form a clearer picture of such diseases in general, and could therefore be useful for developing effective therapeutic strategies for their treatment.

In an even more expansive context, the unresolved issues relating to the field of neuroligins focus attention on more universal matters, such as how synapse development proceeds in general. How do we reconcile observations that presynaptic differentiation occurs prior to and independently of postsynaptic development with data indicating that, for example, preassembled protein complexes accumulate at incipient postsynaptic sites prior to contact with presynaptic terminals, or with evidence showing the recruitment of presynaptic elements by environment-probing dendritic filopodia? More sophisticated visualization of these molecules in live neurons may help clarify this issue by better defining the timing of recruitment of these proteins to excitatory and inhibitory synapses. However, it is likely that synapse development does not occur in a strictly stereotyped manner in all cell types, in all brain regions, at all times. Initial contact formation, as well as synapse maturation, may prove to be highly versatile, adaptable phenomena that change depending on any number of factors, including timing and micro-environment differences.

All of these remaining questions open up the way for exciting new research in the fields of synaptic biology. Pursuing answers to these questions will be important for gaining a more thorough understanding of brain development and function.

4.5 References

- Auranen M, Vanhala R, Varilo T, Ayers K, Kempas E, Ylisaukko-Oja T, Sinsheimer JS, Peltonen L, Jarvela I (2002) A genomewide screen for autism-spectrum disorders: evidence for a major susceptibility locus on chromosome 3q25-27. *Am J Hum Genet* 71:777-790.
- Bao J, Lin H, Ouyang Y, Lei D, Osman A, Kim TW, Mei L, Dai P, Ohlemiller KK, Ambron RT (2004) Activity-dependent transcription regulation of PSD-95 by neuregulin-1 and Eos. *Nat Neurosci* 7:1250-1258.
- Benson DL, Colman DR, Huntley GW (2001) Molecules, maps and synapse specificity. *Nat Rev Neurosci* 2:899-909.
- Bresler T, Ramati Y, Zamorano PL, Zhai R, Garner CC, Ziv NE (2001) The dynamics of SAP90/PSD-95 recruitment to new synaptic junctions. *Mol Cell Neurosci* 18:149-167.
- Bresler T, Shapira M, Boeckers T, Dresbach T, Futter M, Garner CC, Rosenblum K, Gundelfinger ED, Ziv NE (2004) Postsynaptic density assembly is fundamentally different from presynaptic active zone assembly. *J Neurosci* 24:1507-1520.
- Chadman KK, Gong S, Scattoni ML, Boltuck SE, Gandhi SU, Heintz N, Crawley JN (2008) Minimal aberrant behavioral phenotypes of neuroligin-3 R451C knockin mice. *Autism Res* 1:147-158.
- Chih B, Engelman H, Scheiffele P (2005) Control of excitatory and inhibitory synapse formation by neuroligins. *Science* 307:1324-1328.
- Chih B, Gollan L, Scheiffele P (2006) Alternative splicing controls selective trans-synaptic interactions of the neuroligin-neurexin complex. *Neuron* 51:171-178.
- Chih B, Afridi SK, Clark L, Scheiffele P (2004) Disorder-associated mutations lead to functional inactivation of neuroligins. *Hum Mol Genet* 13:1471-1477.
- Comoletti D, Flynn R, Jennings LL, Chubykin A, Matsumura T, Hasegawa H, Sudhof TC, Taylor P (2003) Characterization of the interaction of a recombinant soluble neuroligin-1 with neurexin-1beta. *J Biol Chem* 278:50497-50505.
- Comoletti D, De Jaco A, Jennings LL, Flynn RE, Gaietta G, Tsigelny I, Ellisman MH, Taylor P (2004) The Arg451Cys-neuroligin-3 mutation associated with autism reveals a defect in protein processing. *J Neurosci* 24:4889-4893.
- Dean C, Scholl FG, Choih J, DeMaria S, Berger J, Isacoff E, Scheiffele P (2003) Neurexin mediates the assembly of presynaptic terminals. *Nat Neurosci* 6:708-716.
- El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Brecht DS (2000) PSD-95 involvement in maturation of excitatory synapses. *Science* 290:1364-1368.
- El-Husseini Ael D, Schnell E, Dakoji S, Sweeney N, Zhou Q, Prange O, Gauthier-Campbell C, Aguilera-Moreno A, Nicoll RA, Brecht DS (2002) Synaptic strength regulated by palmitate cycling on PSD-95. *Cell* 108:849-863.
- Elias GM, Funke L, Stein V, Grant SG, Brecht DS, Nicoll RA (2006) Synapse-Specific and Developmentally Regulated Targeting of AMPA Receptors by a Family of MAGUK Scaffolding Proteins. *Neuron* 52:307-320.
- Gale NW, Holland SJ, Valenzuela DM, Flenniken A, Pan L, Ryan TE, Henkemeyer M, Strebhardt K, Hirai H, Wilkinson DG, Pawson T, Davis S, Yancopoulos GD (1996) Eph receptors and ligands comprise two

