NEUROLIGIN FUNCTION IN EXCITATORY AND INHIBITORY SYNAPSES

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DEDICATION

Dedicated to my father Jianjun Zang and my mother Yan Xu

NEUROLIGIN FUNCTION IN EXCITATORY AND INHIBITORY SYNAPSES

by

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DISSERTATION

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Neuroligins (NLs) are postsynaptic cell adhesion molecules which by binding to presynaptic neurexins (NRXs) are thought to mediate synapse formation and function. Both NLs and NRXs are discussed in the genetic correlation to Autism. Over-expression of NLs could induce the formation of synaptic contacts with axons in non-neuronal cells and increase the synaptic density and response in cultured neurons, through binding and recruiting NRXs; however, little is known about NL signaling though NRXs or inside the cell. First,

we hypothesized that NLs signal through their cytoplasmic region. Over-expression of NL1 with cytoplasmic tail truncation abolished the increase of synaptic density by NL1 full length. By yeast two hybrid screening using NL2 cytoplasmic region, we identified potential interaction partners, of which Necab2 and NRP/B (also named as ectodermal cortex 1, EC1) are two promising candidates and the interactions were confirmed. NL1 or NL2 c-tail truncations partially abolished the change in miniature IPSC, but not the evoked responses. NL c-tail binding partners' over-expression does not show any change in evoked responses. It suggested that NL cytoplasmic region is important for some neuronal changes but does not contribute to the major phenotype of NLs. And we investigated the contribution of NL-NRX binding by using NL extracellular NRX binding mutants. The mutants abolished the change of the evoked and miniature inhibitory responses from the NL2 wild type, which suggested the inhibitory responses triggered by NL2 go through NRXs. And the slight change of the paired pulse ratio suggested the change of presynaptic calcium through binding. The study suggested that NL2 facilitate the inhibitory synaptic transmission through extracellular region via neurexin binding, possibly by the increase in presynaptic calcium. We also found Brain-specific Angiogenesis Inhibitors (BAIs), a family of G-protein coupled receptors (GPCRs), will bind to NLs extracellularly and may serve as signaling modules binding to NLs. Over-expression of BAIs do not change evoked IPSCs, but Bai1 decreased evoked EPSCs and increased the burst

duration in the spontaneous responses, possibly because of some secondary responses. Therefore, we found NL-NRX though NL extracellular region is important for NL2 function in synaptic transmission, and BAIs may be potential signaling molecules of NLs.

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Zang T & Südhof TC. The Role of Neuroligin Cytoplamic Tail in Synaptic Function. Conference Poster Session; Society for Neuroscience Meeting 2007 (San Diego, California)

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LIST OF DEFINITIONS

ABP – AMPA-receptor-binding protein

AISs – axon initial segments

AMPA – α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

AMPAR – AMPA receptor

AP – action potential

ARF1 – ADP-ribosylation factor 1

AZ – active zone

BAI – brain specific angiogenesis inhibitor

CASK – calcium/calmodulin-dependent serine protein kinase

CNR1 – cadherin-related neuronal receptor 1

CNS – central nervous system

EC1 – ectodermal cortex 1

EGFRs – epidermal growth factor receptors

Eph – erythropoietin-producing hepatocellular carcinoma

ephexin1 – Eph-interacting exchange protein 1

Ephrin – Eph receptor interacting proteins

EPSC – excitatory postsynaptic current

ERC – ELKS-Rab6-interacting protein-CAST

FGF – fibroblast growth factor

FGFR – fibroblast growth factor receptor 1

FNIII – fibronectin type III

GEFs – guanine nucleotide exchange factors

GPCR – G-protein coupled receptor

GPI – glycosyl phosphatidylinositol

GRIP – glutamate-receptor interacting protein

ICAM – intercellular adhesion molecule

Ig – immunoglobin

IgCAMs – Immunoglobulin-like cell adhesion molecules

IPSC – inhibitory postsynaptic current

L1 – L1 cell adhesion molecule

LMW-PTP – low molecular weight phosphotyrosine phosphatase

MAGUK – membrane-associated guanylate kinase

MAPK – mitogen-activated protein kinase

MIDAS – metal ion dependent adhesion site

NRX – neurexin

NCAM – neural cell adhesion molecule

Necab – neuronal Ca^{2+} -binding protein

NL – neuroligin

NMDA– N-methyl-D-aspartate

NMDAR – NMDA receptor

NMJ – neuromuscular junction

NRP/B – nuclear-matrix-restricted protein/brain

PDZ-RGS3 – regulator of G-protein signaling3

PI3-kinase – phosphoinositide-3-kinase

PICK1 – protein that interacts with C kinase 1

PNS– peripheral nervous system

PPR – paired pulse ratio

PSA – α-2,8-linked polysialic acid

PSD – postsynaptic density

PSD95 – postsynaptic density protein 95

Ptpro – Protein-tyrosine phosphatase receptor type O

RasGAP – Ras GTPase-activating protein

RTKs – receptor tyrosine kinases

SV – synaptic vesicle

Chapter One: Synapse Differentiation In Central Nervous System

Synaptic Machinery

Nervous system processes and transmits signals through the body of all organisms. Nervous systems are found in many multicellular organisms but differ greatly in complexity between species. Mammalian nervous system is composed of central nervous system (CNS) and peripheral nervous system (PNS). The CNS includes the brain and the spinal cord, representing the major part of the nervous system. The PNS includes all the other nervous structures, mostly axonal processes of nerve cells that are called nerves. Neurons are the core components of the nervous system, coordinate multiple functions in organisms. By interconnecting to each other, neurons use electronic or electrochemical signals to transmit to another neuron or the effecter cell. There are cells besides neurons in the nervous system, called glial cells. Glial cells provide support and protection for neurons in ways of holding neurons in the right place, providing nutrition and oxygen, insulating one neuron from another, maintaining homeostasis, forming myelin, destroying pathogens and removing dead neurons, and participating in signal transmission in the nervous system.

Synapses are specialized junction complexes in between cells, a concept first introduced by Charles Sherrington (Fulton, 1938). Neurons communicate with each other or with other non-neuronal cells like muscles via synapses. There

are three types of synapses, chemical, electrical, and immunological. Immunological synapses are mostly for the recognition of immunological cells (Dustin, 2008). Electrical synapses and chemical synapses are two major types of synapses in nervous system. Electrical synapses are compact, usually 2-4nm in between presynaptic and postsynaptic membranes. Neurons communicate through gap junctions in electrical synapses. There are channels formed by connexins (Cx) serving as pores connecting the cytoplasm of two neurons (Beyer et al., 1990; Söhl et al., 2005). The pores are large (16-20Å of diameter) allowing ion, small signaling molecules and metabolic products to pass through. Electrical synapses transmit electrical signals in different patterns, such as low pass, rectifying. Gap junction is predominant in the developing CNS because the chemical synapses are immature at this stage. Electrical synapses contribute to the synchronous rhythmic activity in the CNS, which triggers oscillations in different brain regions (Bennett, 1997; Hormuzdi et al., 2004). Chemical synapse uses neurotransmitter as the messengers to transmit signals from the presynaptic neuron to the postsynaptic neuron (Eccles, 1957). Chemical synapses allow neurons form interconnected neural circuits so that trigger biological computations underlying perception and thought. Chemical synapses were first found in neuromuscular junction (NMJ) where neuron connects to the muscles in peripheral neural system (Katz, 1966). And this thesis is focused on chemical synapses.

Chemical Synapse and Structure

Chemical synapses were first ascribed by Sir Bernard Katz and his colleagues in the last century by using intracellular recordings from stimulation and observed discrete events termed miniature end-plate potentials, which have smaller amplitude but share the same kinetics as the potentials evoked by electronically stimulating the nerves terminating on the muscle (Katz, 1966). Acetylcholine was first identified as a chemical transmitter at NMJ (Cowan et al., 2003). Calcium was found required for neurotransmitter release (Katz and Miledi, 1967b, a).

Synaptic endings are at various locations on the neuron, as on dendrites, soma, or even axons (Kandel et al., 2000). Typical synapses are found at the junction between axon and dendrite, where the axon possesses the presynaptic boutons and the dendrite form postsynaptic density either at protruding dendritic spines regarding as excitatory synapses or dendritic shafts as inhibitory synapses.

Chemical synapses are structurally and functionally asymmetric junctions that contain the presynaptic terminal or synaptic bouton, synaptic cleft and the postsynaptic terminal that can be visualized using an electron microscope (EM). The presynaptic terminal, or synaptic bouton, contains hundreds of small membrane bound spheres called synaptic vesicles (SVs) (~50nm) that have neurotransmitters docking at the presynaptic plasma membrane, and protein complexes composing the active zone (AZ) shown in EM as electron-dense

region (Burns and Augustine, 1995; Phillips et al., 2001). The presynaptic termini usually exist within the axon of the presynaptic cell, but in some cases, could also be in dendrites. The synaptic cleft is about 20-25nm in size and contains cell adhesion molecules and extracellular matrix proteins. The small size of synaptic clefts allow neurotransmitter free diffuse from presynaptic terminal to postsynaptic terminal in high concentration and subject to rapid changes (Schikorski and Stevens, 1997). The postsynaptic region is also recognized as electron-dense and contains clustered scaffolding proteins, synaptic receptors and signaling molecules, usually found on the dendrites or the cell body (soma). Presynaptic neurotransmitters have their corresponding receptors on the postsynaptic membrane.

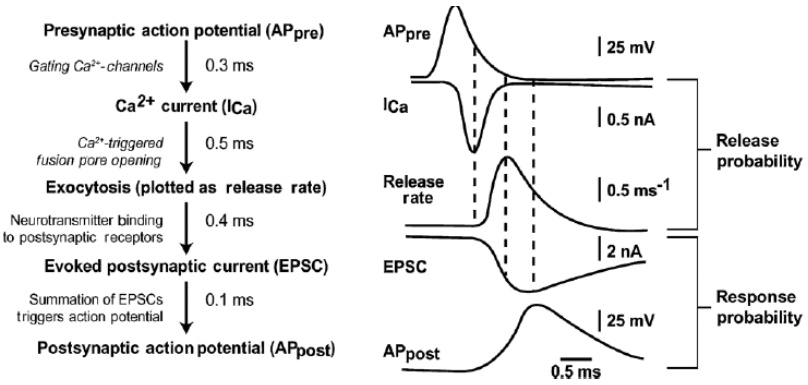


Figure 1-1 Reaction Sequence and Timing of Synaptic Transmission.
The principal reactions with the associated time constants are shown on the left, and traces from the corresponding reactions in the calyx of Held synapses are illustrated on the right. The time calibration bar at the bottom applies to all traces (Meinrenken et al., 2003; Südhof, 2004)

There are nine steps of the synaptic vesicle cycle in the presynaptic

terminal. The arrival of a nerve impulse called action potential (AP) triggers the release of the neurotransmitters to the synaptic cleft. The synaptic vesicles dock at the presynaptic membrane preparing for neurotransmitter release. At the next step priming, ATP is cooperated for the synaptic vesicles to be ready for the exocytosis. The action potential depolarizes presynaptic membrane and produces an influx of calcium from voltage-sensitive channels. Calcium ions trigger a biochemical cascade causing vesicle fusing with the presynaptic membrane and releasing the neurotransmitter from the vesicles rapidly via the SNARE complex, a protein complex that link the synaptic vesicle to the presynaptic membrane. When the synaptic vesicles fuse to the membrane, neurotransmitter is released from the synaptic vesicles to the synaptic cleft for the synaptic transmission. The membrane from the fused synaptic vesicles is recycled by endocytosis, translocated to the intracellular region, and fused with endosome. Then vesicles bud from the endosome, uptake neurotransmitters, and are translocated to the presynaptic membrane ready for the next round of neurotransmitter release and vesicle recycling (figure 1-2) (Südhof, 1995). There is also synaptic vesicle fusion without the action potential, which can be recorded as spontaneous responses. Postsynaptic neurotransmitter receptors open postsynaptic ion channels upon the neurotransmitter binding, causing ions in or out to change the membrane potential, the postsynaptic potential (figure 1-1). Excitatory responses triggered by the binding of the excitatory neurotransmitters (e.g. glutamate) and the postsynaptic

receptors, usually have depolarizing currents, whereas inhibitory responses have hyperpolarizing currents upon neurotransmitter release. To terminate the response, big molecules of neurotransmitters are broken-down, whereas small molecules are mostly recycled (Südhof, 1995, 2004).

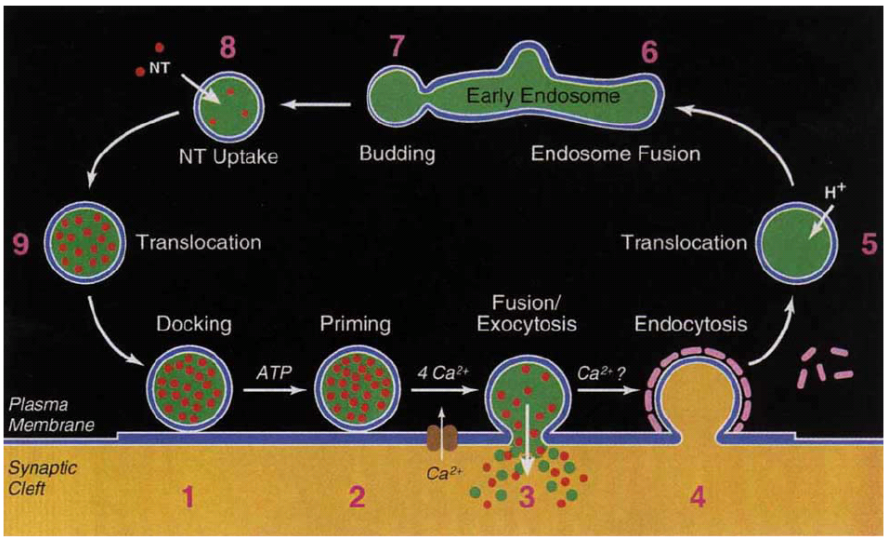


Figure 1-2. The Nine Steps of the Synaptic Vesicle (SV) Cycle in The Presynaptic Nerve Terminal (Südhof, 1995).

Presynaptic and Postsynaptic Organization

Proteins are condensed at presynaptic and postsynaptic regions for the synaptic transmission. At the presynaptic region, complexes are related to synaptic vesicle exocytosis and recycling. One large group of proteins at the presynaptic region are to support the structure of the active zone and align the presynaptic membrane along with the postsynaptic membrane, including cell adhesion molecules, such as neurexins, N-cadherin, synaptic CAM (SynCAM),

NCAM, and cytoskeleton proteins, such as piccolo, bassoon, ERC/Cast, liprin, calcium/calmodulin-dependent serine protein kinase (CASK), veils, mint and spectrin (figure 1-3). In addition, they also interact with other synaptic proteins for other functions. Another group of presynaptic proteins are involved in synaptic vesicle docking and fusion, including syntaptotagmins and SNARE complex, which has syntaxin, SNAP25, synaptobrevin/VAMP (Brünger, 2005; Sorensen, 2005), and Rim, Rab3a, Munc13, Munc18, and N and P/Q type calcium channels (Ryan, 2001; Jin, 2002). SNARE proteins associate with synaptotagmin sensing presynaptic calcium via C2 conserved domain to direct the fusion process (Shao et al., 1996; Rizo and Südhof, 1998). SNARE complex also binds complexin, a small soluble proteins that inserts into the groove of the SNARE complex four-helical bundle in an antiparallel manner (McMahon et al., 1995; Chen et al., 2002), and when fast synaptic response occurs, synaptotagmin competes with complexin to associate with SNARE complex for fast calcium triggered release (Tang et al., 2006). The exocytosis is regulated by Rab3 on the synaptic vesicles (Südhof, 2004). Rim is an important active zone protein that binds to Rab3 on the vesicles and links to other active zone proteins, potential calcium channels (Wang et al., 1997; Wang et al., 2000; Wang et al., 2001; Schoch et al., 2002; Wang et al., 2002; Wang and Südhof, 2003). Rim, Munc13 and Munc18 are crucial for the priming, the step preparing for the synaptic vesicle fusion via the cooperation of ATP. The last group of presynaptic proteins is for

the recycling of fused vesicles, including clathrin, dynamin and a family of SH3-domain-containing adaptor proteins.

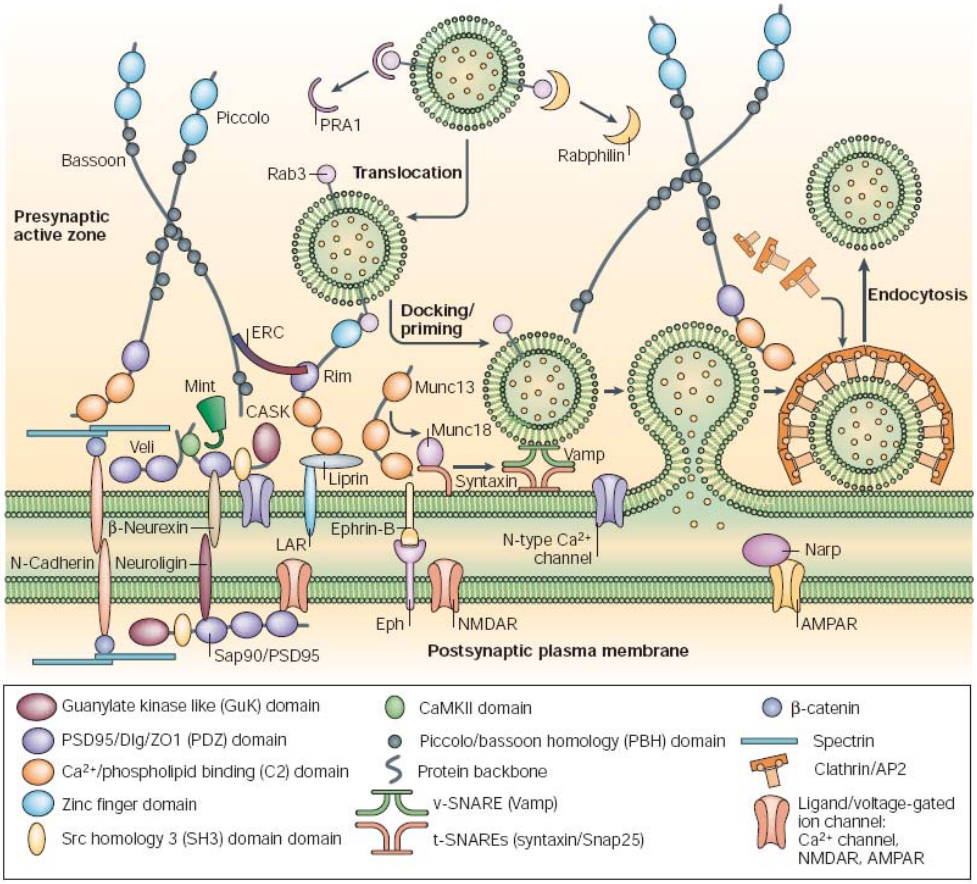


Figure 1-3 Molecular Structure of CNS Glutamatergic Synapses (Ziv and Garner, 2004).

Postsynaptic proteins include postsynaptic receptors of neurotransmitters, synaptic scaffolding proteins or cytoskeletal elements, signaling molecules and cell adhesion molecules, and ion channels (Ziv and Garner, 2004). Postsynaptic

receptors include AMPA receptor, NMDA receptor, metabotropic glutamate receptors, GABA receptor, glycine receptor, histamine receptor, dopamine receptor and others. They are specialized by the neurotransmitter they bind to. Scaffolding proteins anchor the surface molecules at the postsynaptic site, including actin, tubulin, spectrin, PDZ-domain-containing proteins, such as PSD95, PSD93, gephyrin. They are abundant proteins that may bind to multiple partners at one molecule, including NMDAR, AMPAR, neuroligins, etc. So they may contribute to the clustering of the postsynaptic proteins as a functional complex (Irie et al., 1997b; Jarousse and Kelly, 2001; Richmond and Broadie, 2002; Murthy and De Camilli, 2003). Signaling molecules transduct signal from the synaptic surface to the inside of the postsynaptic neuron, including calmodulin, protein kinase C (PKC), protein kinase A (PKA), protein phosphatase-1 (PP1), Fyn tyrosine kinase, receptor tyrosine kinases, BDNF receptor and others (Ziff, 1997). Adhesion molecules align the postsynaptic membrane with the presynaptic membrane. NARP and ephrinB are thought to promote AMPAR and NMDAR clustering, respectively (Garner et al., 2002). Ion channels are not directly regulated by neurotransmitter; rather, they contribute to the postsynaptic membrane excitability sensing voltage changes.

Synapse Formation, Maturation and Differentiation

Axonal growth cone guidance initiates the synapse formation to its dendritic target. Intracellular guidance cues are essential for this step. For example, as for the GABAergic synapse formation on large projection neurons, ankyrin G and neurofascin of L1 immunoglobulin superfamily proteins contribute to the initiation step by establishing gradient in the axon initial segments (AISs) on the purkinje cells (Davis and Bennett, 1994; Davis et al., 1996; Zhou et al., 1998)

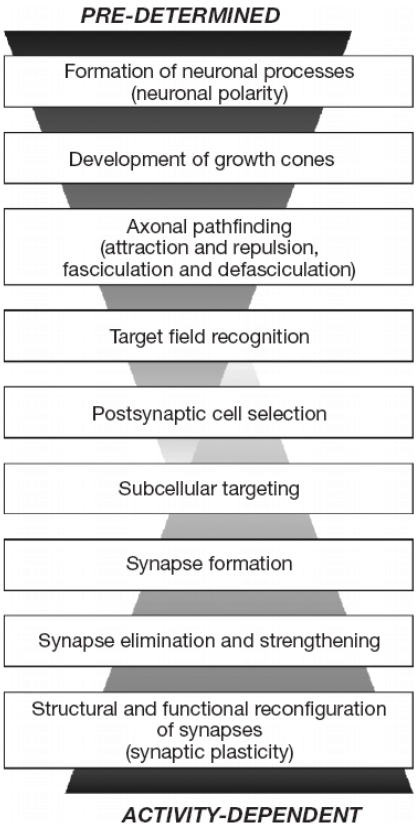


Figure 1-4 Development of neuronal circuits.
The formation of neuronal circuits involves a series of steps. It starts with the formation of axonal and dendritic processes and ends with mechanisms of synaptic plasticity. As development proceeds, activity-dependent processes might be implicated in the formation or modulation of neuronal circuits (Juttner and Rathjen, 2005).