- major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron* 17:9-19.
- Gerrow K, Romorini S, Nabi SM, Colicos MA, Sala C, El-Husseini A (2006) A preformed complex of postsynaptic proteins is involved in excitatory synapse development. *Neuron* 49:547-562.
- Graf ER, Kang Y, Hauner AM, Craig AM (2006) Structure function and splice site analysis of the synaptogenic activity of the neurexin-1 beta LNS domain. *J Neurosci* 26:4256-4265.
- Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM (2004) Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 119:1013-1026.
- Hata Y, Butz S, Sudhof TC (1996) CASK: a novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. *J Neurosci* 16:2488-2494.
- Hines RM, Wu L, Hines DJ, Steenland H, Mansour S, Dahlhaus R, Singaraja RR, Cao X, Sammler E, Hormuzdi SG, Zhuo M, El-Husseini A (2008) Synaptic imbalance, stereotypies, and impaired social interactions in mice with altered neuroligin 2 expression. *J Neurosci* 28:6055-6067.
- Ichtchenko K, Nguyen T, Sudhof TC (1996) Structures, alternative splicing, and neurexin binding of multiple neuroligins. *J Biol Chem* 271:2676-2682.
- Ichtchenko K, Hata Y, Nguyen T, Ullrich B, Missler M, Moomaw C, Sudhof TC (1995) Neuroligin 1: a splice site-specific ligand for beta-neurexins. *Cell* 81:435-443.
- Iida J, Hirabayashi S, Sato Y, Hata Y (2004) Synaptic scaffolding molecule is involved in the synaptic clustering of neuroligin. *Mol Cell Neurosci* 27:497-508.
- Irie M, Hata Y, Takeuchi M, Ichtchenko K, Toyoda A, Hirao K, Takai Y, Rosahl TW, Sudhof TC (1997) Binding of neuroligins to PSD-95. *Science* 277:1511-1515.
- Jamain S, Quach H, Betancur C, Rastam M, Colineaux C, Gillberg IC, Soderstrom H, Giros B, Leboyer M, Gillberg C, Bourgeron T (2003) Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat Genet* 34:27-29.
- Jamain S, Radyushkin K, Hammerschmidt K, Granon S, Boretius S, Varoqueaux F, Ramanantsoa N, Gallego J, Ronnenberg A, Winter D, Frahm J, Fischer J, Bourgeron T, Ehrenreich H, Brose N (2008) Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism. *Proc Natl Acad Sci U S A* 105:1710-1715.
- Kim S, Burette A, Chung HS, Kwon SK, Woo J, Lee HW, Kim K, Kim H, Weinberg RJ, Kim E (2006) NGL family PSD-95-interacting adhesion molecules regulate excitatory synapse formation. *Nat Neurosci* 9:1294-1301.
- Konstantareas MM, Homatidis S (1999) Chromosomal abnormalities in a series of children with autistic disorder. *J Autism Dev Disord* 29:275-285.
- Laumonnier F, Bonnet-Brilhault F, Gomot M, Blanc R, David A, Moizard MP, Raynaud M, Ronce N, Lecomte E, Calvas P, Laudier B, Chelly J, Fryns JP, Ropers HH, Hamel BC, Andres C, Barthelemy C, Moraine C, Briault S (2004) X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *Am J Hum Genet* 74:552-557.
- Levinson JN, El-Husseini A (2005) Building excitatory and inhibitory synapses: balancing neuroligin partnerships. *Neuron* 48:171-174.
- Levinson JN, Chery N, Huang K, Wong TP, Gerrow K, Kang R, Prange O, Wang YT, El-Husseini A (2005) Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. *J Biol Chem* 280:17312-17319.

- Lin JC, Ho WH, Gurney A, Rosenthal A (2003) The netrin-G1 ligand NGL-1 promotes the outgrowth of thalamocortical axons. *Nat Neurosci* 6:1270-1276.
- Mariner R, Jackson AW, 3rd, Levitas A, Hagerman RJ, Braden M, McBogg PM, Smith AC, Berry R (1986) Autism, mental retardation, and chromosomal abnormalities. *J Autism Dev Disord* 16:425-440.
- Missler M, Sudhof TC (1998) Neurexins: three genes and 1001 products. *Trends Genet* 14:20-26.
- Nam CI, Chen L (2005) Postsynaptic assembly induced by neurexin-neurologin interaction and neurotransmitter. *Proc Natl Acad Sci U S A* 102:6137-6142.
- Nguyen T, Sudhof TC (1997) Binding properties of neurologin 1 and neurexin 1beta reveal function as heterophilic cell adhesion molecules. *J Biol Chem* 272:26032-26039.
- Poulopoulos A, Aramuni G, Meyer G, Soykan T, Hoon M, Papadopoulos T, Zhang M, Paarmann I, Fuchs C, Harvey K, Jedlicka P, Schwarzacher SW, Betz H, Harvey RJ, Brose N, Zhang W, Varoqueaux F (2009) Neurologin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. *Neuron* 63:628-642.
- Prange O, Wong TP, Gerrow K, Wang YT, El-Husseini A (2004) A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neurologin. *Proc Natl Acad Sci U S A* 101:13915-13920.
- Radyushkin K, Hammerschmidt K, Boretius S, Varoqueaux F, El-Kordi A, Ronnenberg A, Winter D, Frahm J, Fischer J, Brose N, Ehrenreich H (2009) Neurologin-3-deficient mice: model of a monogenic heritable form of autism with an olfactory deficit. *Genes, Brain and Behavior* 8:416-425.
- Risch N et al. (1999) A genomic screen of autism: evidence for a multilocus etiology. *Am J Hum Genet* 65:493-507.
- Rubenstein JL, Merzenich MM (2003) Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav* 2:255-267.
- Sara Y, Biederer T, Atasoy D, Chubykin A, Mozhayeva MG, Sudhof TC, Kavalali ET (2005) Selective capability of SynCAM and neurologin for functional synapse assembly. *J Neurosci* 25:260-270.
- Scheiffele P, Fan J, Choih J, Fetter R, Serafini T (2000) Neurologin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101:657-669.
- Shapiro L, Colman DR (1999) The diversity of cadherins and implications for a synaptic adhesive code in the CNS. *Neuron* 23:427-430.
- Song JY, Ichtchenko K, Sudhof TC, Brose N (1999) Neurologin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc Natl Acad Sci U S A* 96:1100-1105.
- Tabuchi K, Blundell J, Etherton MR, Hammer RE, Liu X, Powell CM, Sudhof TC (2007) A neurologin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science* 318:71-76.
- Thomas NS, Sharp AJ, Browne CE, Skuse D, Hardie C, Dennis NR (1999) Xp deletions associated with autism in three females. *Hum Genet* 104:43-48.
- Todd PK, Mack KJ, Malter JS (2003) The fragile X mental retardation protein is required for type-I metabotropic glutamate receptor-dependent translation of PSD-95. *Proc Natl Acad Sci U S A* 100:14374-14378.
- Ushkaryov YA, Petrenko AG, Geppert M, Sudhof TC (1992) Neurexins: synaptic cell surface proteins related to the alpha-latrotoxin receptor and laminin. *Science* 257:50-56.
- Varoqueaux F, Aramuni G, Rawson RL, Mohrmann R, Missler M, Gottmann K, Zhang W, Sudhof TC, Brose N (2006) Neurologins determine synapse maturation and function. *Neuron* 51:741-754.

Yamagata M, Sanes JR, Weiner JA (2003) Synaptic adhesion molecules. *Curr Opin Cell Biol* 15:621-632.

Yan J, Oliveira G, Coutinho A, Yang C, Feng J, Katz C, Sram J, Bockholt A, Jones IR, Craddock N, Cook EH, Vicente A, Sommer SS (2004) Analysis of the neuroligin 3 and 4 genes in autism and other neuropsychiatric patients. *Mol Psychiatry*.

Yao WD, Gainetdinov RR, Arbuckle MI, Sotnikova TD, Cyr M, Beaulieu JM, Torres GE, Grant SG, Caron MG (2004) Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioral plasticity. *Neuron* 41:625-638.

Zoghbi HY (2003) Postnatal neurodevelopmental disorders: meeting at the synapse? *Science* 302:826-830.

Appendix A. Additional Publications and Presentations

A.1 List of Additional Published Works

Lui L, **Levinson JN**, Noël G, Handrigan GR, Richman JM, El-Husseini A, Moukhles H. 2009. Synaptic Localization of Neuroligin 2 in the Rodent Retina: a Comparative Study with the Dystroglycan-Containing Complex. *J Neurosci Res.* **88**:837-49.

Krivosheya D, Tapia L*, **Levinson JN***, Huang K, Kang Y, Hines R, Ting AK, Craig AM, Mei L, Bamji SX, El-Husseini A. 2008. ErbB4-neuregulin signaling modulates synapse development and dendritic arborization through distinct mechanisms. *J Biol Chem.* **283**:32944-56. *These authors contributed equally.

Arstikaitis P, Gauthier-Campbell C, Carolina Gutierrez Herrera R, Huang K, **Levinson JN**, Murphy TH, Kilimann MW, Sala C, Colicos MA, El-Husseini A. 2008. Paralemmin-1, a Modulator of Filopodia Induction Is Required for Spine Maturation. *Mol Cell Biol* **19**:2026-2038.

Levinson JN, El-Husseini A. 2007. A crystal-clear interaction: relating neuroligin/neurexin complex structure to function at the synapse. *Neuron* **56**:937-939.

Levinson JN, El-Husseini A. 2005. Building excitatory and inhibitory synapses: Balancing Neuroligin Partnerships. *Neuron* **20**:171-174.