Following the formation, synapses are either stabilized or eliminated, according to synaptic activities. It is well studied in neuromuscular junctions (NMJs); moreover, we are more interested in CNS. The outcomes of maturation are the synaptic size increase and the increase in reliability, magnitude, and efficacy of the synaptic transmission (Jones, 1983; Vaughn, 1989). As of the structural changes during the

maturation process, the immature form of postsynaptic protrusion, filopodia, forms shafts of inhibitory synapses, changing from shaft to spine, or directly to spine of excitatory synapses (Ziv and Smith, 1996; Fiala et al., 1998). And the spines are highly dynamic but the motility declines with the age increase (Fischer et al., 1998; Dunaevsky et al., 1999). For many synapses, the subunit composition of neurotransmitter receptors changes during the maturation process. NMDA receptors have NR2A subunit cooperated into the receptor tetramers or pentamers of the immature composition with only NR1 and NR2B subunits earlier in development (Monyer et al., 1994; Sheng et al., 1994). Different contents show on the synapses at different stages of development. Early postnatal synapses only contain NMDA receptors, but not AMPA receptor; they are called "silent synapse" (Rao and Craig, 1997; Constantine-Paton and Cline, 1998; Nusser et al., 1998; Liao et al., 1999; Petralia et al., 1999; Takumi et al., 1999). Synaptic AMPA receptors appear on the synapses late during the maturation regulated by NMDA receptor activity. GABAergic and glycinergic synapses changed Cl^- reversal potential during development, due to the expression onset of the Cl^- transporter KCC2 that changes the Cl^- equilibrium potential to a more hyperpolarized value, therefore converts early excitatory synapses to mature inhibitory synapses (Cherubini et al., 1991; Boehm et al., 1997; Rivera et al., 1999). Synaptic activities regulate the maturation and plasticity, such as AMPA receptor endocytosis and exocytosis in the long-term depression and potentiation

(Carroll et al., 1999; Li et al., 1999; Shi et al., 1999). Cell adhesion molecules are important in both the synapse formation and maintenance of CNS synapses (Washbourne et al., 2004).

Cell Adhesion Molecules

There are molecules on the synaptic surface to make connections between two neurons. Due to the huge amount and complexity of synapses and connections in the brain, the formation and maintenance are directed largely by the molecules on the synaptic surface. The abundance of the cell adhesion molecules contributes to both the specificity and diversity in the synapse, through mechanisms, such as alternative splicing (Missler et al., 1998b; Schmucker et al., 2000). Families of cell adhesion molecules participate in the function of the synapses at different stages of synaptic development (Benson et al., 2001; Shapiro et al., 2007). Cell adhesion molecules may contribute to cellular clustering into nuclear groups, cortical layering, collection of fibers into tracts, and synaptic terminal fields (Shapiro et al., 2007). We are more interested in the cell adhesion molecules in the synaptic terminals.

There are several major superfamilies of cell adhesion molecules: cadherins, integrins, immunoglobulins, ephrin-eph receptors, the neuroligins and neurexins (figure 1-5). They perform the cell adhesion function in the heterologous cell expressions and the neuronal patterning. The structure and

function of the adhesion molecules are widely discussed, though many processes are still hardly understood.

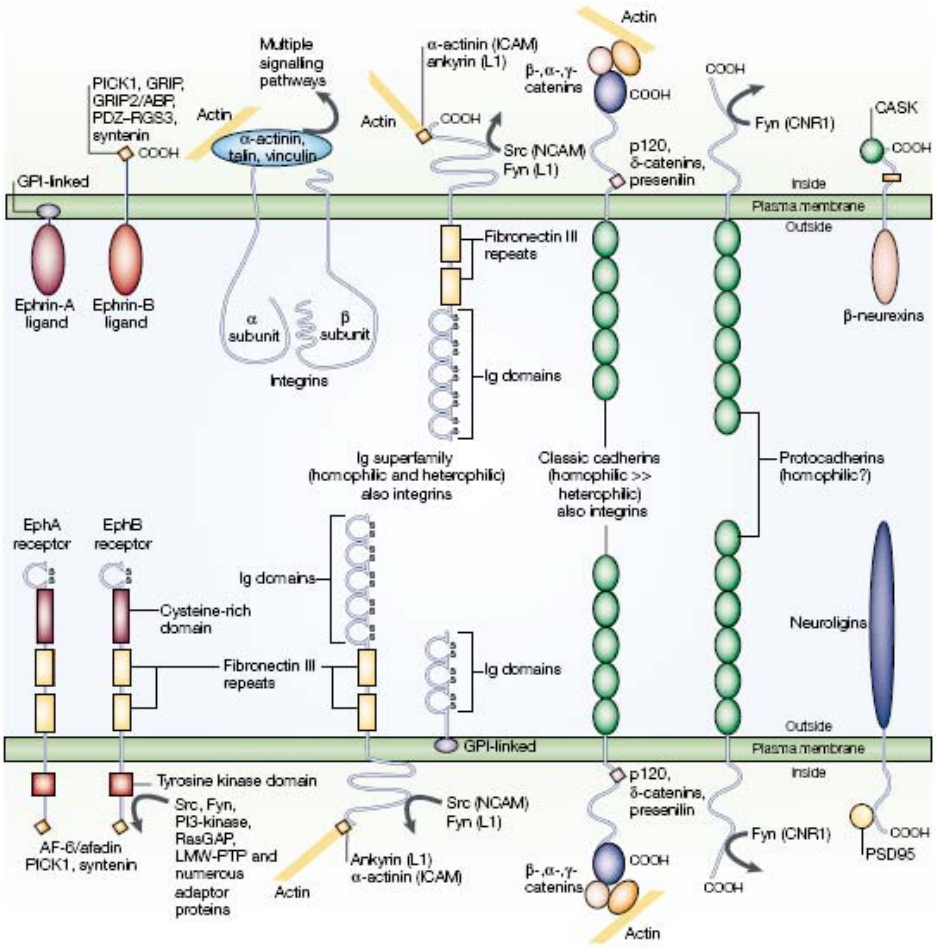


Figure 1-5 Cell Adhesion Molecules and Contact-mediated Recognition. Molecules are shown on the membrane and intracellular signaling pathways are outlined (Benson et al., 2001).

Cadherins and Protocadherins

Cadherins are transmembrane proteins with a large N-terminal extracellular domain, a single transmembrane domain, and a conserved small C-

terminal intracellular domain. They are characterized by the tandemly arranged distinct extracellular motifs called cadherin repeats, or extracellular cadherin domains (EC). These cadherin repeats form β-sandwich domains with Greek-key folding topology (Shapiro et al., 1995b; Shapiro et al., 1995a). Cadherins form strand dimers *in cis* or adhesion dimers *in trans*. Symmetric dimers are formed by exchanging of the N-terminal β-strands between the pair. In the interface of type II cadherins, the critical residues Trp2 and Trp4 insert into a large pocket in the hydrophobic core of the partner molecule. As for type I cadherins, there is only one Trp2 conservative inserting into the interface (Shapiro et al., 1995b; Boggon et al., 2002; Haussinger et al., 2004). A repeated set of dimer interface is common in three lattices to form a linear zipper of molecules that resemble the intracellular filaments that cadherins associate, and the cell-adhesion zipper may provide strong bond between cells (Shapiro et al., 1995b).

The adhesion is calcium-dependent. There is a prodomain N-terminal of those EC domains that must be cleaved by a furin-like protease to activate adhesion. Intracellularly there is a cytoplasmic domain similar to the GPI linkage that anchors to the membrane interacting with catenins and T-cadherin (Shapiro et al., 2007).

There are 13 cadherins in *C. elegans*, and ~100 in human. There are several major classes of cadherins (figure 1-6): R-cadherin which is expressed in retina, E-cadherin which is expressed on epithelial cells (Nagafuchi et al., 1987;

Ringwald et al., 1987), P-cadherin which is expressed on placental cells (Nose et al., 1988; Shimoyama et al., 1989), and N-cadherin which is expressed on neurons (Hatta et al., 1988; Miyatani et al., 1989). They are belong to the classical cadherins, which can be further typed to type I and type II cadherins (Nollet et al., 2000). Classical cadherins are linked to actin cytoskeleton intracellularly through β-catenin binding to the cytoplasmic domains, and β-catenin binds to actin cytoskeleton through α-catenin (Yap et al., 1997). Classical cadherins play a role in the neural structures (Price et al., 2002), especially developmentally (Nakagawa and Takeichi, 1998; Huntley and Benson, 1999), and the organization of the neurons in the brain (Becker and Redies, 2003; Hirano et al., 2003).

Another group of proteins are cadherin-like neuronal receptors (CNRs) that have six EC domains but a distinct cytoplasmic tail (Kohmura et al., 1998). The CNRs actually refer to α-protocadherins (*pcdhα*), and there are two other similar protocadherins, β- and γ- protocadherins. They are 52 genes identified in human and organized into three closely linked clusters (Wu and Maniatis, 1999). They do not have the prodomain for activation.

Each vertebrate genome also encodes one T-cadherin, calsyntenins, and they are synaptic and include two EC domains. They are expressed in both vertebrates and invertebrates. There are homologues of the classic cadherins in *C. elegans*, such as HMR-1A and -1B. Flamingo proteins in *C. elegans* contain EC domains and other extracellular modules. It contains a seven transmembrane

region comparing to the single transmembrane region in other cadherin family proteins (figure 1-6).

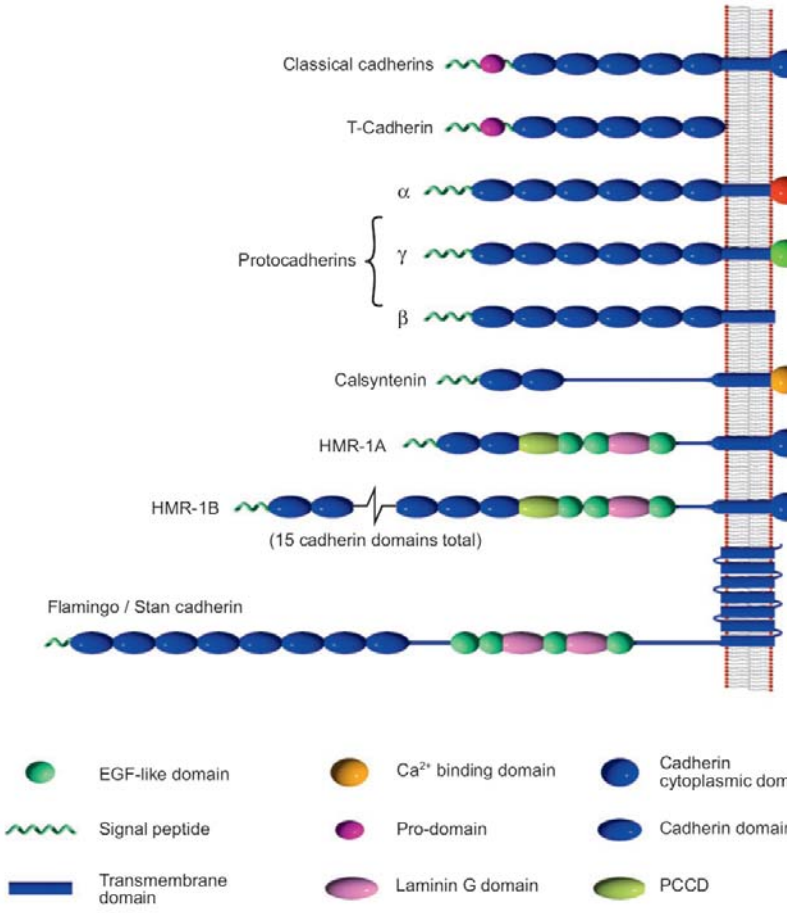


Figure 1-6 Cadherin family proteins.
Domain structures of cadherins expressed in the nervous system are shown schematically. All are type I extracellular proteins, containing an amino-terminal signal sequence. The classic cadherins have five extracellular cadherin (EC) domains, a prodomain that must be cleaved by a furin-like protease to activate adhesion and a cytoplasmic domain known to interact with catenins. T-cadherin is similar but is anchored to the membrane by a GPI linkage. Each vertebrate genome encodes a single T-cadherin. The protocadherins lack a prodomain and include six EC domains in their ectodomains. Calsyntenins, found in both vertebrates and invertertebrates, are synaptically expressed and contain two amino terminal EC domains. HMR-1A and -1B are the classic cadherins of *C. elegans* because they have canonical cytoplasmic regions that interact with the worm β-catenin homolog. HMR-1A and -1B are synthesized by alternative splicing of the same gene. Other cadherin domain–containing proteins, including the flamingo proteins (*starry night* in *C. elegans*), have complex domain structures that include diverse extracellular modules and a seven-transmembrane receptor-like topology in their membrane-spanning segments. Whether these proteins function in cell adhesion remains unclear (Shapiro et al., 2007).

The function of cadherin superfamily proteins includes synaptic adhesion (Fannon and Colman, 1996; Uchida et al., 1996), axon targeting (Iwai et al., 1997; Arndt et al., 1998), and synaptic plasticity (Tang et al., 1998). N-cadherin dimerizes and becomes resistant to the protease cleavage upon synaptic activity independent of the new protein synthesis (Tanaka et al., 2000). The individual interaction between cadherins is weak and flexible; however, the sum of multiple interactions from cadherins on the synapse is strong. The strength of interaction on each of the synapses can be adjusted by changing the organization of cadherins (Tanaka et al., 2000). An axon and the corresponding dendrite express particular types of cadherins so that they recognize each other specifically (Shapiro et al., 2007).

Immunoglobulin Superfamily Cell Adhesion Proteins

Immunoglobulin-like cell adhesion molecules (IgCAMs) are single transmembrane proteins that resemble immunoglobulin (Brummendorf and Rathjen, 1996). The extracellular region of IgCAMs consists of the Ig-like domains near N-terminus and the fibronectin type III (FNIII) repeats or other diverse protein modules as linkers to the cell membrane surface (Williams et al., 1989). However, the arrangements of domains in IgCAMs are quite diverse. Similar as the cadherins, IgCAMs also have the Greek key β-sandwich fold of the

Ig-like domains. Most Ig-like domains are extracellular, though some are intracellular (Hutter et al., 2000; Vogel et al., 2003). There are two major classes of the Ig-like domains: V-type, of which the Ig-like domains are similar to the variable domains of antibodies; C-type, of which the Ig-like domains are similar to the constant domains (Chothia et al., 1998; Stoyanov et al., 2000). Although IgCAMs lack the conserved disulfide bonds in the hydrophobic core structure of the antibodies, IgCAMs still exhibit the hydrophobic core architecture (Shapiro et al., 1996; Al-Lazikani et al., 1997).

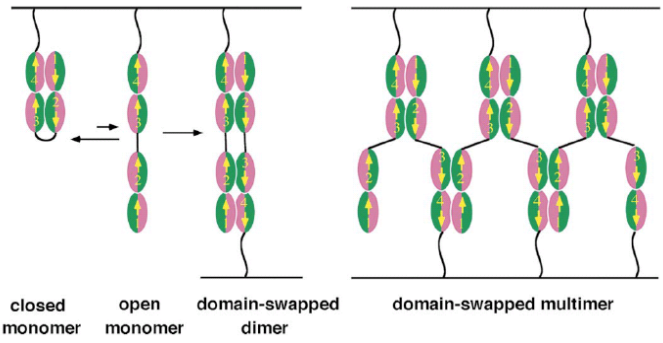


Figure 1-7 Schematic Representation of a Mechanism for Homophilic Adhesion Mediated by 3D Domain Swapping in Hemolin and Related Proteins. On the left, the four NH2-terminal domains of an L1 protein or the hemolin monomer (ABE sheets are green; GFC sheets are purple) are depicted in the closed (bent) conformation. The black line indicates the remaining Ig-like, fibronectin type III, and transmembrane domains in the case of the L1 proteins or attachment to the membrane by posttranslational modification in the case of hemolin. Transient formation of an open form would lead to formation of domain-swapped dimers (middle) or multimers (right) through homophilic interactions with open proteins on another cell (Missler et al., 1998b).

The binding properties of immunoglobulin proteins vary greatly. They bind homophilicly, heterophilicly or to proteins from other cell adhesion classes, such as integrins (Takagi and Springer, 2002). The adhesive function is usually mediated by the Ig-like domains (Brummendorf and Rathjen, 1996). There is a working model for IgCAMs homophilic interaction through the structural studies of hemolin and L1 (Missler et al., 1998b; Schurmann et al., 2001). In this model the four N-terminal Ig-like extracellular domains of IgCAMs form the closed monomeric conformation by the D1-D4, D2-D3 intramolecular pairs, or form the open conformation by the D1-D4, D2-D3 intremolecular pairs. In the closed conformation, the molecule bends between D2 and D3 about 25°, so that the two pairs could form. The variation of positioning of D2-D3 loop triggers different organization of dimers of multimers (figure 1-7) (Missler et al., 1998b). However, this model may not fit all the IgCAMs, such as CD2 and CD58/LFA3 of the heterophilic interaction through D1 domains (Wang et al., 1999).

IgCAMs function either as transmembrane proteins or as secreted soluble proteins, which are usually alternatively splicing variants (Rougon and Hobert, 2003). Many IgCAMs are critical mediators in neural development discovered by genetic studies in *C. elegans* (Cox and Hardin, 2004; Cox et al., 2004; Hardin and Lockwood, 2004).

Neural cell adhesion molecules (NCAMs) are one subfamily of IgCAM that has important functions in migration, survival, axon guidance, synaptic

targeting and plasticity, especially contributes to the construction of a dynamic neural network. It is related to neural disorders such as schizophrenia, biopolar disorder, Alzheimer's disease and to learning and memory (Maness and Schachner, 2007). NCAMs have three major isoforms, NCAM120, NCAM140 and NCAM180, based on their observed molecular weight. They carry an unusual carbohydrate, α-2,8-linked polysialic acid (PSA) that modifies the function during neural migration, axon pathfinding, and synaptic plasticity (Eckhardt et al., 2000; Angata et al., 2004; Kleene and Schachner, 2004; Weinhold et al., 2005). Different isoforms express in different cell types. Their extracellular portion contains five Ig-like domains and two fibronectin type III (FNIII) repeats. Following the extracellular portion, NCAM140 and NCAM180 have a transmembrane domain and then a distinct cytoplasmic domain between them. Instead of a transmembrane domain, NCAM120 has a glycophosphatidyl (GPI) anchor that links it to the membrane (Maness and Schachner, 2007). NCAM140 expresses on both presynaptic and postsynaptic side of migratory growth cones and axon shafts of developing neurons that contributes to neurite outgrowth; and NCAM180 only on postsynaptic side of mature neurons (Persohn et al., 1989). NCAM is essential for mature synaptic vesicle cycling by assisting GTP-dependent ADP-ribosylation factor 1 (ARF1) related AP-3 coated vesicles budding and P/Q type calcium channel related vesicle release; NCAM is necessary for the neuron maturation from the study in the NCAM deficient mice

(Polo-Parada et al., 2001; Polo-Parada et al., 2004). Besides homophilic interactions, NCAM can also mediate heterophilic interaction to other ligands, one of which is FGFR, fibroblast growth factor receptor 1 (Kiselyov et al., 2005). NCAM-FGFR is very important for the FGFR activation. PSA is a switch for the FGFR activation because the big volumn of PSA moiety. Non-polysialylated NCAMs form tight *trans* dimers that form two dimensional compact zipper, resulting in the adhesion in between opposing cells; polysialylated NCAMs form relax *trans* dimers that form one dimensional zipper, and promote FGFR dimerization and increase FGFR concentration for FGF-FGFR signaling (Kiselyov et al., 2003). NCAMs outside lipid rafts activate PKA, PKC and CaMKII-α to signal in the Neurite outgrowth. NCAMs inside lipid rafts activate Fyn and the following Ras-Raf-MEK1/2-ERK1/2 cascade for the similar purpose (figure 1-8) (Kiselyov et al., 2005).

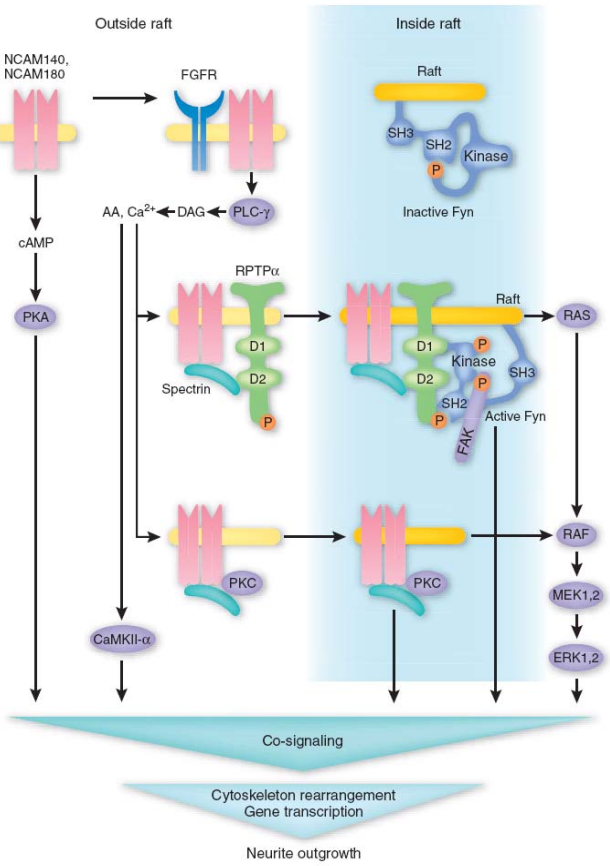


Figure 1-8 Signal transduction pathways activated by 140- and 180-kD NCAM isoforms outside (left) and inside (right) lipid raft compartments of the plasma membrane are presented as a composite map, component parts of which may occur in cell type– or physiological context–specific situations. Outside of lipid rafts, NCAM activates cAMP-dependent protein kinase (PKA) via an unknown mechanism and also interacts with the FGF receptor (FGFR) in some contexts, which leads to activation of phospholipase Cγ (PLCγ) and diacylglycerol lipase (DAG) to generate arachidonic acid (AA) and elevate intracellular calcium. The PLCγ pathway activates AA and Ca2+, separately or in combination, leading to i) the formation of a complex of NCAM140 and receptor protein phosphatase-α (RPTPα; domains D1 and D2 with phosphate group are shown), ii) the association and activation of protein kinase C (PKC) and iii) the activation of CaMKIIα. Inside lipid rafts, Fyn (SH3, SH2, kinase domains) is attached to the raft membrane compartment via palmitoylation, and is inactivated by tyrosine phosphorylation (P) within its C-terminal regulatory region. Clustering of NCAM140 induces PTPα-mediated dephosphorylation and activation of Fyn, recruiting focal adhesion kinase FAK, which triggers the Ras-Raf-MEK1/2-ERK1/2 cascade. Autophosphorylation (P) of active Fyn and FAK are shown. Co-signaling from NCAM inside and outside of rafts is required for cytoskeletal and transcriptional events that culminate in neurite outgrowth. Spectrin binds NCAM180, and to a lesser extent NCAM140, enhancing complex formation with RPTPα and PKC(Maness and Schachner, 2007).