Levinson JN, El-Husseini A. 2005. New players tip the scales in the balance between excitatory and inhibitory synapses. *Mol Pain* **1**:12-18.

Swayze RD, Lise MF, **Levinson JN**, Phillips A, El-Husseini A. 2004. Modulation of dopamine mediated phosphorylation of AMPA receptors by PSD-95 and AKAP79/150. *Neuropharmacology* **47**:764-778.

A.2 List of Presentations

Levinson JN, Li R, Kang R, Moukhles H, El-Husseini A, Bamji SX. 2010. Postsynaptic scaffolding molecules modulate the localization of neuroligins. Cellular and Physiological Sciences Retreat (Vancouver, BC, Canada).

Levinson JN, Li R, Hines R, Kang R, Moukhles H, Bamji SX and El-Husseini A. 2009. Synaptic targeting of neuroligin family members is modulated by postsynaptic scaffolding molecules. Canadian Neuroscience Meeting (Vancouver, BC, Canada).

Levinson JN, Li R, Hines R, Moukhles H and El-Husseini A. 2008. Factors regulating synaptic targeting of neuroligins. Canadian Neuroscience Meeting (Montreal, QC, Canada).

Levinson JN, Li R, Hines R, Moukhles H and El-Husseini A. 2007. Factors regulating synaptic targeting of neuroligin family members. Society for Neuroscience Meeting (San Diego, CA, USA).

Levinson JN, Li R, Moukhles H and El-Husseini A. 2006. Mechanisms underlying the effects of neuroligin 2 at inhibitory synapses. Society for Neuroscience Meeting (Atlanta, GA, USA).

Levinson JN, Chéry N, Huang K, Wong TP, Gerrow K, Kang R, Prange O, Wang YT and El-Husseini A. 2005. Control of synapse formation and specificity by neuroligin family members is mediated by interactions with PSD-95 and neurexin-1beta. Society for Neuroscience Meeting (Washington D.C., USA).

Levinson JN, Chéry N, Huang K, Wong TP, Gerrow K, Kang R, Prange O, Wang YT and El-Husseini A. 2005. Interactions between neuroligins, PSD-95 and neurexin-1beta mediate excitatory and inhibitory synapse formation. Synapse 2005 conference (University of British Columbia, Vancouver, BC, Canada).

Levinson JN, Chéry N, Huang K, Wong TP, Gerrow K, Kang R, Prange O, Wang YT and El-Husseini A. 2005. Involvement of neuroligin family members, PSD-95, and neurexin-1beta in the formation of excitatory and inhibitory synapses. Psychiatry Research Day conference (University of British Columbia, Vancouver, BC, Canada).

Levinson JN, Chéry N, Huang K, Wong TP, Gerrow K, Kang R, Prange O, Wang YT and El-Husseini A. 2005. Involvement of neuroligin family members, PSD-95, and neurexin-1beta in the formation of excitatory and inhibitory synapses. Pacific Northwest Neuroscience Meeting (University of Washington, Seattle, WA, USA).

Levinson JN, Prange O, Gerrow K and El-Husseini A. 2004. PSD-95-dependent clustering of neuroligin 3 in rat hippocampal neurons. Society for Neuroscience Meeting (San Diego, CA, USA).

Appendix B. Supplemental Data

B.1 Localization of Neuroligin 2 in Various Neuronal Cell Types³

B.1.1 Materials and Methods

Neuronal cultures were fixed in -20°C methanol. Retina and brain sections were fixed in 4% paraformaldehyde. Cultures and sections were then washed with PBS (3 x 15 min) before being incubated for 1 hr in a solution containing 2% BSA (Sigma-Aldrich, St. Louis, MO) and 0.2% Triton X-100. Double immunolabeling was performed by incubating cultures and tissue sections sequentially in the presence of the NL2 antibody overnight at 4°C, followed by a 1-hr incubation at room temperature in the presence of antibodies directed against NL2, VGAT or VGluT1. Samples were then washed with PBS (3 x 15 min) and incubated for 1 hr with Alexa Fluor 568 donkey anti-goat IgG and Alexa Fluor 488 donkey anti-rabbit IgG (1/200; Molecular Probes, Eugene, OR). Finally, neuronal cultures and tissue were thoroughly rinsed with PBS and mounted on glass slides and coverslips, respectively, using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Images were acquired on a Zeiss Axiovert M200 motorized microscope using a monochrome 14-bit Zeiss AxiocamHR charge-coupled device camera, and are presented as single-plane images.

For NL2, a polyclonal goat antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; catalog No. sc-14089; 1:50) raised against a peptide mapping within an internal region of rat NL2 was used. The specificity of this antibody was confirmed by immunoblotting of COS cells overexpressing GFP-tagged NL1, NL2, or NL3, showing immunoreactivity in cells expressing GFP-NL2 but not GFP-NL1 or GFP-NL3 (Levinson et al., 2005). For VGAT, a polyclonal rabbit antiserum (Synaptic Systems, Goettingen, Germany; catalog No. 131 002; 1:1,000) raised against amino acid sequence 75–87 of rat VGAT was used. For VGluT1, a polyclonal rabbit antiserum (Synaptic Systems; catalog No. 135 303; 1:1,000) raised against fusion protein (aa 456–560) of rat VGluT 1 was used.