Another subfamily of IgCAMs that is related to neural function is L1. The L1 subfamily includes L1, CHL1 (CALL), neurofascin and NrCAM. It is related to neuronal disorders like X-linked mental retardation, human syndrome of low IQ and developmental delay and schizophrenia (Maness and Schachner, 2007). L1 molecules have six Ig-like domains and four to five FNIII repeats extracellularly, a transmembrane domain followed by a highly conserved cytoplasmic domain of about 110 amino acids, on which a conserved motif (FIGQ/AY) binds to ankyrin that links L1 to the actin cytoskeleton. The sixth Ig-like domain interacts with integrins (α and β subunits) to activate MEK/ERK pathway for axon growth and cell migration (figure 1-9) (Kiselyov et al., 2005).

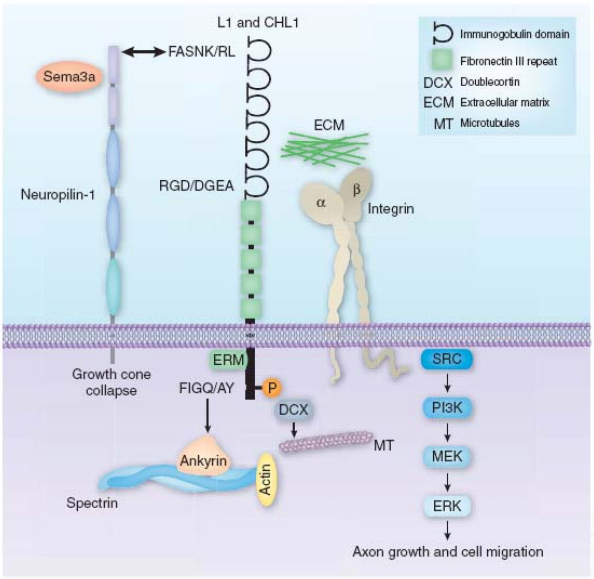


Figure 1-9 Signaling pathways downstream of L1 and CHL1.
Signal transduction induced by L1 or CHL1 involves activation of Src, PI3 kinase, MEK and ERK, which is mediated in some cases by interaction with integrins (α and β subunits) through RGD (L1) or DGEA (CHL1) motifs in their respective Ig6 domains. The Ig1 motif of L1 (FASNKL) or CHL1 (FASNRL) can also bind the semaphoring 3A receptor neuropilin-1 to promote growth cone collapse. A conserved motif in the cytoplasmic domain of L1 (FIGQY) or CHL1 (FIGAY) recruits ankyrin, which couples to F-actin through direct spectrin association. When the motif is tyrosine phosphorylated, the microtubule-associated protein doublecortin (DCX) is recruited, potentially coupling L1 to microtubules. Linkage of L1 family proteins to F-actin, microtubules or both may be important for receptor clustering and signal transduction leading to axon growth, cell migration and growth cone collapse (Maness and Schachner, 2007).

Integrins

Integrins are another family of cell adhesion molecules. Integrins have α- and β- subunits that form heterodimers. Each of the subunits has a big extracellular region, a single transmembrane domain, and a short cytoplasmic tail. The variety of both subunits contributes to the variety of the integrin molecules. In vertebrates, there are eighteen α-subunits and eight β-subunits that form at

least twenty-four different heterodimers with various ligand binding specificities. Also there is an inserted domain of von Willebrand factor, called I-domain, in half of the α-subunits for a dominant ligand binding function. A similar domain exists in β-subunit called I-like domain. Both I-domain in a subunit and I-like domain in β-subunit adopt a Rossmann fold potentially for nucleotide binding (Lee et al., 1995; Bienkowska et al., 1997). There are four (without I-domain) or five (with I-domain) in α-subunit that is up to 1104 amino acids with N-terminal signal sequence; there are eight subdomains in β-subunit that is up to 778 amino acids with N-terminal signal sequence (figure 1-10a) (Humphries et al., 2003; Arnaout et al., 2005; Shapiro et al., 2007).

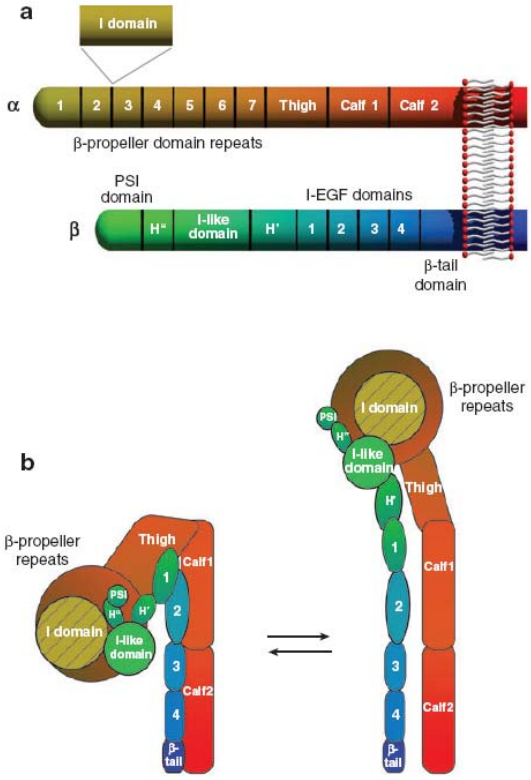


Figure 1-10 Schematic representation of integrin structural organization.
(*a*) Schematic diagram of domain organization in the primary structure of integrins. The α subunit may contain an I domain inserted between blades of the β-propeller. (*b*) Schematic diagram of domain arrangements in the low-affinity (bent) and high-affinity conformations. The positions of the leg regions, which are bound together in the inactive conformation and separate in the high-affinity form, provide a mode of allostery that can be controlled by cytoplasmic signaling (Shapiro et al., 2007).

Ligand binding of integrins is intermediated by the α I domains or the β I-like domains in integrins lacking I domains. Divalent cations needed for ligand binding are initially cooperated by a metal ion dependent adhesion site (MIDAS) in the α I-domain and β I-like domain, completed by further ligand binding involving a solvent-exposed aspartate or a glutamate from the ligand (Tozer et al.,

1996; Craig et al., 2004). Different binding affinity can be adjusted by changing the helix structure in the α I-domain (Xiao et al., 2004). Ligands bound to integrin are soluble and surface-bound proteins; when integrin binds to its ligand on surface, force generates from the location of contact, and it may contribute to the remodeling of extracellular contacts (Ingber, 2003; Ridley, 2004).

Integrin heterodimers employ "inside-out" signaling mechanism. They change the domain arrangement in the structure responding to the signaling from other receptors on the cell surface such as tyrosine kinase and G-coupled receptors (Takagi and Springer, 2002; Humphries et al., 2003; Arnaout et al., 2005). There are currently two models of the inside-out signaling in integrins. In the switchblade model, dissociation of cytoplasmic regions from α and β subunits as in the low affinity state changes the ectodomain conformation and activates integrin. The activation is achieved by the conformation change from the "bent" form to the "extended" form of integrins, because the dissociation of the cytoplasmic region destabilizes the ectodomain and promotes it to adopt the extended conformation (figure 1-10b) (Springer and Wang, 2004; Arnaout et al., 2005). In the deadbolt model, the allosteric inhibitor lovastatin is removed and the integrin transmembrane and cytoplasmic regions are deleted upon activation and the F-α7 interface on α subunit is exposed to be transformed into activate state (Humphries, 2000).

The well-known functions of integrin are in the neuronal muscular junctions (Burkin et al., 2000; Cohen et al., 2000; Burkin et al., 2001) and the attachment to the extracellular matrix of the Schwann cells in the peripheral nervous system (Einheber et al., 1993; Feltri et al., 1994). There are also integrins in the central nervous system (CNS), there are β1 and α8 subunit containing integrins in neurons, glia, meningeal and endothelial cells (Einheber et al., 2001) or β2 in microglia (Koenigsknecht and Landreth, 2004). Integrins are also related to the oligodendrocyte proliferation and migration (Baron et al., 2002) and synapse formation promoted by an astrocyte product thrombospondin that binds to integrin (Christopherson et al., 2005).

Ephrin-Eph Receptors

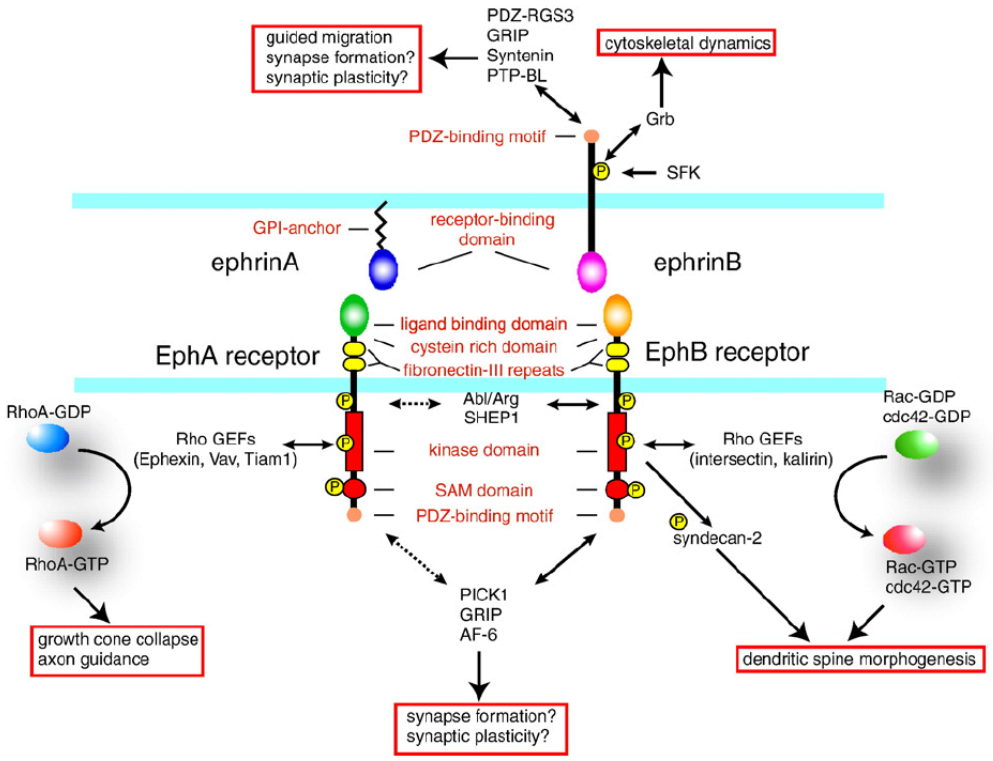


Figure 1-11 Schematic drawing of the ephrin and Eph domain structures and summary of protein interactions involved in forward and reverse signaling. Discrete functional domains are labeled in red and interacting proteins are labeled in black.↔with solid line depicts established interactions while ↔ with dashed line depicts predicted interactions (Aoto and Chen, 2007).

Erythropoietin-producing hepatocellular carcinoma (Eph) receptor and its ligand, Eph receptor interacting proteins (ephrin) were identified as important receptor tyrosine kinases (RTKs). There are sixteen Ephs in vertebrates classified in two groups, EphA1-10 and EphB1-6, based on the similarity of the extracellular domain sequences and the affinity to the ligand ephrins. The extracellular part includes an N-terminal ligand binding domain, a cysteine-rich region, and two fibronectin type III (FNIII) repeats (Yamaguchi and Pasquale,

2004). Following the transmembrane region is the cytoplasmic segment that includes a tyrosine kinase domain, a sterile-α-motif (SAM), and a type-II PDZ-binding motif at the C-terminus (Kullander and Klein, 2002). Ephs form homo- or hetero- dimers via the cysteine-rich region, FNIII repeats (Lackmann et al., 1998), and SAM motif (Stapleton et al., 1999). Ephrins are divided into two groups, ephrinA1-6 and ephrinB1-3, based on the way of their linkage to the plasma membrane. Ephrin-As are bound to the membrane via a glycosylphosphoinositol (GPI) anchor, whereas Ephrin-B are type-I transmembrane proteins. In both classes of ephrins, there is an Eph receptor binding domain in the ectodomain, about 180 amino acids. There is a small cytoplasmic tail of about 80 amino acids and a type-II PDZ binding motif at the C-terminus of ephrin-Bs (Kullander and Klein, 2002; Martinez and Soriano, 2005; Nikolov et al., 2005). In most cases, ephrin-As interact with EphA receptors, and ephrin-Bs with EphB, with one exception that EphA4 and EphB2 interact with ephrinB2/3 and ephrinA5, respectively (Grunwald et al., 2004; Himanen et al., 2004).

Bidirectional signaling by ephrin-Eph is unique. Forward signaling is activated upon the ligand binding through Eph receptors. Reverse signaling is the signaling from ephrins upon binding to Eph receptors. Forward signaling through Eph receptors activates autophosphorylation and phosphorylation of other proteins, then activates Rho guanine nucleotide exchange factors (GEFs), such as Ephexin, Vav, Tiam1, to switch the Rho GTPase from the GDP binding form to

the GTP binding form. The Rho GEF ephexin1 (Eph-interacting exchange protein 1) constitutively binds to EphA receptors and have different function when EphA is inactivated or activated. Ephexin 1 is unphosphorylated when EphA is inactivated in the absence of ephrins, and it activates RhoA, Rac1 and Cdc42 to promote axon outgrowth; ephexin 1 is tyrosine phosphorylated upon ephrin binding to Eph receptors to specifically active RhoA to promote axon growth cone collapse (Shamah et al., 2001; Knoll and Drescher, 2004; Sahin et al., 2005). Vav2 does not distinguish EphA and EphB receptors and ligand binding phosphorylates Vav2 to promote local Rac1-dependent endocytosis of the ephrin-Eph complex as repulsion (Cowan et al., 2005). Protein-tyrosine phosphatase receptor type O (Ptpro) dephosphorylates EphA and EphB receptors after activation to convert them back to the autoinhibited inactive state (Shintani et al., 2006). In the reverse signaling of ephrinB, ephrins transduct signal to the cell where ephrins reside upon the complex formation between ephrins and Eph receptors. Upon activation, ephrinBs are phosphorylated to serve as docking sites to adaptor proteins that activate signaling pathways leading changes in the actin cytoskeleton and focal adhesions (Cowan and Henkemeyer, 2001), promoting maturation of spines (Zhang et al., 2005a; Segura et al., 2007), reducing motility and reorganizing focal adhesions of smooth muscle cells by cleaving the cytoplasmic region to produce a intracellular peptide (Georgakopoulos et al., 2006).

High affinity interaction between ephrins and Ephs controversially induces repulsion instead of adhesion, by *cis*- or *trans*- cleavage of A-Disintegrin-And-Metalloprotease (ADAM) 10, the mammalian homolog of *Drosophila* Kuzbanian (KUZ), on ephrins of certain ephrin-Eph combinations (Hattori et al., 2000; Janes et al., 2005). Repulsion is also triggered by an alternative mechanism of the ephrin-Eph complex rapid endocytosis. The endocytosis exhibits three different manners as reverse, forward and bidirectional, based on different cellular context and the intracellular domain organizations of ephrins and Eph receptors (Mann et al., 2003; Marston et al., 2003; Zimmer et al., 2003). The manner of binding between ephrins and Eph receptors also affects the result of binding, masking Eph receptors by *cis* ephrin, blocking forward signaling of Eph receptors by *cis* ephrin, or independent signaling by co-expressed ephrin and Eph that enable forward and reverse signaling in the same cell (Egea and Klein, 2007).

Neuroligin and Neurexins

Neurexins are brain-specific proteins that were found as α-latrotoxin receptors. There are α and β isoforms from each of the three neurexins which differ in their N-terminal sequences. Five splice sites exist on the ectodomain of neurexin-α and the last two are on neurexin-β (figure 1-12), and trigger the polymorphism that neurexins may express in thousands of splice variants, including truncated/secreted forms, in different subtypes of cells in the brain

(Ushkaryov et al., 1992; Ushkaryov and Südhof, 1993; Ullrich et al., 1995). There are two different types of LNS domains on the extracellular region of neurexins, LNS(A) and LNS(B). LNS domains are loose conserved and about 190 amino acids in length(Ushkaryov et al., 1992). LNS domains may be cell-surface recognition elements (Missler and Südhof, 1998b). Neurexins are high N- and O-glycosylated (Ushkaryov et al., 1994) and its cytoplasmic tail PDZ binding motif bind to the PDZ domain of an abundant neuronal protein kinase CASK that binds ATP and catalyze phototransfer without Mg^{2+} and may phosphorylate neurexin (Hata et al., 1996; Atasoy et al., 2007; Mukherjee et al., 2008). By binding to Mint1, the homologue of *C. elegans LIN-10*, and veli1, 2, 3, the homologue of *C. elegans LIN-7*, via the CaM kinase domain and veli-binding domain of CASK respectively, CASK links neurexin to the presynaptic machinery assembly and potential vesicle trafficking in the presynaptic terminals (Butz et al., 1998). In the meantime, calcium-independent receptors (CLs/lactrophilins) were found of the similar affinity to latrotoxin as neurexins and employ different pathways for the neurotransmitter release (Davletov et al., 1996; Krasnoperov et al., 1996; Geppert et al., 1998; Ichtchenko et al., 1998). Because only neurexin-1α binds α-latrotoxin in the presence of Ca^{2+}, and α-latrotoxin triggers neurotransmitter release independent of Ca^{2+} (Missler and Südhof, 1998b), it suggests that neurexin may have major function despite of the binding to latrotoxin. Other proteins bound to neurexins besides latrotoxin are neurexophilins, CASK,

neuroligins (Ichtchenko et al., 1995b; Hata et al., 1996; Butz et al., 1998; Missler et al., 1998a; Missler and Südhof, 1998a).

Neuroligins were found as post-synaptic binding partners of both α- and β-neurexins that lacks splice site 4 in a calcium-dependent manner (Ichtchenko et al., 1995a; Ichtchenko et al., 1996; Krasnoperov et al., 1996; Boucard et al., 2005). There are five neuroligins in human, neuroligin 1 to 5, among which neuroligin 3 and 4 are on X-chromosome, neuroligin 5 is on Y-chromosome (Bolliger et al., 2001; Varoqueaux et al., 2006b; Bolliger et al., 2008). Neuroligins have a large extracellular segment, a highly conserved transmembrane region and a small cytoplasmic segment. The extracellular segment contains an N-terminal signal peptide, an inactive acetylcholine esterase domain in which the active catalytic site is substituted to glycine from serine, multiple N-glycosylation sites and an O-linked glycosylation region that is serine/threonine rich (Ichtchenko et al., 1995b; Missler and Südhof, 1998b). Two splice sites has been found on neuroligins located on the ectodomain, splice site A and splice site B; neuroligins with splice site B only bind to β-neurexin, however neuroligins without splice site B can bind to both α- and β-neurexins (Ichtchenko et al., 1995b; Boucard et al., 2005). From the NL1-N1β structure, the binding interface is changed by the insertion of splice site B, and explained the change of affinity to neurexins with and without splice site B (Arac et al., 2007). Recently neuroligin 3 missense and neuroligin 4 nonsense mutations has been found in some autism patients (Jamain et al., 2003;

Comoletti et al., 2004; Laumonnier et al., 2004; Yan et al., 2005), and also neurexins have been reported to be related to autism (Alarcon et al., 2008; Arking et al., 2008; Kim et al., 2008). This deficiency may due to the defects in protein processing (Comoletti et al., 2004; De Jaco et al., 2006). Especially NL3 Arg^{451} to Cys^{451} mutation (R451C) as an autism mice model has been reported to have increased inhibitory responses and enhanced special learning in addition to the impairment of social interaction (Tabuchi et al., 2007).

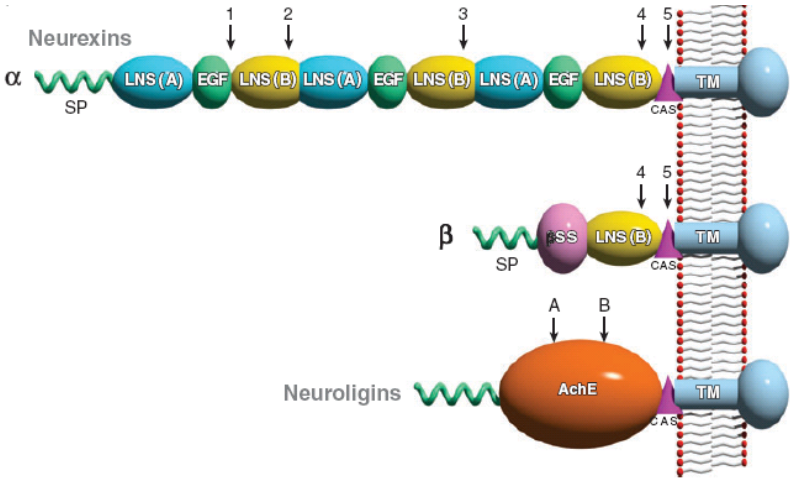


Figure 1-12 Domain structure and alternative splicing of neurexins and neuroligins.
The α- and β-neurexins are transcribed from alternative promoters from the same genes. α-neurexins contain an N-terminal signal peptide (SP), which is removed from the mature protein, followed by three neurexin repeats, each consisting of two LNS domains (A and B) flanking an EGF-like domain. A membrane-proximal carbohydrate attachment site (CAS) precedes a single transmembrane segment (TM), and a short cytoplasmic region. The β-neurexins contain a different signal peptide and a short β-neurexin specific sequence (βss) and share membrane proximal regions, including the LNS(B) domain with their α counterparts. Positions of the five alternative splice sites of α-neurexins are numbered and indicated by arrows. Two of these (4 and 5) are shared with the β-neurexins. The neuroligin ectodomain is characterized by the presence of a large domain with homology to acetylcholinesterases (AchE). Neuroligin 1 has two alternative splice sites, A and B. Splicing at site B determines affinity for α-neurexins, but has little effect on β-neurexin binding (Shapiro et al., 2007).