³ A version of this work has been published. Lui L, **Levinson JN**, Noël G, Handrigan GR, Richman JM, El-Husseini A, Moukhles H. 2010. Synaptic localization of neuroligin 2 in the rodent retina: comparative study with the dystroglycan-containing complex. *J Neurosci Res* **88**:837-849.

B.1.2 Results

In this study, we analyzed the localization of NL2 at both inhibitory and excitatory synapses. Double immunolabeling was performed for NL2 and either VGAT or VGLuT1 in mature (DIV14) hippocampal and cortical neuronal cultures. Immunofluorescence analysis showed that most NL2 puncta are apposed to VGAT-positive terminals (Fig. 5.1A–C, G–I) and that little apposition was seen between these puncta and VGLuT1-positive terminals.

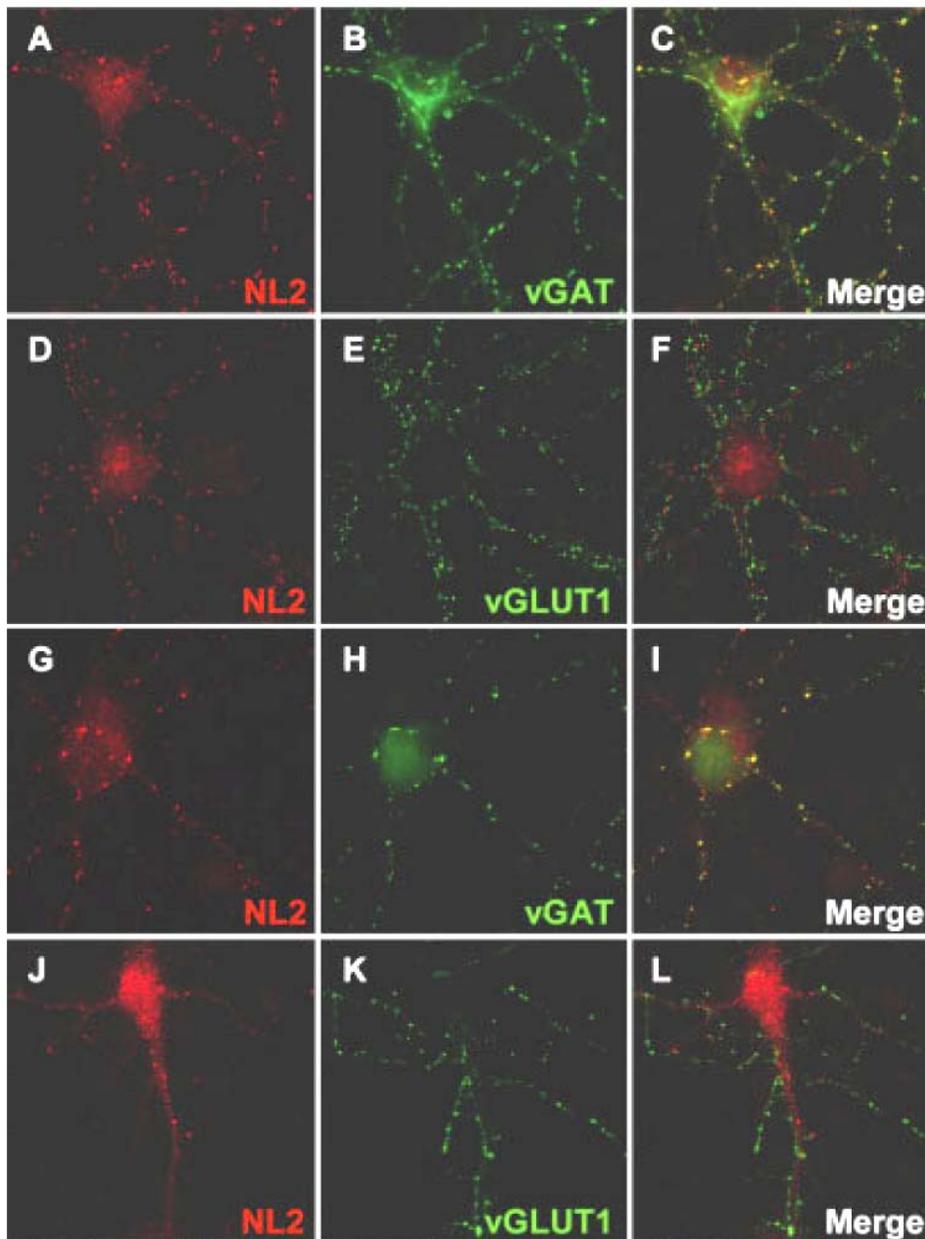


Fig. B.1. Neuroligin 2 localization in rat hippocampal and cortical cultures. DIV 14 hippocampal (A-F) and cortical (G-L) neuronal cultures were double immunolabeled for NL2 and either VGAT or VGLuT1. The merged images in C and I show that the majority of NL2 clusters are apposed to VGAT-containing terminals, whereas those in F and L show little apposition between NL2 clusters and VGLuT1-containing terminals. Scale bar = 10 μ m.

B.2 Role of ErbB4 in Synapse Formation and Dendritic Branching⁴

B.2.1 Methods and Materials

To suppress the expression of endogenous ErbB4, the pFUGW vectors expressing short hairpin RNAs specifically directed against rat ErbB4 (Li et al., 2007) were transfected into hippocampal neurons using Lipofectamine 2000 (Invitrogen) 3 or 5 days before. The target sequences of two short hairpin RNAs for erbB4 are 5'-CCAGACTACCTGCAGGAATAC-3' (hp2) and 5'-GCCCGCAATGTGTTGGTGAAA-3' (hp3). A vector expressing GFP was used as a control.

Dissociated primary neuronal cultures were prepared from hippocampi of embryonic day 18/19 Wistar rats. Cells were dissociated by papain digestion followed by brief mechanical trituration and plated on poly-D (or L)-lysine (Sigma)-treated coverslips at a density of 10⁵ per 8-mm glass coverslip in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), glucose (Sigma), sodium pyruvate, GlutaMAX, and penicillin/streptomycin (Invitrogen). After 2 h the medium was replaced with NeuroBasal medium (Invitrogen) supplemented with B-27, GlutaMAX, and penicillin/streptomycin (Invitrogen) as previously described (Brewer et al., 1993). Every 3–4 days, half of the volume of maintenance medium was taken out and replaced with fresh solution. Cultures were transfected by lipid-mediated gene transfer using Lipofectamine 2000 reagent following the manufacturer's protocol (Invitrogen) or by the calcium phosphate technique (Clontech) as previously described (Jiang et al., 2004).

Coverslips were fixed in -20°C methanol for staining for synaptic proteins or in 4% paraformaldehyde with 4% sucrose (Sigma) and permeabilized with 0.3% Triton-X-100 in phosphate-buffered saline. The following primary antibody solutions were used: GFP (chicken, 1:1000, Abcam), synaptophysin (mouse, 1:1000, Sigma, and rabbit, 1:500, Pharmingen). Secondary antibodies were generated in goat and conjugated with Alexa 488 (1:1000) or Alexa 568 (1:1000, Molecular Probes). All antibody reactions were performed in blocking solution containing 2% normal goat serum for 1 h at room temperature or overnight at 4°C.

⁴ A version of this work has been published. Krivosheya D, Tapia L, **Levinson JN**, Huang K, Kang Y, Hines R, Ting AK, Craig AM, Mei L, Bamji SX, El-Husseini A. 2008. ErbB4-neuregulin signaling modulates synapse development and dendritic arborization through distinct mechanisms. *J Biol Chem* **283**:32944-32956.

Images were acquired on a Zeiss Axiovert M200 motorized microscope with a 63x 1.4 NA ACROMAT oil immersion lens and a monochrome 14-bit Zeiss AxioCam HR charge-coupled camera with 1300 x 1030 pixels. The exposure time was adjusted per individual experiment to achieve maximal brightness without saturation; for intensity measurement experiments, all pictures were taken at equal exposure for all experimental conditions. To correct for out-of focus areas within the field of view, focal plane (z) stacks were collected, and maximum intensity projections were compiled. Images were scaled to 16 bits and analyzed in Northern Eclipse (Empix Imaging Inc., Mississauga, ON, Canada) using custom written software routines as previously described (Flames et al., 2004). In brief, images were processed at a constant threshold level (of 32,000 pixel values), and dendrites visualized by immunofluorescence signal were outlined. Only clusters with average pixel values three times greater than background (diffuse dendritic shaft pixel values) were selected for analysis. The number of dendritic clusters per unit length was measured as a function of dendritic length and normalized to controls. For intensity analysis, the average background intensity was subtracted from the average intensity of individual puncta and multiplied by the puncta area to obtain integrated intensity. Two-tailed Student's *t* test was performed to calculate the statistical significance of results between experimental groups.

B.2.2. Results

Presynaptic Terminal Intensity Is Decreased in Neurons Expressing ErbB4 shRNA: To assess a role for endogenous ErbB4 in synapse maturation, we employed a knockdown approach. The number and intensity of presynaptic terminals synapsing onto neurons expressing shRNAs specifically directed against ErbB4 was measured. The efficacy and specificity of these shRNA in reducing ErbB4 expression has been previously demonstrated (ref). Because ErbB4 is specifically expressed in inhibitory GABAergic neurons, only ErbB4 shRNA-transfected neurons immunopositive for GAD65 were analyzed. Complementary to the effects seen after ErbB4 overexpression, reduction in endogenous ErbB4 expression resulted in an ~16% decrease in the intensity of SYN clusters associated with knockdown cells as compared with GFP-expressing control cells (Fig. 5.2). Consistent with overexpression studies, the density of presynaptic terminals associated with knockdown cells remained unchanged (Fig. 5.2B).