The c-terminal of neuroligin 1 can bind to PSD95, an abundant synaptic scaffolding protein (Irie et al., 1997a). PSD95 is a member of membrane-associated guanylate kinase (MAGUK) family(Sheng, 1996), the first and second PDZ domain interact with NMDA receptor subunit and K^+ channels (Kornau et al., 1995; Kim et al., 1996). And PSD95-neuroligin complex may modulate pre-synaptic release (Futai et al., 2007). NL1-3 triple knockout mice showed the impairment of spontaneous responses, especially inhibitory responses (Varoqueaux et al., 2006a). It has been shown that neuroligin 1 mostly located at excitatory synapses, and neuroligin 2 at inhibitory synapses (Graf et al., 2004; Varoqueaux et al., 2004; Chih et al., 2005), but not limited to them (Prange et al., 2004). There is a balancing effect of the distribution of neuroligins to excitatory or inhibitory synapses by proteins such as PSD95 and neurexins (Levinson et al., 2005), and neurexins are related to presynaptic calcium in regulating synaptic transmission (Zhang et al., 2005b). Exogenous neuroligin applied to neuronal culture are able to recruit and cluster neurexins (Dean et al., 2003). Although initial synapse formation could be observed in a neuroligin-independent manner (Scheiffele et al., 2000; Gerrow et al., 2006), neuroligins are needed to validate these transient synapses (Varoqueaux et al., 2006a; Chubykin et al., 2007). The neuroligin trafficking may be contributed by some cytoplasmic region besides PSD95 binding motif (Dresbach et al., 2004).

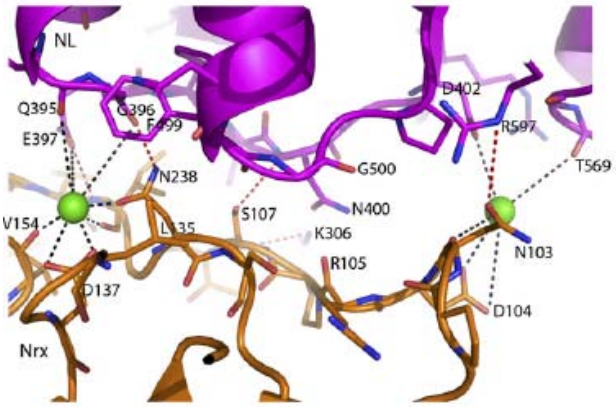


Figure 1-13 Close-up View of the Binding Interface between NL1 and NRX1β. Ca^{2+} is shown as green spheres. Hydrogen bonds are shown by red dashes. The Ca^{2+} coordination is shown by black dashes. Residues at the binding interface are shown as sticks (Arac et al., 2007).

The structures of NL1 and neuroligin-neurexin complex suggest the splice variants of either neuroligin or neurexin have different conformations especially affects the binding interface. Mutations of the amino acids on the interface changed the affinity between neuroligins and neurexins and potentially affect the physiological function (figure 1-13) (Arac et al., 2007). This finding provides us with the tool for study the structure-function relationship of neurexin-binding and neuroligin function.

G-protein Coupled Receptors

G-protein coupled receptors (GPCRs) are a large superfamily of cell-surface proteins that have seven transmembrane domains, and are able to activate

the intracellular heterotrimeric GTP-binding proteins (G proteins) for signaling (Krupinski et al., 1989; Taussig et al., 1993; Hamm and Gilchrist, 1996). The G-protein is composed of one α-, one β-, and one γ- subunits. Fifteen α-subunits, which contain the guanine nucleotide binding site, are classified into four different subfamilies as G_s, G_i, G_q and G_{12}. There are five β and fourteen γ different subunits that form tight βγ complexes (figure 1-14). Upon receptor activation, Gα exchanges GDP to GTP, Gβγ dissociates from the trimeric complex, and then Gα and Gβγ both activate or inhibit the second messengers, promote intracellular Ca^{2+} increase, and open or close the channels. Usually the effect of GPCRs are integration of the activation of an intracellular signaling network (Marinissen and Gutkind, 2001; Gainetdinov et al., 2004). Mitogen-activated protein kinases (MAPKs) play important roles in multiple pathways in linking the GPCR signaling to the nucleus and regulating transcription upon the activation of GPCRs (Davis, 1995). GPCR activation also leads to the activation of receptor tyrosine kinases (RTKs), such as epidermal growth factor receptors (EGFRs) (Daub et al., 1997). GPCRs also release the membrane-bound pro-hormones by provoking the proteolytic cleavage (Prenzel et al., 1999).

GPCR signaling shows plasticity that high activation of GPCRs triggers desensitization whereas low triggers sensitization (Hausdorff et al., 1990). In this way, the signaling strength is regulated by changing the number of receptors on the cell surface by recycling, which usually shows net internalization of receptors

after activation, or by changing the efficacy of receptors responding to the signals (Bohm et al., 1997).

GPCRs have various kinds of ligands, ranging from amines (dopamine, noradrenalin, serotonin, histamine), amino acid transmitters (glutamate, GABA), peptides (opiods, tachykinins, neurotensin, somatostatin, cholecystokinin), lipid-derived products (lysophosphtidic acid, sphingosine-1 phosphate, eicosinoids), hormones, growth factors, to odorant molecules and light (Marinissen and Gutkind, 2001).

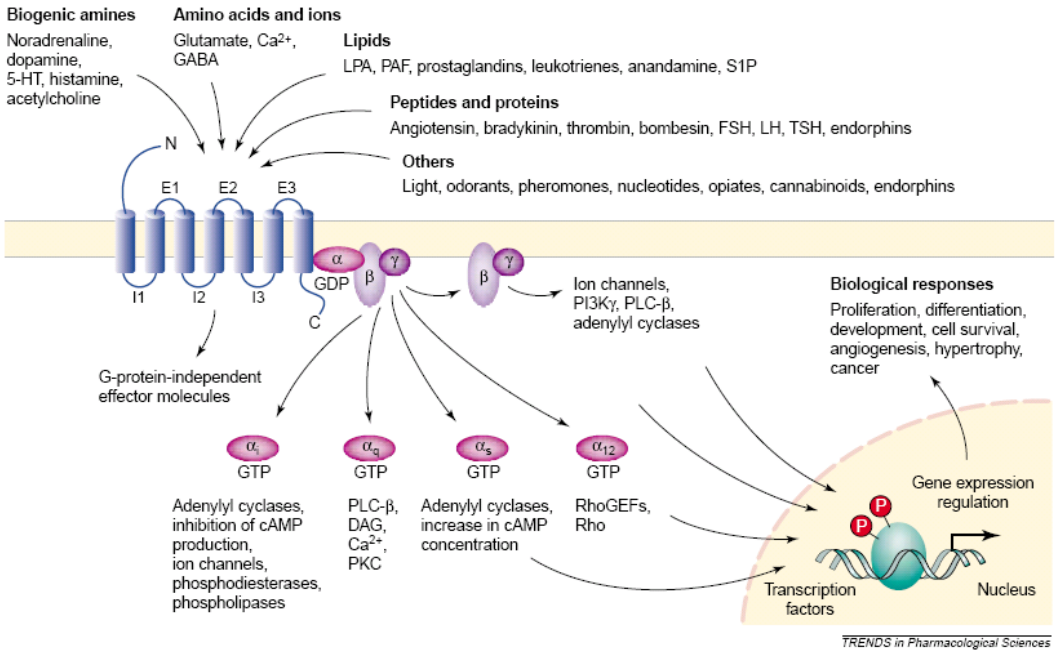


Figure 1-14 Diversity and Signaling of GPCRs (Marinissen and Gutkind, 2001).

Brain-specific Angiogenesis Inhibitor (BAI)

Brain-specific Angiogenesis Inhibitors (BAIs) belong to the GPCR superfamily. There are three members in this family, BAI1, BAI2 and BAI3 (Nishimori et al., 1997; Shiratsuchi et al., 1997). BAI1 was found as a novel DNA fragment that has p53 binding site (Tokino et al., 1994; Nishimori et al., 1997). BAIs are big transmembrane proteins that have about 1,500 amino acids, has a long extracellular region that about 900 amino acids, and a seven-transmembrane hydrophobic region and a cytoplasmic tail about 400 amino acids (Duda et al., 2002; Kee et al., 2002; Kee et al., 2004). Although some studies were done in cancer and ischemia, the neuronal function of BAIs is still unknown. It still remain unclear what ligands bind to BAIs and what the downstream signal pathways are.

Central Questions

The brain development is fascinating. Especially the synapse, the small unit which performs the transmission from one neuron to the other, builds up the whole signaling system that eventually carries out recognition, computation, learning and memory. Although there are huge amount of research and exciting new findings every day in the neuroscience field, lots of issues are still remain unclear and need to be investigated in order to understand the mechanism of the brain work. Development of the synapses, which represents that of the brain, still

needs to be studied more. What are the cues leading to the different types of synapses? And how the synapses are maintained in the time scale of human life? What are the triggers for the aging of human brain, especially in cases of neuronal diseases, such as Alzheimer's and Parkinson's disease? Another open question is about the signal network in the presynaptic and postsynaptic neuron. So far we only know a small portion of the whole network that controls the complex function in between neurons. What are the mechanisms of the different signaling of a similar context in different conditions? And what is the fine network connecting the surface receptors and the cellular changes? Last, since one neuron contains thousands of synapses, and one human brain contains millions of neurons, it would be interesting to see how the synaptic activities integrate into the big picture of brain function. How similar neurons are as building blocks contributing to the different functions in the different contexts? And how the brain organizes the neurons for specific purposes? There are lots of intriguing questions remained to be answered regarding the synaptic machinery and function. It is especially interesting to trace the specific molecule, such as neuroligins that we are studying, from the detailed functions in the synaptic excitatory and inhibitory response, to the overall contributions to the development and functionality of the brain circuitry.

Chapter Two: Neuroligin Cytoplasmic Tail Function

Introduction

Neuroligins (NLs) are transmembrane molecules on the postsynaptic membranes (Ichtchenko et al., 1995b; Scheiffele et al., 2000). The four amino acids as PDZ binding motif at the cytoplasmic terminal of NL1 and NL3 can bind to PDZ95, an abundant postsynaptic scaffolding protein. However, other aspects of NL function on signal transduction or trafficking are still unknown. So we hypothesized that some region of NL cytoplasmic tail works as a signal transduction motif or a trafficking signal. Here we used the yeast two-hybrid screening to find the potential interaction partners of NL intracellular region and then verified the interaction in both the yeast two-hybrid assay and the GST-pull down. Necab2 and EC1 (NRP/B) are two candidates that are potential interact with NL cytoplasmic tail and exert function on signal transduction or trafficking. However, when we test the function in electrophysiology, we found that NL cytoplasmic tail is not necessary to trigger the electrophysiological changes, neither are the two candidates. It is difficult to explain the function of NL cytoplasmic tail; however, for the electrophysiological properties, it is certain that the extracellular portion is more important. However, the intracellular portion may play a role in trafficking, which need to be further determined.

Results

Neuroligin Cytoplasmic Tail is Necessary in Postsynaptic Assembly

NL1 has been shown to have the ability to increase the spine density and the pre- and post- synaptic representative puncta densities when over-expressed in the neurons (Chih et al., 2005). This is because of the synaptic formation or stabilization promoted by NLs. In order to investigate the function of the NL cytoplasmic tail, we truncated the 115 amino acids from the NL1 cytoplasmic tail and fused it with mVenus after the truncation to test its properties in the synapse formation assay. In the transfection, we also transfected ActinGFP as a spine marker, and fixed the cultured neurons DIV14-16.

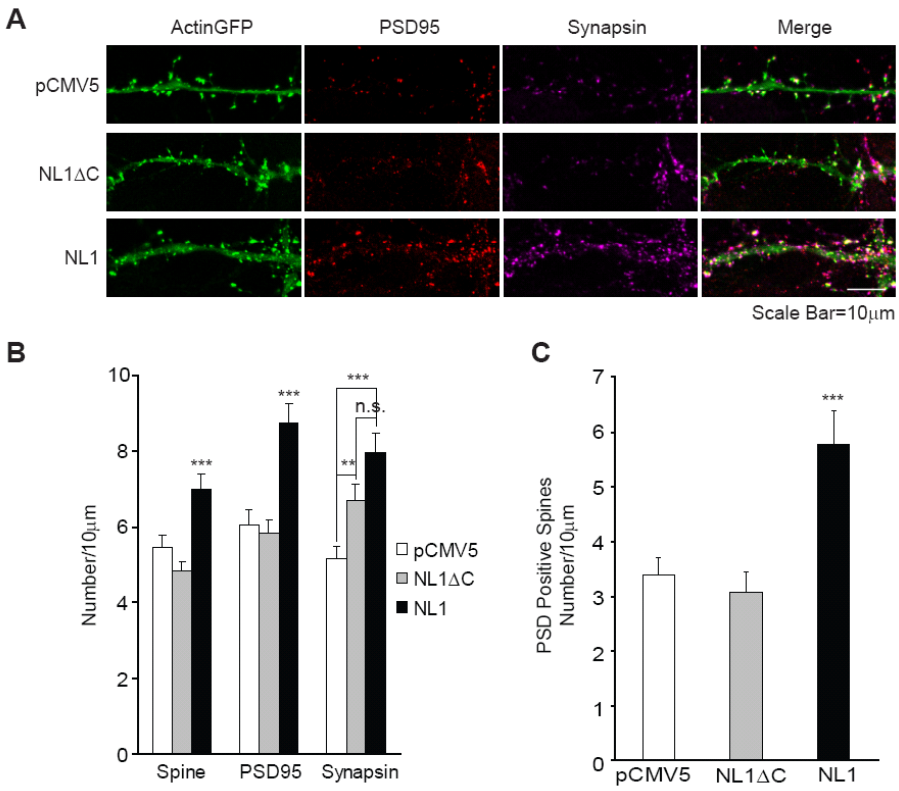


Figure 2-1. Neuroligin with Cytoplasmic Tail Truncation Abolished the Increase by the Full-length NL1 on Postsynaptic Organizations.
(A) Representative images from dissociated hippocampal cultured neurons over-expressed NL1 full length, NL1ΔC115, which is the c-tail truncation, and control vector pCMV5. (B) Quantitation of synaptic densities of the spine, PSD95 staining, and synapsin staining. (C) Comparison of PDS95 positive spines which represent the more matured spines in the image.

As for the postsynaptic density, the spine density is significantly increased in NL1 full-length over-expression neurons, but NL1 with the cytoplasmic tail truncation and the control vector are of the same synaptic density; PSD95 density showed similar phenotype that only NL1 full-length could increase the density, but not NL1 with the cytoplasmic tail truncation and the control. It can be

explained that the signals from outside the cell to form/stabilize synapses do not fully enter the cell; therefore, the postsynaptic properties hardly change accordingly as the NL1 full-length. As for the presynaptic density, we stained neurons with synapsin antibody that will label all the presynaptic terminals. Here we saw the significant increase from NL1 full-length, but NL1 with the cytoplasmic tail truncation mildly increased presynaptic density, however, it is difficult to conclude the effect of NL1 cytoplasmic tail on the presynaptic density (figure 2-1). It is necessary to investigate this phenomenon in more detail.

NL1 Cytoplasmic Tail Affects Inhibitory Synapses

Now we want to dissect the effect of NL1 cytoplasmic tail on the different types of synapses, whether excitatory or inhibitory or both. We immunostained the cultured dissociated hippocampal neurons with the presynaptic marker vGLUT1, which labels the excitatory synaptic vesicles, and vGAT, which labels the inhibitory synaptic vesicles. Here we found that the contribution by the NL1 cytoplasmic tail to the density increase in the different types of synapses is different. The NL1 with the cytoplasmic tail truncation only abolished the increase of vGAT staining, which represents the inhibitory presynaptic terminals. For the excitatory synaptic density, NL1 with the cytoplasmic tail truncation did not change (figure 2-2). Therefore, the effect from NL1 over-expression is triggered differently in the excitatory and inhibitory synapses, and NL1

cytoplasmic tail only affects the inhibitory side. However, because of the limitation of the imaging assay, it is difficult to further investigate this problem in the assay.

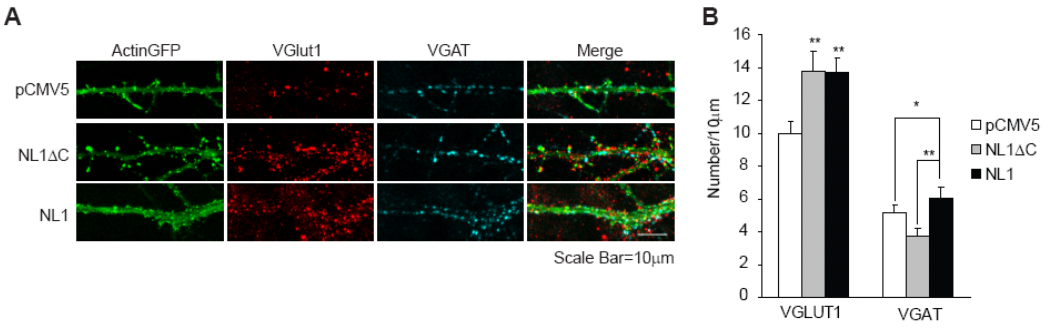


Figure 2-2. Dissection of NL1 cytoplasmic tail contribution in excitatory and inhibitory synapses.
(A) Representative images of dissociated cultured hippocampal neurons over-expressed with NL1 full-length, NL1 cytoplasmic tail truncation and control vector. Neurons were also immunostained with PSD95 and synapsin antibody to label post- and pre- synaptic puncta, respectively. (B) Quantitation of the vGLUT1 (excitatory) and vGAT (inhibitory) densities.

NL1 Cytoplasmic Tail Affects the Miniature Inhibitory Responses

Furthermore, we were interested in the physiological effects from the neuroligin cytoplasmic tail. The same NL1 construct with the cytoplasmic tail truncation, NL1ΔC115, was over-expressed in the cultured dissociated hippocampal neurons along with the NL1 full-length construct as the positive control and the empty vector as the negative control. The frequency of miniature IPSC was changed with the over-expression of NL1 full-length construct with no change in the amplitude (figure 2-3). This reflexes that NL1 may promote the synaptic vesicle release, via the increase of the amount of the presynaptic vesicles,

or the increase in the release probability. NL1 with the cytoplasmic tail truncation partially increased the mIPSC frequency with no change of the mIPSC amplitude (figure 2-3).

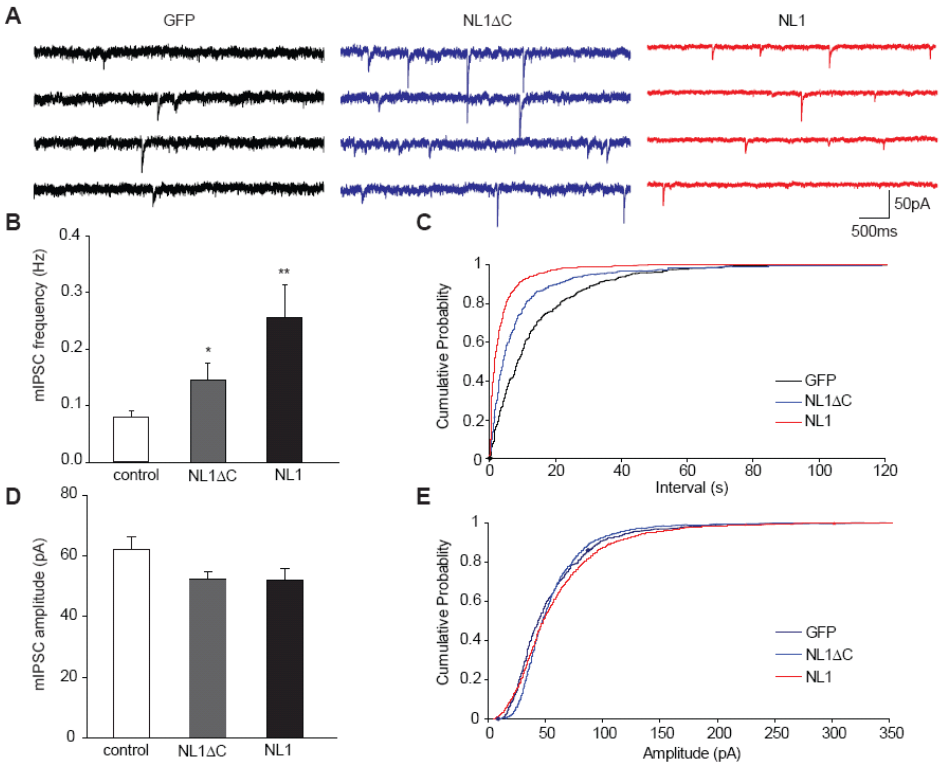


Figure 2-3 NL1 Cytoplasmic Tail Partially Contributes to the Increase of the Miniature IPSC.
(A) Representative traces shows the miniature IPSC from NL1 full-length, NL1 c-tail truncation and the vector alone over-express cultured dissociated hippocampal neurons. (B-C) The frequency of miniature IPSC frequency changed. (D-E) The amplitude of miniature IPSC was not changed.

Neuroligin Cytoplasmic Tail Affects the Miniature NMDA Responses

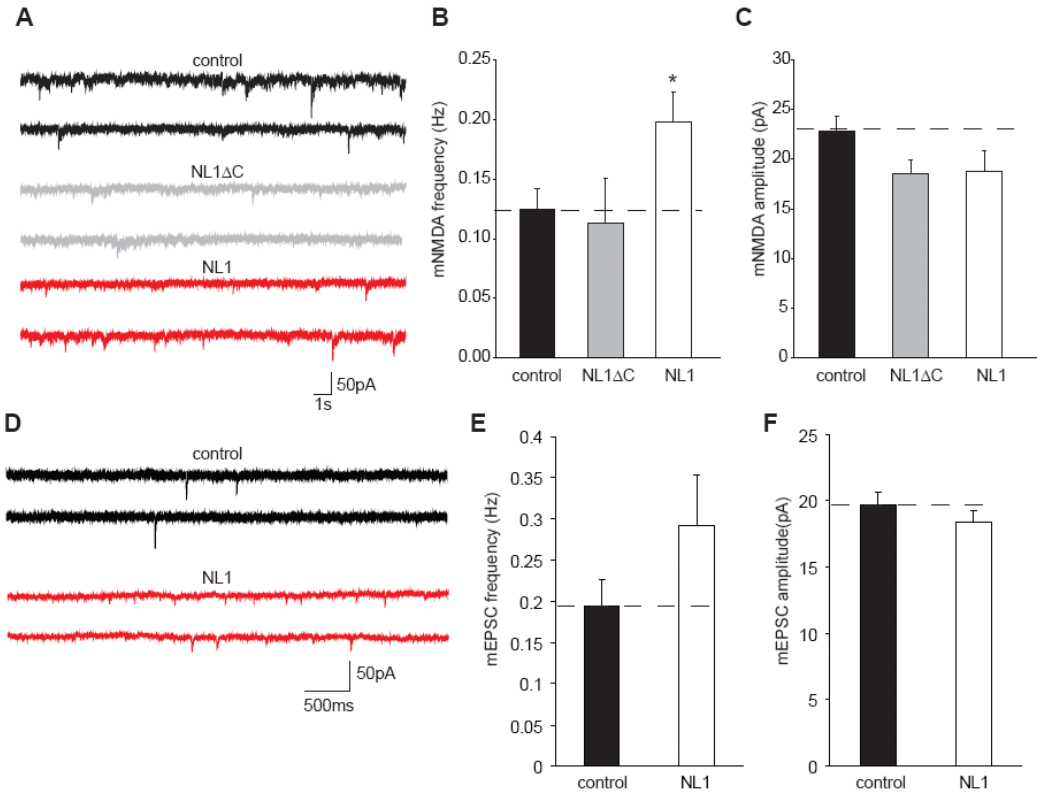


Figure 2-4 NL1 Increased the Miniature NMDA Frequency but not NL1 with the Cytoplasmic Tail Truncation.
(A) The representative traces of the miniature NMDA responses of the cultured dissociated hippocampal neurons over-expressing NL1, NL c-tail truncation and the control vector. (B) The frequency of the miniature NMDA responses. (C) The amplitude of the miniature NMDA responses. (D) The representative traces of the miniature AMPA responses of the cultured dissociated hippocampal neurons over-expressing NL1 and the control vector. (E) The frequency of the miniature AMPA responses showed a trend of increase but not significant. (F) The amplitude of the AMPA responses remained unchanged.