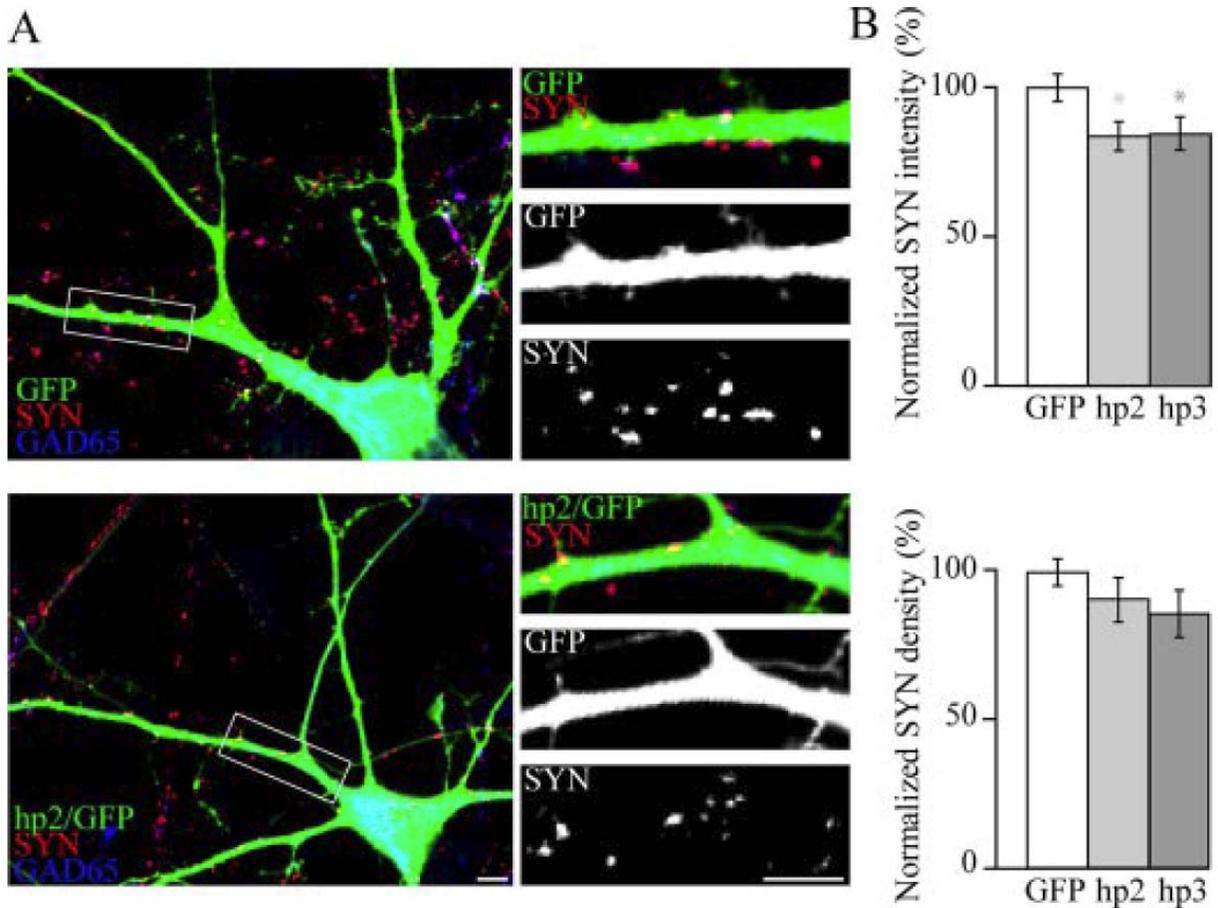


Fig. B.2. shRNA-mediated knockdown of ErbB4 in GABAergic neurons reduces presynaptic maturation in contacting axonal terminals. (A) Hippocampal neurons were transfected with either GFP alone or a vector expressing both GFP and shRNA directed against ErbB4 (hp2 or hp3) and immunostained for SYN as well as GAD65 to identify inhibitory neurons. (B) Quantification of changes in SYN cluster intensity and density. Although SYN cluster density remains unchanged upon ErbB4 knockdown (hp2, n = 40 cells; hp3, n = 23 cells), cluster size is significantly reduced as compared with cells transfected with GFP only (n = 46 cells). *, p < 0.05 (scale bars, 5 μm).