As for the other aspect of physiological function, we investigated the effects of the excitatory responses. There is no significant change on the miniature AMPA responses; however, there is a trend of increase in the NL1 over-expressing neurons. As for the NMDA responses, we found the NL1 full-length

increased the frequency of miniature responses without changing the amplitude. NL1 with the cytoplasmic tail truncation partially triggered the increase, which means the cytoplasmic tail partially contributes to this effect.

Necab2 and EC1 as Interaction Candidates of NL Cytoplasmic Tail

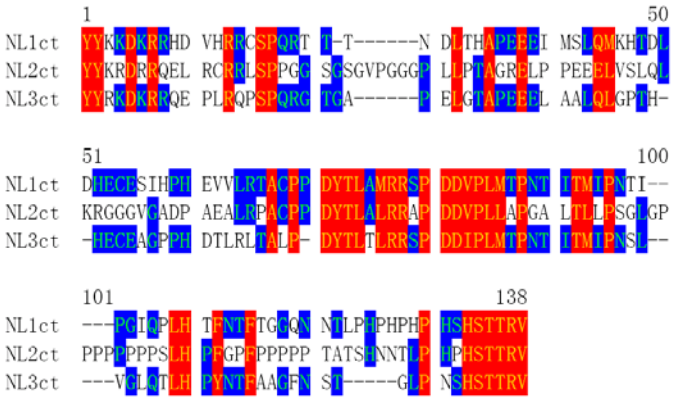


Figure 2-5 Neuroligin Cytoplasmic Tail Protein Sequence Alignment.

In order to find the pathways of NL function, we used the yeast two-hybrid screening to search for the interaction partners of the NL cytoplasmic tail. Since the last four amino acids were already found interacting with PSD95 (Irie et al., 1997b), we excluded this region in our screenings.

A

B

Bait	NL2 699-832	NL2 699-797
Scaffolding Protein	ProfilinIIa POSH MAGI-2 MAGI-3	MAGI-2 MAGI-3
Ubiquitin Ligase	SMAD, specific E3 ubiquitin protein ligase 2 NEDL2 NEDD4 Itchy homolog E3 ubiquitin protein ligase WW domain containing E3 ubiquitin protein ligase 1	NEDD4 Itchy homolog E3 ubiquitin protein ligase WW domain containing E3 ubiquitin protein ligase 1
Others	dystrophin-related protein 2 spinophilin neuronal calcium binding protein NECAB2 Sorbs1 protein Similar to angiogenic factor with G patch and FHA domains 1 Myosin Va variant GRP75 Endophilin B1b	neuronal calcium binding protein NECAB2 GRP75 Endophilin B1b Ectodermal Cortex 1 (NRP/B)

Figure 2-6. NL Cytoplasmic Tail Interaction Candidates were Found in the Yeast Two-Hybrid Screening.
(A) The diagram showing the region of the baits in the screening. (B) Positive candidates from the two screening.

Neuroligin cytoplasmic regions are highly conserved among the three isoforms (figure 2-5). We used NL2 cytoplasmic tail because NL1 and NL3 cytoplasmic tails have high auto-activities in the yeast two-hybrid assay, which decrease the possibility of finding possible interaction partners. First, we used NL2 699-832, which is the complete cytoplasmic tail except the last four amino acids, as the bait for the screening (figure 2-6A). The library we used is rat E14 brain cDNA library. From the first screen, we found potential candidates as following: profilinIIa, POSH, MAGI-2, MAGI-3, SMAD (specific E3 ubiquitin protein ligase 2), NEDL2, NEDD4, itchy homolog E3 ubiquitin protein ligase, WW domain containing E3 ubiquitin protein ligase 1, dystrophin-related protein2, spinophilin, neuronal calcium binding protein NECAB2, Sorbs1 protein, similar

to angiogenic factor with G patch and FHA domains 1, myosin Va variant, GRP75, endophilin B1b (figure 2-6B). By looking at these candidates, we found there were quite a few SH3 domain or WW domain containing proteins. Close to the end of the cytoplasmic tail of NL2, there is a proline-rich region that may induce the binding with SH3 domain or WW domain containing proteins, and the bindings are most likely artificial. So in the next screening, we used shorter bait NL2 699-797. This bait covered the whole NL2 cytoplasmic tail except the proline-rich region and the PDZ binding motif. From the second screening, we found potential candidates as following: MAGI-2, MAGI-3, NEDD4, itchy homolog E3 ubiquitin protein ligase, WW domain containing E3 ubiquitin protein ligase 1, neuronal calcium binding protein NECAB2, GRP75, endophilin B1b, ectodermal cortex 1 (NRP/B) (figure 2-5).

We selected two candidates that are highly neuronal specific in the brain atlas map (http://www.brain-map.org; data not shown).

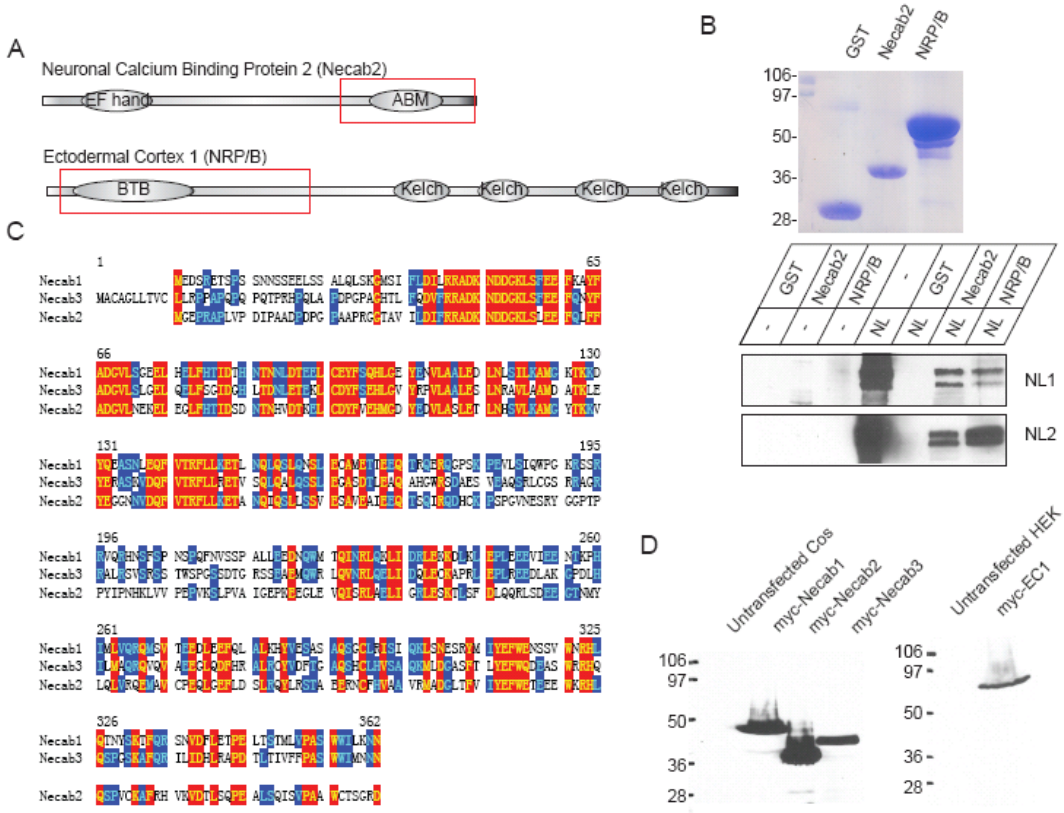


Figure 2-7. Necab2 and EC1 were Verified as the Interaction Partner of NL2 Cytoplasmic Tail.
(A) Diagrams showing the domain structure of Necab2 and EC1. (B) Alignment of all three family members of Necabs. (C) GST pull down from NL-transfected cell membranes. (D) The expression of Necab1-3 and EC1 in Cos-7 cells.

One is ectodermal cortex 1 (EC1) or nuclear-matrix-restricted protein/brain (NRP/B), which is a highly conserved protein and is a component in the nuclear matrix in neurons. It has a BTB/POZ domain at N-terminal, and four kelch-repeats at C-terminal (Hernandez et al., 1997; Kim et al., 1998). EC1 is necessary for the neurite growth of PC12 cells upon NGF stimulation (Kim et al., 2005). Another kelch repeat protein actinfilin is as a binding partner of GluR6, a

kainite-type glutamate receptor subunit, for its degradation. Kelch repeat protein may act as a substrate adaptor for E3 ubiquitin ligase complex (Salinas et al., 2006). So we hypothesized that EC1 is involved in the degradation of NL2.

The other one is neuronal Ca^{2+} -binding protein 2 (Necab2). There are three family members of Necab, Necab1, Necab2, and Necab3. Necabs have an EF-hand domain which has a single Ca^{2+} binding site at the N-terminal and an ABM domain which is a bacterial domain of unknown function at the C-terminal (Burgoyne et al., 2004). Adjacent to the EF-hand domain is a coiled-coil domain called NHR domain that is highly conserved in all three members of Necab family (figure 2-4C). Necab1 and Necab2 are expressed mostly in the brain, and Necab3 is more ubiquitous that has been found in both the brain and the muscle (Sugita et al., 2002). The function of the Necab family is not clearly known yet. Necab1 was reported being co-purified with synatotagmin 1 (Syt1) in the affinity chromotography (Sugita et al., 2002). Necab2 binds to adenosine A_{2A} receptor to regulate its surface expression and affects the related MAPK pathway (Canela et al., 2007). Necab3 from *hXB51* gene has been shown to bind to X11/Mint from the yeast two-hybrid screening (Sumioka et al., 2003). Here we hypothesized that Necab2 may trigger downstream signal transduction in a Ca^{2+} dependent manner.

GST recombinant EC1 and Necab2 were made and the expression was shown (figure 2-6B). Cos-7 cells were transfected with NL1 or NL2 with an extracellular flag tag and after transfection cell membrane portion was obtained

and incubated with GST recombinant EC1 or Necab2. Both NL1 and NL2 could be pulled down by GST recombinant EC1 or Necab2 detected by the flag antibody (figure 2-6B). Full-length EC1 and Necab1-3 were purchased from ATCC and subcloned into pCMV5 expression vector with the myc tag. Expressions of pCMV5-myc-EC1 and pCMV5-myc-Necab1, pCMV5-myc-Necab2, pCMV5-myc-Necab3 were tested in Cos-7 cells (figure 2-6D). Those expression constructs were used in the following experiments.

EC1 and Necab2 do not Change the Property of NL2 in Evoked IPSC

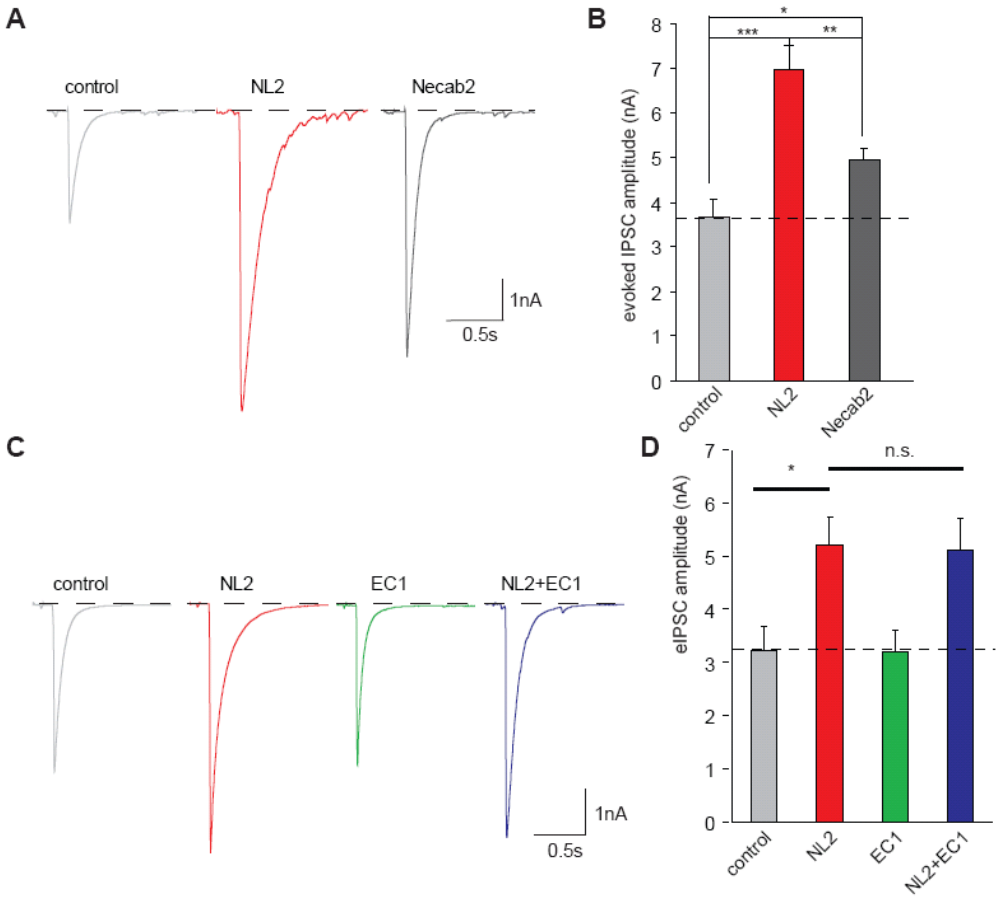


Figure 2-8 EC1 and Necab2 does not Contribute to the Evoked Inhibitory Responses.
(A) The representative traces of the evoked IPSC from the cultured dissociated hippocampal neurons over-expressing NL2 or Necab2 or the control vector. (B) The average amplitude of the evoked IPSC from the neurons over-expressing NL2 or Necab2 or the control vector. (C) The representative traces of the evoked IPSC from the cultured dissociated hippocampal neurons over-expressing NL2 or EC1 or both or the control vector. (D) The average amplitude the evoked IPSC from the neurons over-expressing NL2 or EC1 or both or the control vector.

In order to observe the physiological effects of the binding partner EC1 and Necab2, we transfected them into the culture dissociated hippocampal

neurons along with the NL2 full-length construct as the positive control and the control vector (pCMV5 or pCMV5-mVenus) as the negative control. Over-expressing Necab2 increased the evoked IPSC mildly, not as much as NL2 (figure 2-7 A- B). For EC1, first we found that it did not trigger any increase in the evoked IPSC by over-expressing it. However, since it could be the substrate adaptor of an E3 ubiquitin ligase complex, it is possible that the evoked IPSC increase by NL2 will be diminished when we express NL2 and EC1 together in the same neuron, because the access amount of EC1 could degrade the increase the level of NL2. However, we did not find any "quench" effect from the co-expression condition (figure 2-7 C-D). Therefore, the interaction of Necab2 and EC1 do not contribute to the eIPSC changes triggered by NL2.

Neuroligin Cytoplasmic Tail does not Contribute to the Evoked Response Changes triggered by Neuroligins

From the previous experiments, we found that Necab2 and EC1, the binding partners of NL2, do not contribute to the evoked IPSC changes by NL2. But at this point, we have not investigated the effect of neuroligin cytoplasmic tail function in evoked responses. So we decided to justify the contribution of the cytoplasmic tail. By over-expressing NL1 and NL2 with the cytoplasmic tail truncations comparing to the full-length NL1 and NL2, with the control vector as the negative control, we found neuroligins with the cytoplasmic tail truncation

showed very similar phenotype in the evoked responses as the full-length NL1 or NL2 (figure 2-8).

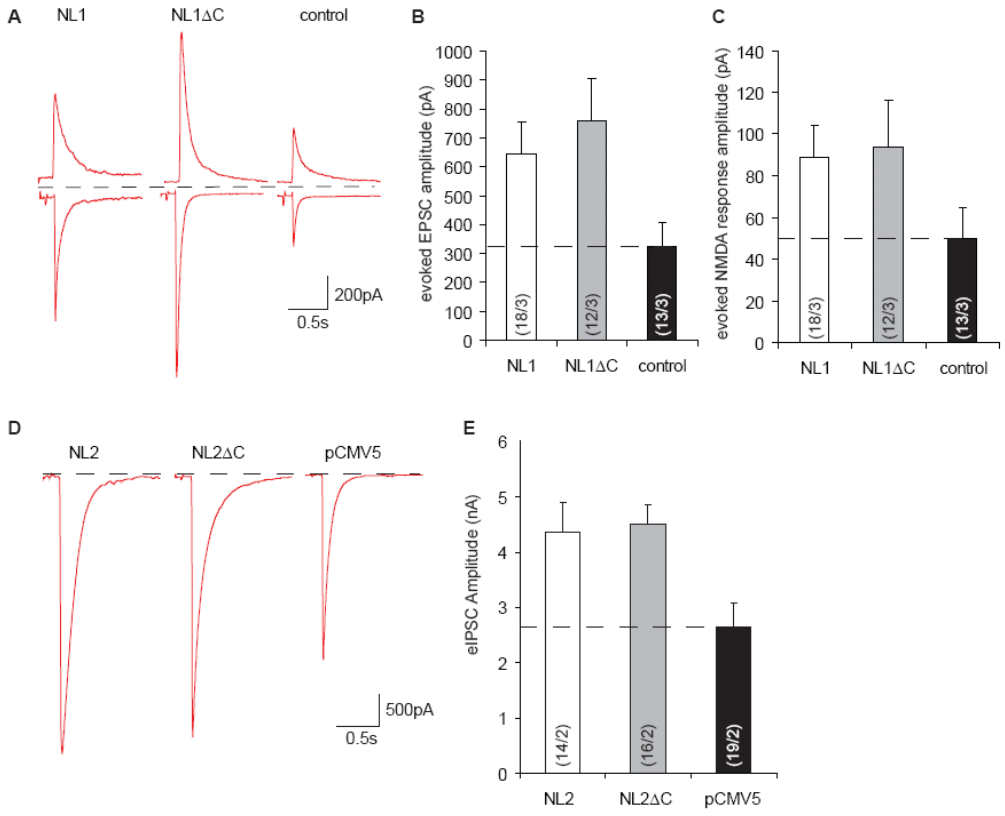


Figure 2-9 Neuroligin Cytoplasmic Tails do not Contribute to the Changes in Evoked Responses.
(A) The representative traces of the evoked EPSC of the NL1 full-length, NL1 c-tail truncation, and the control vector over-expressing neurons. (B) The average evoked AMPA amplitude of the NL1 full-length, NL1 c-tail truncation and the control vector over-expressing neurons. (C) The average evoked NMDA amplitude of the NL1 full-length, NL1 c-tail truncation and the control vector over-expressing neurons. (D) The representative traces of the evoked IPSC of the NL2 full-length, NL2 c-tail truncation and the control vector over-expressing neurons. (E) The average evoked IPSC amplitude of the NL2 full-length, NL2 c-tail truncation and the control vector over-expressing neurons.

Conclusion and Discussion

Through the study of the neuroligin cytoplasmic tail in biochemistry, imaging and electrophysiology, we found the neuroligin cytoplasmic tail does not significantly contribute to the major physiological function of neuroligins, although there are some aspects reflecting the contribution of the cytoplasmic tail, such as the formation or stabilization of the synapses and the miniature changes in the transfected neurons. The problem is complicated because the major function of neuroligin is still not clear, and it is difficult to predict what function the cytoplasmic tail contributes. One weak point of the whole study is that although we found several preys as the ubiquitin ligases or the ubiquitin ligase related proteins in the yeast two-hybrid screening, we did not really pursue them in the following studies. It is highly possible that the neuroligin cytoplasmic tail contributes to the recycling of the neuroligins linking to the ubiquitin complex. Another weak point is that we should have considered more "housekeeping" functions of the neuroligin cytoplasmic tail, because it may participate in the trafficking to target neuroligins to the synapses, especially to discriminate the excitatory and inhibitory synapses. However, because all the studies were done in wild type neuronal cultures, which have the considerable amount of the endogenous neuroligins, and because neuroligins have the ability to form dimmers, it is difficult to observe the effect of the neuroligins with the cytoplasmic tail truncations unless the effect is dominant negative. To rule out the disguise of the endogenous wild type neuroligins, we could use the NL1 knockout

neuronal cultures to study NL1 with the cytoplasmic tail truncation, and use the NL2 knockout neuronal cultures to study NL2 with the cytoplasmic tail truncation. And in the case of discriminating the targeting effect between the excitatory and the inhibitory synapses, it is better to pick specific interaction partners for a single neuroligin isoform to investigate.

It is difficult to exclude the neuroligin cytoplasmic tail from triggering the signal transduction. Because the last four amino acids bind to PSD95, the postsynaptic scaffolding protein that also binds to NMDA receptor subunit, potassium channel, and other synaptic proteins, PSD95 is able to bring some proteins in the close vicinity. It may not be necessary for neuroligins to directly interact with the downstream signal molecules to transduct the signal. Even if there is binding involved, it may not be found by the yeast two-hybrid screening, because the interaction for the signal transduction maybe dynamic and weak.

However, it is certain that the major function of neuroligins, which is the increase of the functional synapses number, lies in the extracellular region of neuroligins. We are more curious to investigate how the neuroligin extracellular region promote the appearance of the functional synapses, and because of the classic neurexin-neuroligin binding, we really want to know what the role of neurexins is in the function of neuroligins.

Chapter Three: Neuroligin-Neurexin Binding is Necessary in the NL2 Function in the Inhibitory Synapses

Introduction

Neuroligins were first found as the binding partner of the latrotoxin receptor, neurexins. Both neuroligin and neurexin were found in the patients' rare genetic defects related to Autism. However, we still do not know how this interaction affects the major function of neuroligins. With the structure of the neuroligin and neurexin complex resolved, it is feasible to make mutations that potentially change the interaction and then to investigate the change in the neuroligin function. According to the structure, we made mutations in NL2 that could potentially change the affinity to neurexins. We hypothesize that the neurexin interaction is essential for the NL2 function in synapses.

NL2 mostly affects the inhibitory synapses. By over-expressing NL2 in the cultured dissociated neurons, we found the NL2 over-expression increased the inhibitory responses, but not the excitatory. However, the increase was abolished when the NL2 was mutated into the neurexin binding deficient forms.

Results

NL2 Neurexin Binding Mutants

According to the structures of NL1 and NL1-N1β complex, the L399A, N400A, D402N mutation set (NL1-LND, #5) changes the hydrogen bond between

NL1 N400 and N1β S107, also disrupts the Ca^{2+} coordination via NL1 D402. The L399A, N400A, D402N, Q395A, E397A set (NL1-LNDQE, #35) disrupts the Ca^{2+} coordination mediated by NL1 E397. The L399A, N400A, D402N, E297A, K306A set (NL1-LNDEK, #32) is made on top of #5 and additionally changed the two residues flanking the splice site B (SSB) (Arac et al., 2007). As for the affinity of binding between NL1 and N1β, wild type is at the nanomolar level, #5 and #32 decreased to the micromolar level, and #35 even less, which means the binding affinity between the NL1 mutants and N1β is greatly decreased (Arac et al., 2007). It was found that the NL1#5 decreased the binding affinity with N1β, abolished binding with N1α. NL1#32, NL2#35, and NL2#37 which have additional mutations based on NL1#5, abolished binding with both N1β and N1α (Antony Boucard, unpublished).

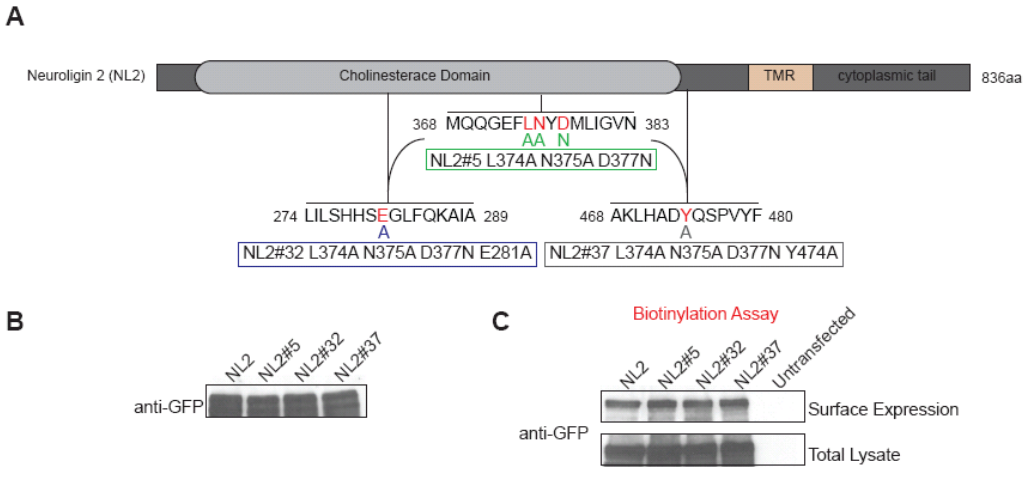


Figure 3-1 The Diagram and Expression of Neurexin Binding Mutants on NL2.

(A) The diagram showing the neurexin binding mutants on NL2. (B) The expression in transfected HEK-293T cells comparing the mutants with NL2 wild type. (C) Biotinylation assay shown the surface expression of the NL2 wild type and mutants.

Although the NL2 structure has not been published, due to the high homology between the sequence of NL1 and NL2, we speculated the structure of NL2 be very similar to NL1. So we found the correlate amino acids to the neurexin binding deficiencies in NL2 as in NL1. For the N1β decreased affinity mutant, we did L374A, N375A, D377N in NL2 (NL2#5). We also did two additional mutants, L374A, N375A, D377N, E281A (NL2#32) which changed one amino acid flanking SSB the same manner as the NL1-LNDEK mutation; L374A, N375A, D377N, Y474A (NL2#37) which was not reported in the paper but also abolished both N1β and N1α binding in NL1. All the mutants were made with an intracellular GFP tag (figure 3-1A).

After finished the mutations, we transfected them into HEK-293T cells. Membrane portions from transfected cells were obtained and blotted with GFP antibody. The expression levels of all mutants were as good as the NL2 wild type (figure 3-1B). By the biotinylation assay, all the mutants showed similar surface expression as the NL2 wild types.

NL2 Mutants Changed the Binding with Neurexins

First we want to know whether those NL2 mutants changed the binding with neurexins similar as the NL1 mutants. HEK-293T cells were transfected with the NL2 wild type, the NL2 mutants or the control construct. To see the binding with neurexins, IgC, IgN1β and IgN1α were purified and incubated with the transfected HEK-293T cells. Excess Ig proteins were washed away and attached Ig proteins were stained with polyclonal IgG antibody.

Both wild type NL1 and NL2 showed normal binding to IgN1β and IgN1α. The different intensities between NL1 and NL2 were due to the different expression levels of NL1 and NL2 in HEK293-T cells (figure 3-2 A-C). NL2#5 showed greatly reduced binding to IgN1β, and binding to IgN1α is almost gone. Both NL2#32 and NL2#37 abolished the binding to either IgN1β or IgN1α (figure 3-2 D-F).

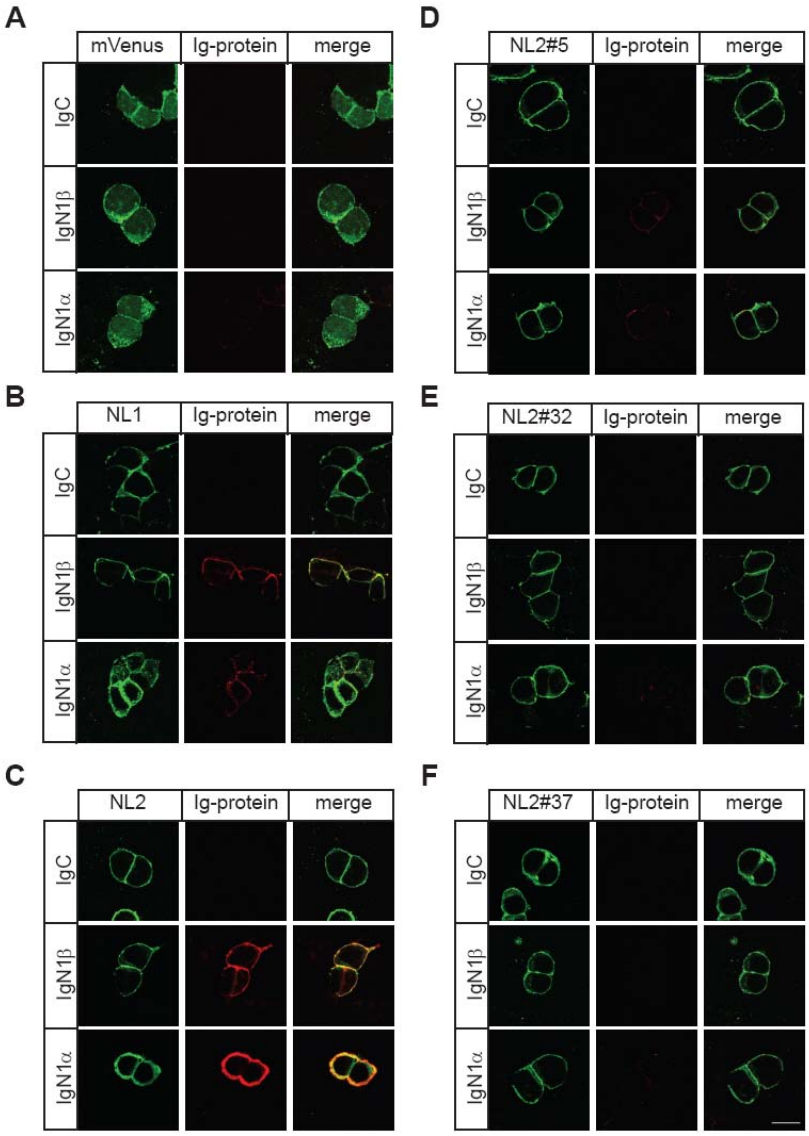


Figure 3-2 Surface Labeling of NRXs on the Cells Transfected with NL2 Wild Type and Mutants.

(A) HEK-293T cells transfected with mVenus and labeled with IgC, IgN1β, or IgN1α. (B) HEK-293T cells transfected with NL1 and labeled with IgC, IgN1β, or IgN1α. (C) HEK-293T cells transfected with NL2 and labeled with IgC, IgN1β, or IgN1α. (D) HEK-293T cells transfected with NL2#5 and labeled with IgC, IgN1β, or IgN1α. (E) HEK-293T cells transfected with NL2#32 and labeled with IgC, IgN1β, or IgN1α. (F) HEK-293T cells transfected with NL2#37 and labeled with IgC, IgN1β, or IgN1α. Scale bar=10μm

NL2 Mutants Abolished the Evoked IPSC Increase Caused by NL2

NL2 over-expression in neurons increased the evoked inhibitory responses, and NL2 knockout mice showed decreased amplitude in evoked IPSC from neurons in brain slices (Chubykin et al., 2007). Here, we transfected the neurons with the NL2 wild type and the NL2 mutants. We want to observe the effect of the neurexin binding to the electrophysiology property. All the mutants, NL2#5, NL2#32, NL2#37, showed similar phenotype that failed to increase the evoked IPSC responses (figure 3-3). Therefore, the neurexin binding to NL2 may intermediate the increase of the evoke responses in the inhibitory synapses. The detailed mechanism is not clear at this point, but there are several possibilities, one is that NL2 interacts with the presynaptic neurexin, either β or α, to enrich the presynaptic proteins and increase the synaptic strength; another possibility is that NL2 recruits more postsynaptic receptors via neurexins, and the increase in receptor amount directly increased the amplitude of evoked IPSC. In order to determine whether the increase is from the presynaptic side or the postsynaptic side, miniature IPSCs were recorded. There is another possibility that neurexins interact with NL2 to send NL2 to the right synaptic region so that it will trigger the correct responses. NL1 only affects the evoked excitatory responses, not the inhibitory; NL2 only affects the inhibitory responses, but not the excitatory (Chubykin et al., 2007). It is because they have different mechanisms to activate different downstream synaptic machineries in order to trigger different responses;

also it could be due to the different localizations but the similar downstream mechanism. So now it is helpful to determine whether neurexin binding contributes to the localization of NL2 or to the function of NL2.

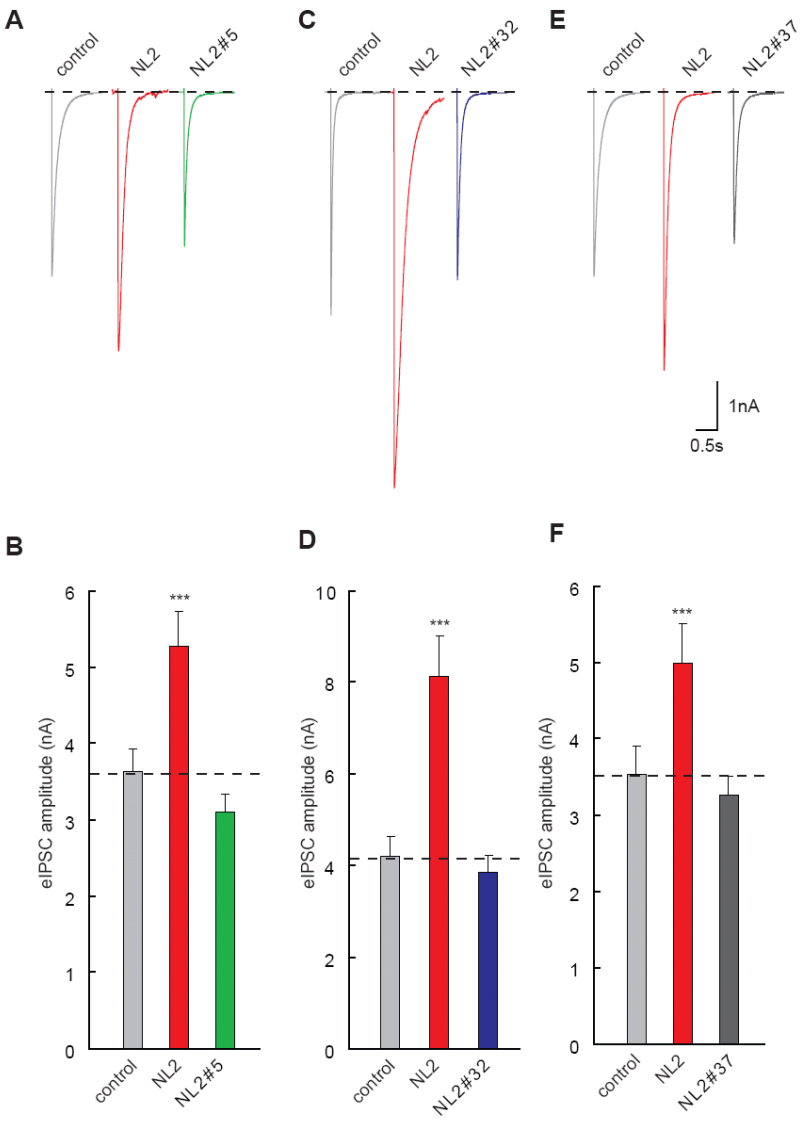


Figure 3-3 Neurexin Binding Mutants in NL2 Abolished the Evoked Inhibitory Response Increase Triggered by NL2 Over-expression.
(A) Representative traces of evoked IPSC from the NL2 wild type, NL2#5 and the control vector over-expressing neurons. (B) The average amplitude of evoked IPSC from the NL2 wild type, NL2#5 and the control vector over-expressing neurons. (C) Representative traces of evoked IPSC from the NL2 wild type, NL2#32 and the control vector over-expressing neurons. (D) The average amplitude of evoked IPSC from the NL2 wild type, NL2#32 and the control vector over-expressing neurons. (E) Representative traces of evoked IPSC from the NL2 wild type, NL2#37 and the control vector over-expressing neurons. (F) The average amplitude of evoked IPSC from the NL2 wild type, NL2#37 and the control vector over-expressing neurons.

NL2 Mutants did not Change the Excitatory Evoked Responses

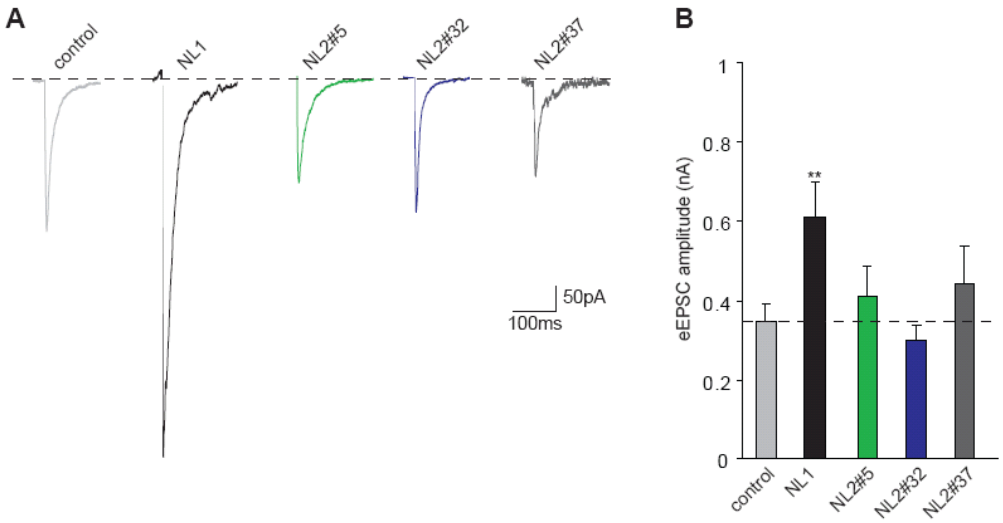


Figure 3-4 NL2 Mutants did not Change the Excitatory Responses.
(A) Representative traces of the evoked excitatory responses from the neurons over-expressing NL1, NL2#5, NL2#32, NL2#37 or the control vector. (B) The average eEPSC amplitude of the evoked excitatory responses from the neurons over-expressing NL1, NL2#5, NL2#32, NL2#37 or the control vector.

In order to answer the question that neurexin binding contribute to the targeting of NL2 to the inhibitory synapses or to the function of NL2, we went to record the evoked synaptic responses. If the neurexin binding contributes to the targeting of NL2 to the inhibitory synapses, and the untargeted NL2 could go into the spines, then we should have seen the increase in the evoked excitatory synapses. If the untargeted NL2 could not go into the spines, then we should not see any increase in both the excitatory and the inhibitory responses. By over-expressing the NL1 wild type and the NL2 mutants, we found that those neurexin binding mutants did not increase the evoked excitatory responses.

Then there are two possibilities, one is that the NL2 neurexin binding mutants could not be targeted to synapses, neither excitatory nor inhibitory, in order to function in the synapses; the other is that the NL2 function through the neurexin binding, no function could be conducted by NL2 without binding to neurexins. In the meantime, another interesting point is which side of the synapse (pre- or post- synaptic) neurexins are located binding to NL2.

NL2 Mutants Changed mIPSCs Frequency Increase Caused by NL2

In order to dissect the mechanism of NL2 responses, we recorded miniature IPSCs from the NL2 wild type and the NL2 mutants over-expressing neurons. We observed the increase from the NL2 wild type in mIPSC was not shown in the NL2 mutants, on the contrary, NL2#32 and NL2#37 even showed decreased frequency compared to the control vector expressing neurons, which means these two neurexin binding mutants have dominate negative effects (figure 3-5 A-B). The change in the mIPSCs frequency suggests there are more synaptic vesicles released. It is mostly because of the changes on presynaptic side, not postsynaptic. There are two possibilities of the change, one is that there are more synapses; the other is that the dynamics of the existing synapses is changed. The mIPSCs amplitude usually reflects the postsynaptic organizations, if the amplitude changes, then the postsynaptic strength also changes. NL1 or NL2 wild type did not show any change in the mIPSCs amplitude. But NL2 mutants showed

decrease in the mIPSCs amplitude about 30% comparing to the control vector (figure 3-5 A, C). It is probably because another kind of interaction with the neurexins changed the postsynaptic organization. NL2#5 does not have the dominant negative effect on the mIPSCs frequency. It is possible that neurexin-α changes the amplitude, but β changes the frequency. But it needs to be further investigated.

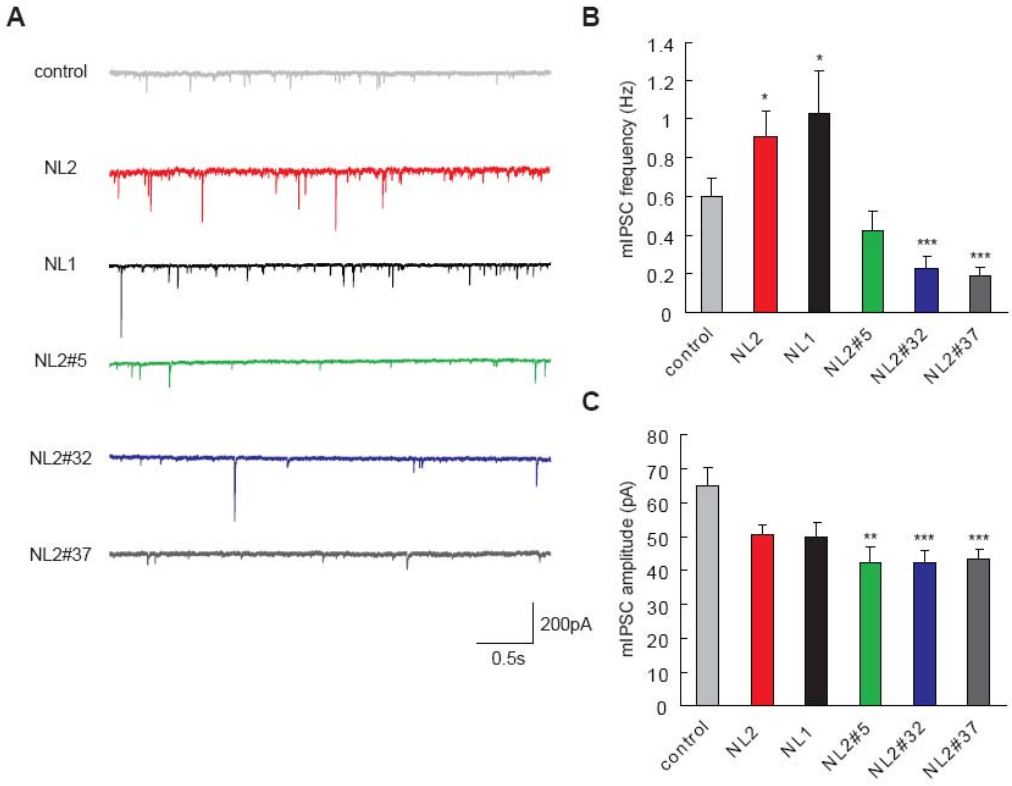


Figure 3-5 NL2 Mutants Changed the mIPSCs to the Opposite Direction from the NL1 And NL2 Wild Type.
(A)Representative traces of the miniature inhibitory responses from the neurons transfected with the NL1 or NL2 wild type, the NL2 mutants, or the control vector. (B) The average frequency of mIPSC from the neurons transfected with the NL1 or NL2 wild type, the NL2 mutants, or the control vector. (C) The average amplitude

of mIPSC from the neurons transfected with the NL1 or NL2 wild type, the NL2 mutants, or the control vector.