Endogenous ErbB4 regulates neurite outgrowth: To support a role for endogenous ErbB4 in the extension of primary neurites, ErbB4 levels were depleted in inhibitory neurons using shRNAs. Inhibitory neurons have a simple morphology compared with excitatory cells, typically extending ~5 primary neurites. In ErbB4 knockdown cells, however, primary neurite number was decreased to 3–4 (Fig. 5.3). This modest yet highly significant reduction in primary neurite number is in accord with the similarly modest yet significant increase in neurite number after NRG1 treatment of inhibitory neurons.

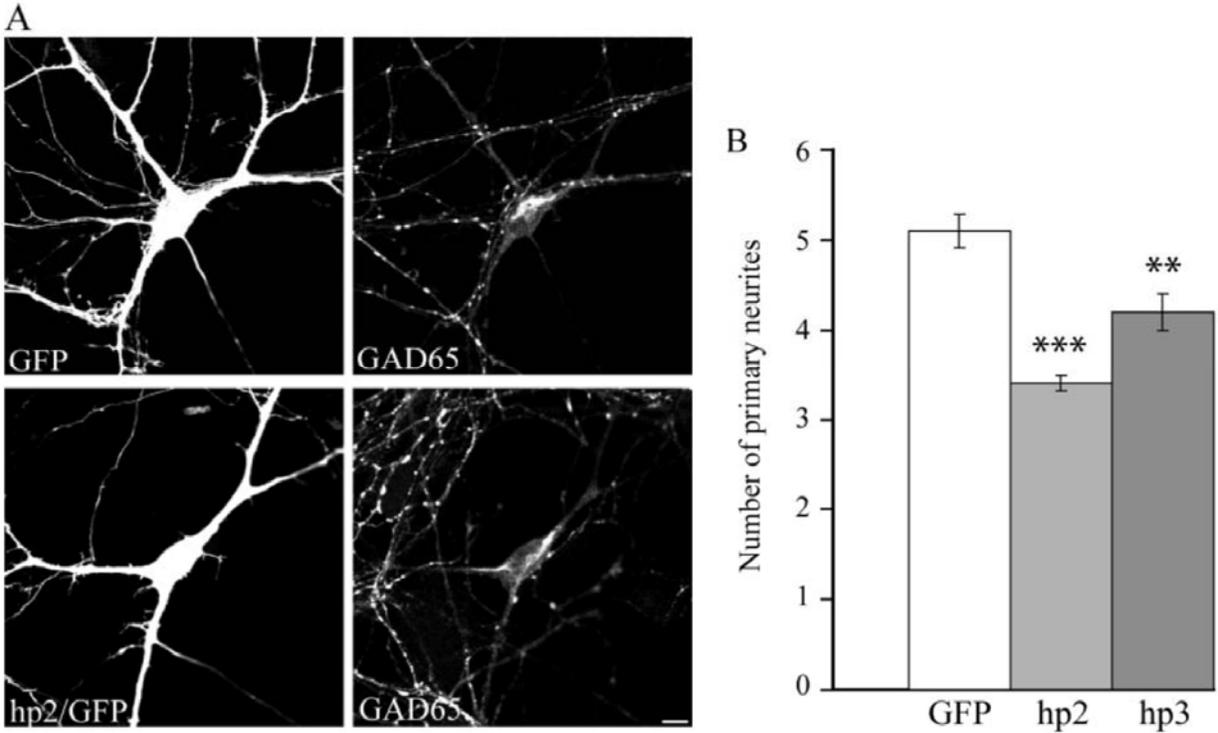


Fig. B.3. Decreased number of primary neurites in inhibitory GABAergic neurons expressing ErbB4 shRNA. (A) Hippocampal neurons were transfected with either GFP alone or a vector expressing both GFP and ErbB4 shRNA (hp2 or hp3) and immunostained for GAD65 to identify inhibitory neurons. (B) Quantification of the number of primary neurites. Cells transfected with ErbB4 shRNA (hp2, $n = 52$ cells; hp3, $n = 30$) showed a statistically significant reduction in the number of primary neurites as compared with GFP control cells ($n = 51$ cells). **, $p < 0.01$; ***, $p < 0.001$ (scale bars, $10 \mu\text{m}$).

B.3 References

- Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 35:567-576.
- Flames N, Long JE, Garratt AN, Fischer TM, Gassmann M, Birchmeier C, Lai C, Rubenstein JL, Marin O (2004) Short- and long-range attraction of cortical GABAergic interneurons by neuregulin-1. *Neuron* 44:251-261.
- Jiang M, Deng L, Chen G (2004) High Ca(2+)-phosphate transfection efficiency enables single neuron gene analysis. *Gene Ther* 11:1303-1311.
- Levinson JN, Chery N, Huang K, Wong TP, Gerrow K, Kang R, Prange O, Wang YT, El-Husseini A (2005) Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1beta in neuroligin-induced synaptic specificity. *J Biol Chem* 280:17312-17319.
- Li B, Woo RS, Mei L, Malinow R (2007) The neuregulin-1 receptor erbB4 controls glutamatergic synapse maturation and plasticity. *Neuron* 54:583-597.