NL2 and Mutants Changed the Synaptic Dynamics

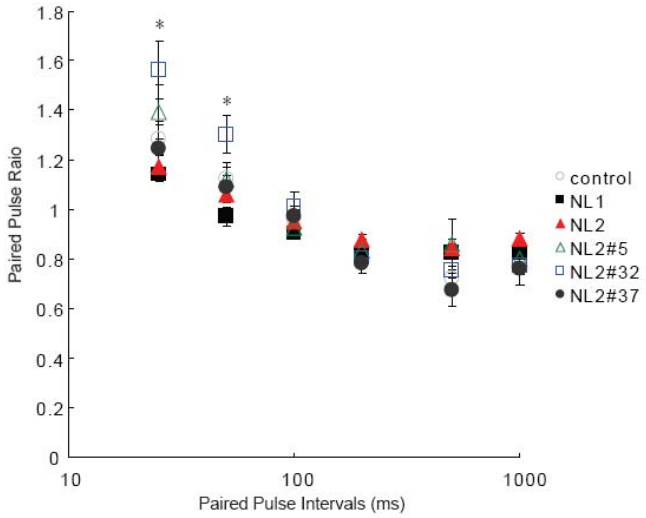


Figure 3-6 Paired-Pulse Ratios of the NL2 Wild Type and Mutants.
IPSCs were recorded from the neurons transfected with the NL2 wild type, the NL2 mutant or the control vector. Paired pulse recordings were at different time intervals: 25ms, 50ms, 100ms, 200ms, 500ms, 1000ms. Paired pulse ratio was calculated as the ratio of the amplitude of the second IPSC to the amplitude of the first.

From the miniature IPSCs change of NL2, it is suggested that there are more synaptic vesicles released due to the over-expression of the NL2 wild type, but not the mutants. However, it is not clear whether the increase is due to the increase of synaptic numbers, or the increase in release probability. In order to dissect this mechanism, the inhibitory paired-pulse responses in the neurons over-expressing the NL2 wild type, the NL2 mutants, or the control vector, were

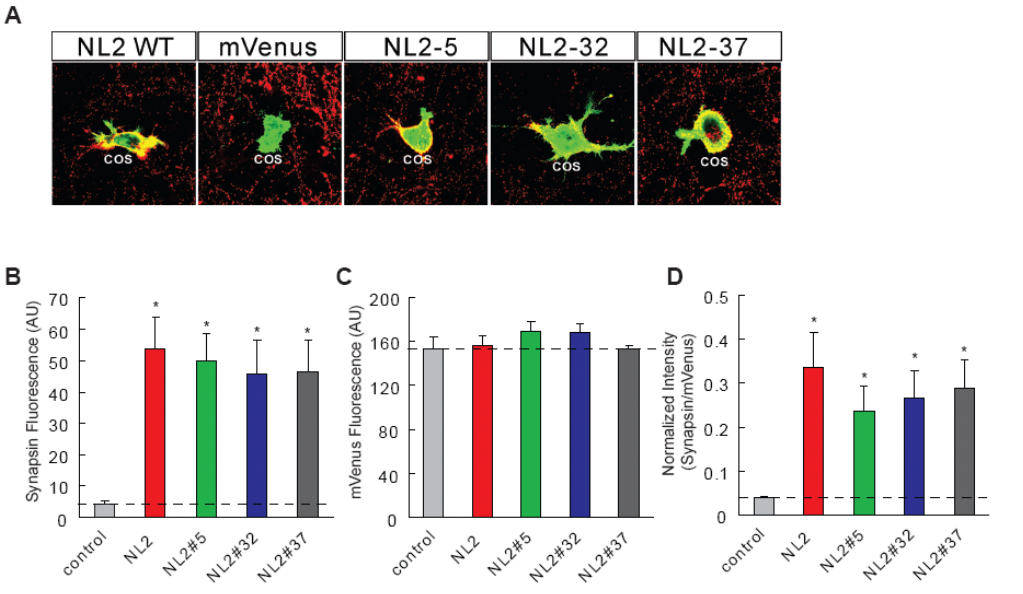
recorded. Paired-pulse recording is to give two stimuli (paired pulse) to the neuron to observe the relative ratio of the two responses. Paired-pulse ratio is a reflection of the release probability, if the vesicles are easily depleted which means the release probability is high, the second response is much smaller than the first one, recognized as “depression”; if the vesicles are not easily depleted which means the release probability is low, the second response is bigger than the first one, recognized as “facilitation”, because the first response helped Ca^{2+} and other factors to be ready at the synapses for the further responses (Thomson, 2000). Here we give two stimuli at different intervals (25ms, 50ms, 100ms, 200ms, 500ms, 1000ms) and observe the ratio of the second IPSCs amplitude to the first, as a measurement of the release probability. With the shorter intervals, we saw facilitations from all the neurons; at the longer intervals, mild depressions were observed. At two early time points (25ms, 50ms), NL1 and NL2 showed a trend of decrease paired-pulse ratio (PPR), which reflected the increase of the release probability. And the NL2 mutants, #5 and #32, showed a trend of the increase PPR, which reflected a decrease in the release probability. The increase of PPR by the over-expression of the NL2#32 mutant is statistical significant (figure 3-6). This decrease in the release probability explains, at least partially, the decrease in the evoked IPSC amplitude and the decrease in the miniature IPSC frequency of the NL2 mutants compared to the wild type. It suggests that the electrophysiological effects from NL2 may go through neurexins on the

presynaptic side, change the Ca^{2+} efficiency and change the dynamics of the synapses.

NL2 and Mutants are Normal in Forming Artificial Synapses

Because the NL2 mutants failed to trigger the changes that the NL2 wild type does, it is interesting to know whether those neurexin binding mutants are able to form the artificial synapses as the NL2 wild type. Neuroligins were able to form synapses in the artificial co-culturing system (Scheiffele et al., 2000). In the previous studies, HEK-293T cells were extensively used in the co-culturing system. However, we found Cos-7 cells were easier to be observed because they have extended membrane surfaces. To obtain a cleaner background, we used Banker Culture which is a low-density culture with neurons and glial cells grow on two separate surfaces. Cos-7 cells were transfected with the NL2 wild type and the NL2 mutants. Transfected Cos-7 cells were replated onto the neurons grown separately from the glial cells. Then the Cos-7 cells with the neurons were stained with synapsin antibody to visualize the presynaptic organizations. GFP signals were visualized by immunostaining. If the artificial synapses are formed, overlaps on Cos-7 surfaces of GFP signal and synapsin should be observed, which means the artificial synapses were formed on the Cos-7 surfaces with the NL2 expression. To quantify the ability to form the artificial synapses, we quantify the synapsin intensity of the overlapping region, either the absolute intensity, or the relative

intensity normalized by the mVenus intensity, which represents the expression level of the NL2 protein. Here we found surprisingly that both the NL2 wild type and the NL2 mutants were able to form the artificial synapses equally well (figure 3-7). However, whether those synapses are functional is still unknown.



(Jaewon Ko)

Figure 3-7 Both the NL2 Wild Type and Mutants were able to Form Artificial Synapses.
(A) Representative images from Cos-7 cells co-cultured with neurons. Red signal is synapsin; green signal is mVenus tagged NL2 or NL2 mutants. (B) The absolute fluorescence intensity of the synapsin staining. (C) The absolute fluorescence intensity of the mVenus. (D) The synapsin intensity normalized by mVenus intensity.

Conclusion and Discussion

We investigated NL2 and its neurexin binding mutants of the function at the synapses. According to the structure of NL1, we made the similar neurexin binding mutants in NL2: NL2#5, NL2#32, and NL2 #37. By the surface labeling

assay, we found that NL2#5 bound neurexin-β with decreased affinity, and it did not bind to neurexin-α. NL2#32 and NL2#37 totally abolished the binding to both neurexins. Currently we only know that the neuroligins will increase the synaptic responses, but not the underlying mechanisms. Here we are able to investigate the contribution of the neurexin binding to the electrophysiological effects. All mutants showed total abolishment on the electrophysiological responses. In the miniature responses, the mutants even showed dominant negative effects. It could be explained that the endogenous wild type neuroligins form heterodimmers with the transfected mutants, and the heterodimers do not act as the wild type neuroligin dimmers.

In the meantime, the effects of the neurexin binding mutants suggested that the neuroligins, at least NL2 in the inhibitory synapses, change the synaptic properties through the neurexin binding. From the reduction in the release probability from the mutants, it is suggested that the binding is essential for the Ca^{2+} efficacy. The number of synapses is increased from the neuroligin over-expression, as observed in the NL1 over-expression neurons (Chih et al., 2005; Chubykin et al., 2007), and may be affected by the neurexin interaction. If the neurexin binding mutants fail to increase the synaptic number as the wild type, it will explain the effect of the frequency decrease of the miniature IPSCs.

Neurexins are likely to be presynaptic in order to explain the effect of the neurexin binding to the neuroligins. In other words, neurexins interact with NL2

in trans from the presynaptic side, and trigger the electrophysiological responses by releasing more synaptic vesicles. The increase in release may due to the increased cooperation of calcium as well as the increase of the synaptic number. However, it is possible that neurexins are postsynaptic in other circumstances.

Developmentally, NL2 may be trafficked to the inhibitory synapses, by interacting with neurexins on the presynaptic side. NL2 induces the formation of the functional inhibitory synapses, stabilizes the functional inhibitory synapses, or facilitates the release of the synaptic vesicles, which is supported by the finding that neurexins interact with the calcium channels.

To discriminate the effect of neurexin-α or -β, further mutants need to be made, because all the mutants used here affect the binding affinity of both neurexins. The impairment from the NL2 mutants can be due to the loss of binding to neurexin-α, or to the decreased affinity to neurexin-β. Two NL2 mutants, which completely abolished affinity to both neurexin-β and –α, have more sever abolishment of the mIPSCs frequency, comparing to the NL2 mutant that only have decreased affinity to neurexin-β and no affinity to –α. It suggests that the amount of neurexin-β bound to NL2 is important to the function of NL2 in the inhibitory synapses.

Chapter Four: GPCR protein BAI in Synapses

Introduction

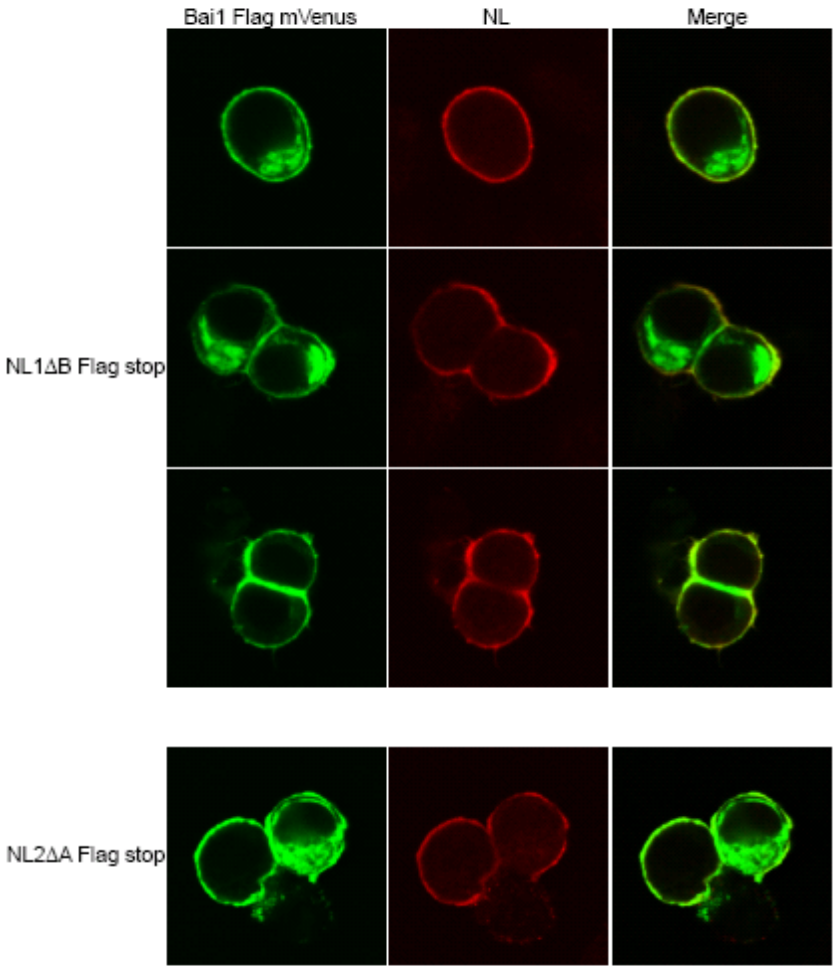
From the studies of neuroligin, there are still open questions about neuroligins' signaling partners and how the signals are transducted to change the neuronal responses and morphology. It is unlikely that the cytoplasmic region of neuroligins directly conduct the signal pathway, however, we found that an important family of G-protein coupled receptors (GPCR), brain specific angiogenesis inhibitors (BAIs), may participate in the signaling. Similar as other members of GPCRs, BAIs have seven transmembrane domains, an extracellular N-terminal region, and a short intracellular cytoplasmic region. BAIs are specifically expressed in brain. By surface labeling assay, we found BAIs bound to neuroligins. There is no clear function being observed of BAIs from the current literatures. We found some changes in the excitatory synapses postsynapticly. Since there are enormous signal pathways related to the GPCRs, it is intriguing to investigate the potential signaling from neuroligins through BAIs.

Results

Bai1 was Found as a Potential Ligand to Neuroligins

From the Ig-neurexin pull down from the brain homogenate, we found Bai1 from the proteins being pulled down. However, there is no interaction

between Bai1 and neurexin (Antony Boucard, data not shown). Since neurexin and neuroligin interacts with nanomolar affinity, it is possible that Bai1 was pulled out by the neurexin-neuroligin complex. So we used the surface labeling assay to see whether neuroligins interact with Bai1. HEK-293T cells were transfected with flag- and mVenus- tagged Bai1, and the expression was well on the surface, except that there is some retention inside the cell, probably in ER. IgC as the control, Ig-NL1ΔB and Ig-NL2ΔA that contain extracellular domain of NL1ΔB and NL2ΔA, were purified and quantified, then incubated with the cells transfected with Bai1. Bai1 was visualized by the mVenus tag in the green channel, and the Ig-proteins, IgC, Ig-NL1ΔB and Ig-NL2ΔA, were visualized by immunostaining with IgG antibody in the red channel. We found Bai1 retained both Ig-NL1ΔB and Ig-NL2ΔA on the cell surface (figure 4-1), which means that Bai1 can bind to both Ig-NL1ΔB and Ig-NL2ΔA extracellularly. Bai1 is the only other protein we found potentially bound to neuroligin extracellular region. It is interesting to know how the BAIs play the role in the physiology of neuroligins.



(Antony Boucard)

Figure 4-1 NL1 and NL2 Bind to Bai1 in the Surface Labeling Assay.
HEK-293T cells were transfected with flag- and mVenus- tagged Bai1. Ig- NL1ΔB or NL2ΔA were incubated with the cells and immunostained with IgG antibody to visualize in the red channel.

BAIs do not Affect the Postsynaptic Inhibitory Responses

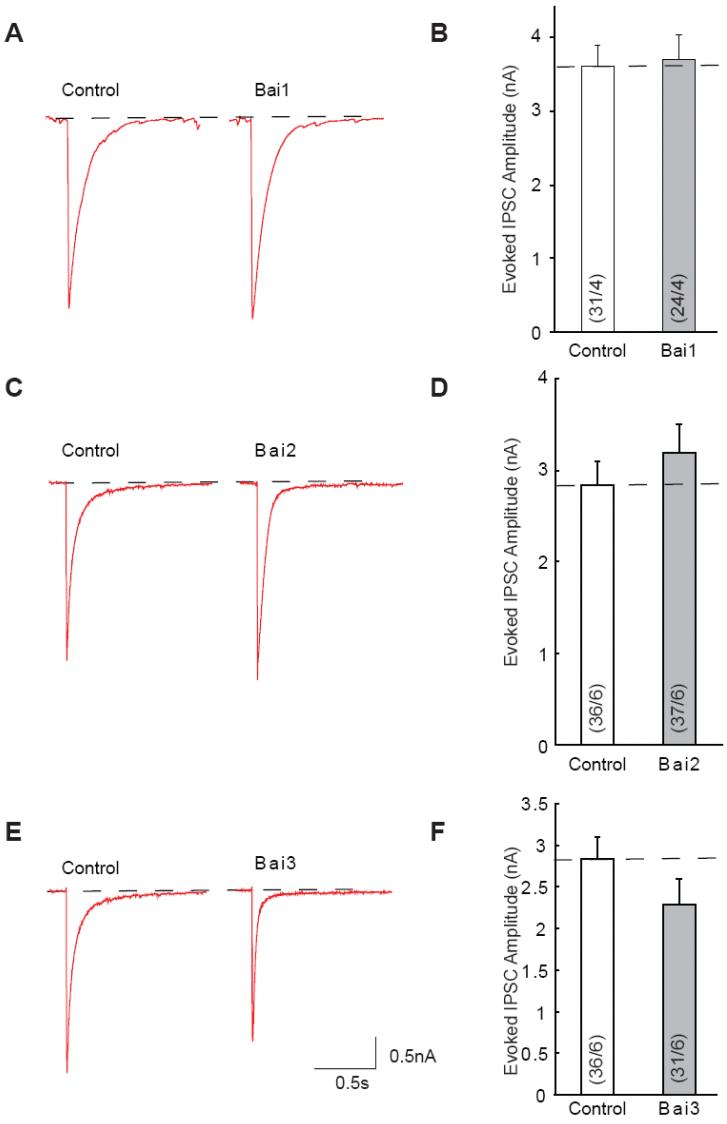


Figure 4-2 Bai1, 2, and 3 Over-expressions do not Change the Evoked IPSCs. (A) Representative traces of the evoked IPSCs from the Bai1 or the control vector over-expressing neurons. (B) The average amplitude of eIPSCs from the Bai1 or the control vector over-expressing neurons. (C) Representative traces of the Bai2 or the control vector over-expressing neurons. (D) The average amplitude of eIPSCs from the Bai2 or the control vector over-expressing neurons. (E) Representative traces of the Bai3 or the control vector over-expressing neurons. (F) The average amplitude of eIPSCs from the Bai3 or the control vector over-expressing neurons.

Different neuroligin affects different types of synapses. NL1 affects the excitatory synapses, whereas NL2 affects the inhibitory synapses. The binding of Bai1 to NL1 and NL2 were similar in the surface labeling assay (figure 4-1), so we decided to investigate both excitatory and inhibitory synaptic properties. We used the transfection system to observe any postsynaptic effect from the BAIs over-expression in the neurons.

First, we looked at the inhibitory responses from the Bai1, 2, and 3 over-expressing neurons. eIPSCs from the transfected neurons of both Bais or the control vector were recorded and analyzed. There is no significant difference in between Bai1, 2, or 3 comparing to the control vector. It suggested that the BAI proteins do not have significant effects on the evoked inhibitory responses postsynapticly.

Bai1 Have Prolonged Bursts in Spontaneous EPSCs

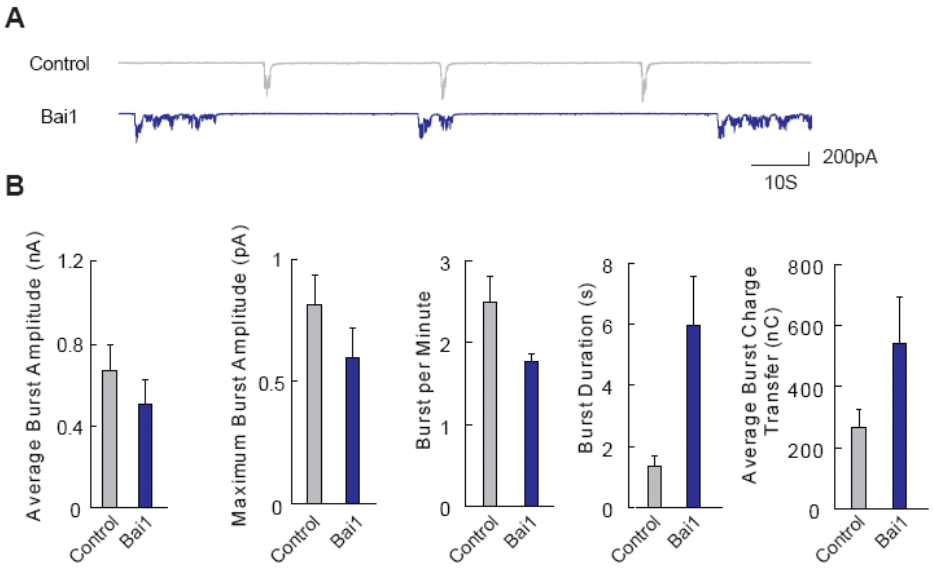


Figure 4-3 The Spontaneous Excitatory Responses from the Bai1 Over-expressing Neurons.
(A) Representative traces of the spontaneous EPSCs from the neurons over-expressing Bai1 or the control vector. (B) Quantitation of the average burst amplitude, the maximum burst amplitude, the burst frequency (burst per minute), the burst duration and the average burst charge from the neurons over-expressing NL3, Bai1, or the control vector.

In order to observe the excitatory effects from Bai1, we transfected Bai1 into the neurons and recorded the spontaneous EPSCs. In the spontaneous EPSCs, there are bursts that represent the spontaneous excitatory activity. We quantify the average and maximum burst amplitude, the burst frequency and duration, and the average burst charge from the neurons over-expressing Bai1 or the control vector. We found there is a trend of decrease in the average and maximum burst amplitude and the burst frequency, which suggested a change in the synaptic organizations. More dramatically, the bursts from Bai1 over-expressing neurons were significantly prolonged compared to the control vector (figure 4-3). It

suggested that the Bai1 molecule may act as an effecter of delayed responses, such as extending the postsynaptic receptor opening time. It is interesting to know the trigger of the prolonged burst spans.

Bai1 Decreased the Evoked Excitatory Responses

Because of the decrease trend in the spontaneous response amplitude and frequency, we went on to measure the evoked excitatory responses. The eEPSCs from the Bai1 transfected neurons showed much decreased amplitude compared to the control vector transfected neurons (figure 4-4). Along with the observation that Bai1 over-expression prolonged the burst duration, it is possible that Bai1 prolongs the excitatory responses, and asynchronizes the responses. However, it is too early to draw the conclusion. It is still unclear how BAIs work in the synapses.

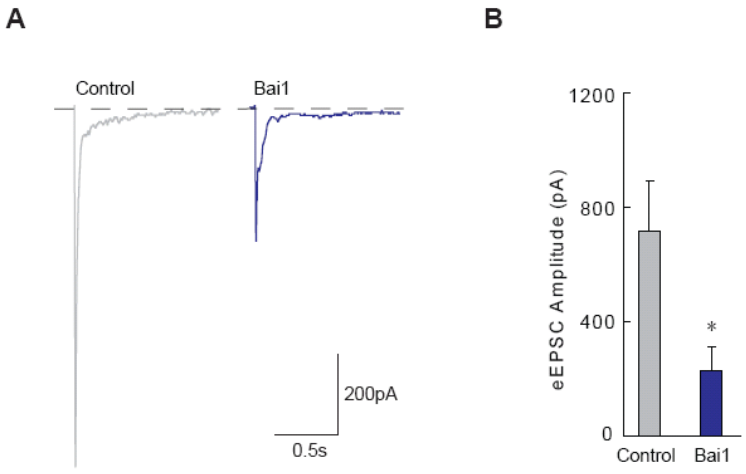


Figure 4-4 The Evoked EPSCs from the Bai1 Over-expressing Neurons. (A) Representative traces from the neurons transfected with Bai1 or the control vector. (B) The average amplitude of eEPSCs from the neurons transfected with Bai1 or the control vector.

Conclusion and Discussion

Here we did preliminary studies on the function of BAIs, a family of G-protein-coupled receptors. We found that the BAIs bound to the neuroligin extracellular region, so they are potential ligands of neuroligins besides neurexins.

The fundamental question of BAIs localization on the synapse is not answered due to the lack of antibody. They can be presynaptic or postsynaptic interacting with neuroligins *in trans* and *in cis*, respectively (figure 2-5). The surface labeling experiment suggested the *trans* interaction. In the transfection system, which we studied the neuroligin physiological function, we studied the BAIs function, given they are postsynaptic, because the low efficiency of transfection. In this way, we can observe one neuron with most of the postsynaptic compartments (dendrites) over-expressing BAIs from the transfected neuron, but most of the presynaptic compartments (axons) with wild-type expression of all the proteins from the untransfected neuron. However, we need to use infection by lentivirus to introduce the over-expression of BAIs in the axons to study the BAIs function presynapticly.

For the postsynaptic physiological effects from BAIs in neurons, Bai1 showed reduced evoked excitatory responses, reduced spontaneous responses, and prolonged spontaneous burst duration. But BAIs did not change the inhibitory responses. It is suggested that BAIs may play important roles in the excitatory

synapses along with NL1 postsynapticly. As a GPCR, Bai1 may receive signal from NL1 and transduct into the cell, trigger intracellular pathways and affect the synaptic properties. Moreover, BAIs could be presynaptic and may relate to both the inhibitory and excitatory synapses. We still do not know much about the BAIs function. Further experiments, to first determine the synaptic localization and interaction pattern with neuroligins, then investigate the morphological and physiological contributions to the neurons, and dissect the intracellular signaling pathways, are greatly needed to answer the questions.

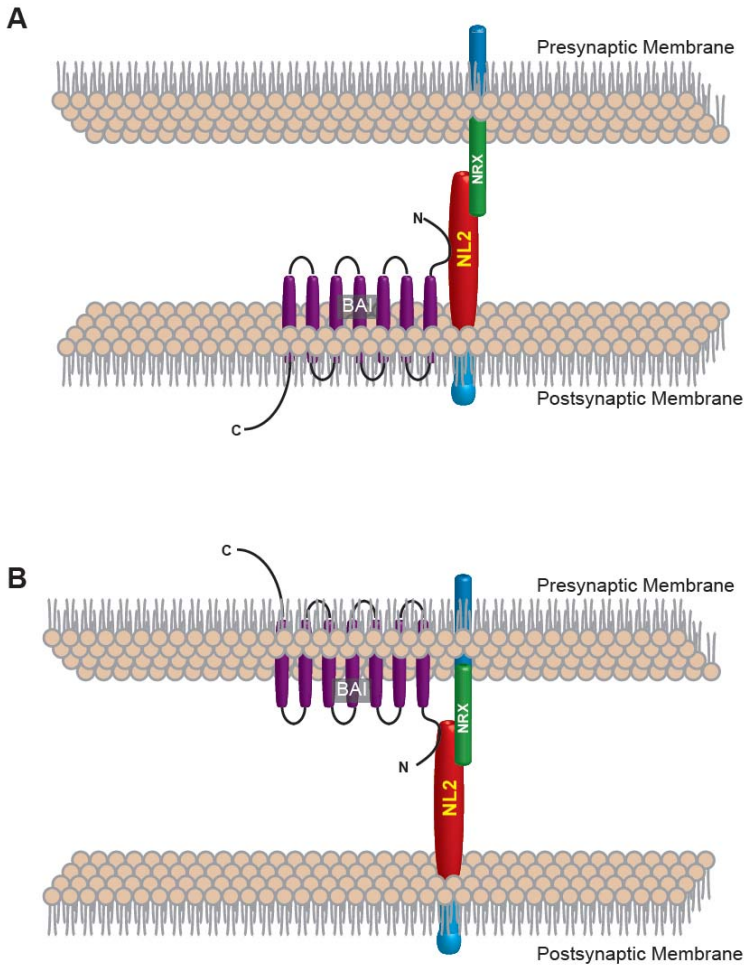


Figure 4-5 Two Models of BAI-NL2 Interaction.
(A) Postsynaptic Localization of BAIs and *cis* interaction with NL2. (B) Presynaptic Localization of BAIs and *trans* interaction with NL2.

Chapter Five: Conclusion and Discussion

From the studies of neuroligins, we found neuroligins are important cell adhesion molecules in both excitatory and inhibitory synapses. Neuroligin cytoplasmic regions may be important anchoring neuroligins in the right location on the postsynaptic membrane by binding to the scaffolding protein PSD95; however, other functions may not be significant, though it may direct the trafficking and recycling of neuroligins. The major function of the neuronal responses and signaling is contributed by the extracellular region of neuroligins, and different isoforms of neuroligins contribute to different type of synapses. NL1 exclusively strengthens the excitatory synapses and NL2 to the inhibitory synapses. According to the structure of NL1 and the complex of NL1 and neurexin1β (N1β), we predicted the binding sites to neurexins in NL2 and made subsequent mutants to abolish the binding and dissociate the complex. Those mutants abolished the electrophysiological functions contributed by NL2, suggested that neurexins are important in mediating the neuronal activity contributed by NL2 at inhibitory synapses. It is still needed to investigate the excitatory synapses to find out whether the phenomena are specific to the inhibitory synapses. Also it is necessary to know whether the localization of neuroligins is changed by the loss of binding to neurexins. We also found the change in electrophysiology may go through the presynaptic changes, so it is

interesting to know how the presynaptic terminal is reorganized upon the neuroligin-neurexin binding. The following are several main questions remained to be answered.

What is the function of neuroligins?

There are observations that neuroligins trigger synapse formation in non-neuronal system, increase the number of synapses, and promote the synaptic transmission, it is not certain what the exact functions of neuroligins in the synapses are. Neuroligins could stabilize the synapses during development in ways of forming the structural stable linkage with neurexins, and/or assembly the presynaptic and/or postsynaptic protein complexes for functional synapses, or employ specific signal pathways to promote the synaptic transmission.

How is the differentiation of synapses related to the neuroligins?

It is very unique that different isoforms of neuroligins contribute to different type of synapses, especially that the sequences between these isoforms are highly homologous and the structures of them are similar. There are several possibilities of the ways that the differentiation of synapses related to neuroligins. First, it is unknown how neuroligins are targeted to the different types of synapses, excitatory or inhibitory. It is possible that there is a default targeting and the other location is a regulated process. It is also possible that there are two distinct

pathways leading to the excitatory and inhibitory synapses. On the other hand, different neuroligin isoforms may have totally different function by employing distinct pathways. And it is unclear whether the synapse differentiation occurs first, and then different neuroligins are transported to the right type of synapses, or neuroligin promote the synapse differentiation. It will be hilarious if we find out the answer.

How are neurexins involved?

We also studied similar neurexin binding mutants in NL1; however, we did not observe any loss of function in electrophysiology from them (Antony Boucard & Chen Zhang, unpublished). It is interesting that similar mutants have distinct effects in NL1 comparing to NL2. It suggests that the effects of the neurexin binding to NL1 and NL2 are different. Here in the NL2 study, we found that the neurexin binding was greatly involved. It is possible that neurexin binding only affects the function of inhibitory synapses. As for the manner of the interaction, it is most likely to be trans-synaptic, which means neuroligins and neurexins are located at the postsynaptic and presynaptic membrane, respectively, and bind to each other *in trans*. However, it is difficult to rule out the possibility that *cis* interaction exists between neuroligins and neurexins and has some different function.

Is there any other binding partner to neuroligin?

Although still uncertain of the binding to neuroligins, BAIs are potential partners of neuroligins, and may be in charge of the signaling. It is possible that other binding partners also exist. The neuroligin isoforms have distinct functions, suggesting that they may employ different signal pathways. *In vivo*, BAIs may interact with a certain isoform of neuroligins to specifically trigger a certain function. To differentiate the two models of BAIs localization in the synapse, we still need to use antibody to determine the synaptic localization from EM.

Chapter Six: Materials and Methods

Neuroligin Constructs

NL1 and NL2 full length without both splice sites constructs express full length NL1 or NL2, with or without mVenus tag at RsrII site in the cytoplasmic region. Neurexin mutantions were made using QuickChange site directed mutagenesis (Stratagene). NL1 and NL2 with the cytoplasmic tail truncation were made by PCR only the eleven amino acids (NL1ΔC) or the eight amino acids (NL2ΔC) after the transmembrane region and then subcloned back into the expression vector; mVenus is tagged after the truncation at the C-terminus. The pCMV5-Ig-neurexin constructs are: pCMV5-IgC; pCMV5-IgN1β, residue 1-299 of rat neurexin 1β without splice site 4; pCMV5-IgN1α, residue 1-1361 of bovine neurexin 1α without splice site 4. pPDGF-EGPF-β-Actin encodes EGFP-tagged full-length β-Actin, and is provided by Y. Goda (University College, London, UK) (Morales et al., 2000).

Antibodies and Western Analysis

SDS-PAGE gels were made as following. For stacking gel, pH6.8, 3% Acrylamide, 0.125mM Tris, 0.1% SDS, 0.1% APS, 1000X TEMED. For separating gel, 6-12% Acrylamide, 0.38mM Tris, pH8.8, 0.1% SDS, 10% glycerol, 0.1% APS, 1000X TEMED. Samples were loaded with high molecular weight

marker (Bio-Red, Prestained SDS-PAGE High Range, catalogue #161-0309) or low molecular weight marker (Bio-Red, Prestained SDS-PAGE Low Range, catalogue #161-0305). Gels were stained with Commassie (50% Methanol, 10% HAc, 0.125% Commassie Blue) or transfer to nitrocellulose membrane, and blotted with primary antibody then secondary antibody in 5% dry milk, 5% goat serum.

GST-protein Expression and Pull down

Desired region of recombinant protein was cloned into pGexKG. Expression vector and pGexKG alone were transformed into BL21 *E. coli* stain. Single colony was picked and inoculated in LB medium for protein expression. 10ml starting culture was shaked overnight and transferred into 1L LB medium, start shaking at 37°C from $O.D._{600}$ 0.2. When $O.D._{600}$ came to 0.6, 50mM IPTG was added to induce protein synthesis for 4-6hrs at 18°C. Bacteria was spinned down at 3,500rpm for 30min, wash with ~100ml PBS and vortex the pellet in 25ml PBS with 5μg/ml leupeptin, 2μg/ml aprotinin, 1mM PMSF, 0.15ml 1M DTT. Mixture was sonicated using 30s brief episodes with 30s interval in between. TritonX-100 was added to the final 1% and mixture was rotated at 4°C for 30min to solublize. Then 40-50μl benzonase was added to mixture and rotated at 4°C for 1-2hrs to get rid of DNA/RNA, especially potential dnaK band on the gel. The mixture was spinned down at 15,000rpm for 30min. 0.6ml 50% glutathion

agarose beads was added to the mixture and rotated at 4°C for 4hr to overnight. Beads were washed with PBS and PBS/1M NaCl alternatively for three times each, and finalize with a wash with PBS. Beads were directly run on the gel in 2XSDS sample buffer, or eluted with glutathione solution.

Cos-7 cells were transfected with NL expression contructs pCMV5-flag-NL1A, pCMV5-flag-NL2, pCMV5-flag-NL3. Three days after transfection, cells were washed once by cold PBS (Sigma, Phosphate Buffered Saline, Catalogue No. P4417), then scraped in 5ml cold PBS with proteinase inhibitors (5μg/ml leupeptin, 2μg/ml aprotinin, 1mM PMSF) to be centrifuged at 3,500rpm for 25min. Pellet was retained as the membrane fraction and solublized in buffer E (20mM Hepes, 100mM NaCl, 2mM CaCl2, 0.1mM EDTA, 1% TritonX-100, pH7.4) at 4°C for 2hrs rotating. Insoluble proteins were removed by centrifugation at 13,000rpm for 30min.

The supernatants were incubated with 100μl GST-protein on beads at 4°C for 2-4hrs. Beads were washed five times with 0.5% TritonX-100, 50mM Hepes, 100mM NaCl, pH7.5 with 5μg/ml leupeptin, 2μg/ml aprotinin, 1mM PMSF. Beads were then dissolved in 2XSDS loading buffer and run on SDS-PAGE gel.

Yeast-two-hybrid Screen and Interaction Study

Yeast-two hybrid bait constructs were made on pLexN vector using cytoplasmic regions of NL2. L40 yeast strain was used for all transformation.

Background β-galactosidase activities of the bait constructs were tested on a series of 3-AT (Sigma, 3-Amino-1,2,4-triazole, catalogue No. 09540) concentration (1mM, 2mM, 3mM, 4mM, 5mM, 8mM, 10mM, 20mM) on selection plates lacking Uracil, Tryptophan, and Leucine (-UTL), and 5mM 3-AT concentration was determined for both NL2 bait constructs. Yeast-two hybrid screens were performed using a rat brain cDNA library in pVP16-3. Large scale transformation mixes were plated on 50-60 150mm selection plates lacking Uracil, Trypotophan, Leucine, Histidine, and Lysine (-THULL). Positive clones were picked four days after incubation at 30°C and inoculated in –THULL medium for 1-2 days at 30°C. Extracted yeast DNA was transformed to HB101 bacteria strain. Purified prey DNA was obtained and co-transformed to yeast, selected on –THULL plates for growth assay, -UTL plates for β-gal assay. Positive clones were sequenced and further selected for GST-pull down.

Biotinylation Assay

HEK-293T cells two days after transfected with NL constructs were washed with cold PBS twice carefully. 1mg/ml Biotin (Pierce, EZ link sulfo-NHS-LC Biotin, catalogue No. 21335) dissolved in cold PBS were incubated with cells for 30min on ice. Reaction was quenched with TBS (150mM NaCl, 50mM Tris pH7.4) by washing 3-4 times for 5min each. Cells were collected in RIPA buffer (65mM Tris pH7.4, 150mM NaCl, 1% triton, 0.1% SDS, 1mM EDTA,

0.5% deoxycholate salt, 50mM NaH_2PO_4, 10mM $Na_4P_2O_7$, 1mM Na_3VO_4, 50mM NaF, 5μg/ml leupeptin, 2μg/ml aprotinin, 1mM PMSF), homogenized with syringe ten times, extracted for 1hr at 4°C. Cell debris was spined down at 14,000rpm for 15min at 4°C. 50μl of 50% Neutravidin Biotin Binding beads (Pierce, catalogue No. 29200) were incubated with the supernatant overnight at 4°C rocking gently. Beads were loaded on the gel after 3-4 times of PBS washes.

Cell Culture and Transfection

HEK-293T and Cos-7 cells were maintained at 37°C and 5%CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% penicillin (100units/ml) and streptomycin (100units/ml). To passage the cell, cells were trypsinized by 0.05% trypsin-EDTA (Gibco, 1X 0.05% Trypsin-EDTA, catalogue No. 25300). Cells were transfected using Fugene 6 (Roche Molecular Biochemicals) using 2-3μl Fugene 6 per 1μg DNA. Usually cells were harvested two days after transfection.

Surface Labeling

To produce Ig-proteins, HEK-293T cells were transfected by Fugene 6 and pCMV5-IgC, pCMV5-Igβ-NRX1, pCMV5-Igα-NRX1. Then medium was harvested in four days. Medium were centrifuged at 3,200rpm for 5min at 4°C to remove the cell debris. Supernatant were transferred and supplemented 10mM

Hepes pH8.0, 1mM EDTA pH8.0, 5μg/ml leupeptin, 2μg/ml aprotinin, 1mM PMSF as final concentration. 20-50μl PBS-pre-washed slurry 50% protein A sepharose was incubated by rotating overnight at 4°C in every 50ml medium. Beads were washed three times by centrifugation at 800g for 3min at 4°C, then eluted with 0.1M glycine pH2.2 by incubate for 5min at room temperature. Supernatant which contained the Ig-protein was obtained by quick centrifugation through the column at 13,000rpm for a few seconds. Immediately the flow through supernatant was neutralized by add 15μl 1M Tris-HCL pH 9.0 per 100μl volume. Ig-protein concentration was evaluated on SDS-PAGE gel through commassie staining by comparing to 2μg, 5μg, and 10μg BSA bands on the same gel.

To surface label the cells transfected with NL contructs, HEK-293T cells were replated onto poly-D-lysine coated coverslips in 12-well plates one day after transfection, and 48hr after transfection medium was depleted and 3μg IgC or Igβ-NRX1, or 10mg Igα-NRX1 per well were added onto the cells in DMEM with phenol red, 20mM Hepes-NaOH pH7.4, 0.1%BSA. After shaking very gently at 4°C for 16hr, cells were washed three times with cold DMEM to remove the excess Ig-protein. Cells were fixed by 4% paraformaldehyde (PFA) for 10min on ice and washed three times by PBS. Then cells were blocked in PBS with 3% milk. Two hours prolonged incubation with rabbit-anti-human IgG antibody

(1:500) was followed by the 1hr incubation with 2nd Alexa fluorescent goat anti-rabbit 546nm antibody (1:500) (Invitrogen, Alexa Fluor 546 goat anti-rabbit IgG (H+L), catalogue No. A11035). Coverslips were mounted on slides and image was acquired by Zeiss inverted two-photon microscope. Pinhole was set low about 85μm. Adjust the 546nm acquisition so that it is just no to see any signal in the IgC incubated condition. Adjust the 488nm acquisition so that there is no cross-talk can be observed from the red channel. Acquisition parameters were not changed after the first image. Z-series images were taken and projected maximally.

Primary Hippocampal Neurons Cultured at High Density

Mouse or rat hippocampus were dissected from P1 pups, followed by papain (Worthingon, catalogue No. LS003126) digestion, and then plated on poly-D-lysine-coated glass coverslips. Neurons were maintained *in vitro* for 14-18 days in MEM (Gibco, minimum essential medium, catalogue No. 51200-038) supplemented with B27 (Gibco), glucose, transferring, fetal bovine serum, and Ara-C (Sigma).

Neuronal Transfection by Calcium Phosphate

Neurons maintained at 8-10 days *in vitro* were transfected by desired DNA. 4μg DNA, 2μl 2M $CaCl_2$, and H_2O to make up the volume to 15μl/well

were mixed in a polystyrene tube, then was slowly and gently dropped to another tube with flicking which has 15μl 2HBS (274mM NaCl, 10mM KCl, 1.4mM Na_2HPO_4, 15mM glucose, 42mM HEPES, adjust pH to 7.05 with NaOH, filter, aliquot, and store at -20°C). Neurons were washed with DMEM (Invitrogen, DMEM (1X), catalogue No. 31053-036) 500μl each well once, and DNA/2HBS mix was slowly dropped to the wells with 500μl DMEM in each well. After incubation for 25min at 37°C, neurons were washed three times with DMEM and then original medium was put back to each well.

Immunocytochemistry and Confocal Image Acquisition

Transfected hippocampal neurons were fixed in methanol for 10min at -20°C 14-16 days *in vitro*. Fixation was quenched by 10mM Glycine in PBS for 10min at room temperature. Neurons were blocked in PBS with 3% milk, 0.1% saponin. vGLUT1 (Millipore, anti-vesicular glutamate transporter 1 guinea pig antibody, catalogue No. AB5905), vGAT (Synaptic System, anti-vesicular GABA transporter rabbit polyclonal, catalogue No. 131003), PSD-95 (Affinity BioReagents, anti-PSD-95 antibody mouse, catalogue No. MA1-045), or Synapsin (homemade antibody from rabbit, E028) was used to label desired synaptic regions for 2hr at room temperature separated from light. Subsequent secondary fluorescent antibodies were incubated for 30min at room temperature

separated from light. Coverslips were mounting on slides and proceeded to image acquisition.

Images were acquired using inverted Zeiss two-photon microscope. For the first image, fluorescence and other parameters were adjusted so that the signals fell into the linear range. Fluorescence gain from 488nm was adjusted according to the different expression levels of the transfected neuron to avoid over-saturating problem, while 546nm and 633nm acquisition parameters remained unchanged all through the acquisition of the whole culture. Imaging analysis was done by Image J, using a universal threshold through all the images from one channel and quantifys the size and densities by using the function of analyze particles.

Cultured Neuron Electrophysiology

Synaptic responses were triggered by 1ms current injection (100μA) through a local extracellular electrode (FHC concentric bipolar electrode, Catalogue No. CBAEC75) with a Model 2100 Isolated Pulse Stimulator (A-M System, Inc.) 100-150μm from the soma of the recorded neuron, and recorded in a whole-cell mode using a Multiclamp 700A amplifier (Axon Instruments, Inc.). Data was digitized at 10kHz with a 2kHz low-pass filter. Intracellular whole-cell patch solution contained 135mM CsCl, 10mM Hepes, 1mM EGTA, 4mM Mg-ATP, 0.4mM Na-GTP, 10mM QX-314, pH7.4. Extracellular bath solution

contained 140mM NaCl, 5mM KCl, 2mM $CaCl_2$, 0.8mM $MgCl_2$, 10mM Hepes, and 10mM glucose, pH7.4. Inhibitory responses were isolated pharmacologically by adding 50μM D-AP5 and 20μM CNQX to the bath solution; excitatory responses were isolated pharmacologically by adding 100μM picrotoxin. Miniature responses were further isolated by 1μm tetrodotoxin (TTX). Giga seal was achieved for each patch, and access resistance was kept below 15MΩ, in some cells by compensating 10-80%. Data was analyzed using Clampfit 9.02 (Axon Instruments, Inc.). Synaptic responses were recorded one minute after the patch was set up. For evoked responses, 5-10 sweeps were recorded from each cell; for miniature response, 3min was recorded from each cell.

Statistical Analysis

All data were presented as mean±SEMs. Unpaired or paired student *t*-test was used to evaluate the significance.

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