THE FUNCTION OF NEUROLIGIN-2
IN NEURONAL DEVELOPMENT
AND NEUROPSYCHIATRIC DISORDERS

A Dissertation in
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by
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ABSTRACT

A delicate balance between excitation and inhibition is crucial for brain functions, and the disturbance of this balance is an emerging hypothesis underlying many neuropsychiatric disorders. Neuroligins are a family of cell adhesion molecules found at the postsynaptic sites of both excitatory glutamatergic and inhibitory GABAergic synapses. The trans-synaptic interactions between neuroligins and their presynaptic receptor neurexins regulate synapse formation and function. Accumulating genetic studies have implicated neuroligin mutations in neuropsychiatric disorders, such as autism spectrum disorders. However, the functional relevance of neuroligins and the molecular mechanisms of their contribution to disease onset are largely unknown.

First, I investigated the functional defects of novel neuroligin-2 mutations linked to schizophrenia. Among neuroligins, neuroligin-2 is selectively localized at GABAergic synapses and is critical for regulating inhibitory synaptic transmission. Since GABAergic deficits have been implicated in schizophrenia by both human postmortem and genetic studies, we hypothesized that neuroligin-2 is a potential risk gene for schizophrenia. In a cohort of 584 schizophrenia patients, we identified novel neuroligin-2 missense point mutations. Among them, I identified R215H as a loss-of-function mutation applying a heterologous GABAergic synapse induction assay. The R215H mutant was defective in mediating cell adhesion and in promoting GABAergic synapse formation. Mechanistically, the R215H mutant showed significantly reduced cell surface expression possibly due to incomplete glycosylation. My work suggests that defect in GABAergic synapse formation may be a potential risk factor for schizophrenia.
In the second part of my thesis, I reported a novel function of neuroligin-2 in regulating GABA functional switch from excitation to inhibition through KCC2. KCC2 is a neuron specific potassium-chloride co-transporter that exports chloride. The developmental up-regulation of KCC2 mediates GABA functional switch by decreasing intracellular chloride concentration. Surprisingly, KCC2 expression was significantly reduced after knockdown of neuroligin-2 by shRNA-mediated RNA interference. As functional consequences of decreased KCC2, knockdown of neuroligin-2 abolished GABA functional switch in developing neurons and reversed GABA action to excitatory in mature neurons. Overexpression of shRNA proof neuroligin-2, but not neuroligin-1, rescued both decreased KCC2 expression and delayed GABA functional switch induced by shRNAs. Using gramicidin-perforated patch clamp recordings, I further demonstrated that neuroligin-2 expression level directly regulates GABA equilibrium potential. It has been reported that knockdown of neuroligin-2 decreased the number of both GABAergic and glutamatergic synapses, but the mechanism was unknown. I showed that KCC2 overexpression rescued glutamatergic synapse loss induced by knockdown of neuroligin-2, suggesting that neuroligin-2 regulates glutamatergic synapses through KCC2.

In summary, my findings uncovered a new function of neuroligin-2 in regulating GABA functional switch and glutamatergic synapse formation. Therefore, in addition to its conventional role of cell adhesion at GABAergic synapses, neuroligin-2 may serve as a master regulator in balancing excitation and inhibition in the brain. Dysfunctions of neuroligin-2 may cause excitation/inhibition imbalance and contribute to the etiology of neuropsychiatric disorders, as indicated by the case of neuroligin-2 R215H mutant in schizophrenia.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>a.u.</td>
<td>arbitrary unit</td>
</tr>
<tr>
<td>ASD</td>
<td>autism spectrum disorders</td>
</tr>
<tr>
<td>BIC</td>
<td>bicuculline</td>
</tr>
<tr>
<td>DIV</td>
<td>days in vitro</td>
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<tr>
<td>DNQX</td>
<td>6,7-dinitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>$E_{\text{GABA}}$</td>
<td>GABA reversal/equilibrium potential</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GABA$_A$R</td>
<td>GABA$_A$ receptor</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamate</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>KCC2</td>
<td>potassium-chloride cotransporter 2</td>
</tr>
<tr>
<td>mEPSCs</td>
<td>miniature excitatory postsynaptic currents</td>
</tr>
<tr>
<td>mIPSCs</td>
<td>miniature inhibitory postsynaptic currents</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>n.s.</td>
<td>not significant</td>
</tr>
<tr>
<td>NKCC1</td>
<td>sodium-potassium-chloride cotransporter 2</td>
</tr>
<tr>
<td>NL, NLGN</td>
<td>neuroligin</td>
</tr>
<tr>
<td>NLmiR</td>
<td>shRNA targeting neurolgin1, 2, and 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>-------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>nonTF</td>
<td>non-transfected</td>
</tr>
<tr>
<td>NRXN</td>
<td>neurexin</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin RNA</td>
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<tr>
<td>TF</td>
<td>transfection</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>vGlut1</td>
<td>vesicular glutamate transporter 1</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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Chapter 1

Introduction
Human brain is mainly composed of neurons and glial cells. Neurons are remarkable in that they form extensive connections, termed synapses, with each other and function through network activities. There are billions of neurons in a human brain and each neuron forms thousands of synapses with others. There are two types of synapses: electrical synapse and chemical synapse. While electrical synapses couple the activity of connected neurons by gap junctions, chemical synapses utilize neurotransmitters to conduct directional information from presynaptic to postsynaptic cell. In the adult mammalian central nervous system, glutamate and GABA (γ-aminobutyric acid) are the two major types of neurotransmitters that mediate excitatory and inhibitory neurotransmissions, respectively. Glutamatergic neurons release glutamate from their axon terminals and depolarize postsynaptic neurons; GABAergic neurons release GABA and hyperpolarize neurons. The changes in membrane potential of neurons largely determine the probability of firing action potentials and regulate the overall neural network activity. The modulations in both the number and the strength of synapses have long been proposed as the cellular and molecular basis of brain function, which defects are also implicated in brain disorders.
1.1 Neurexin and neuroligin

Neurexin is originally discovered as a receptor for α-latrotoxin (Ushkaryov et al., 1992), a major toxin found in the venom of black widow spider that evokes massive neurotransmitter release. Not surprisingly, neurexins play a role in coupling calcium channel activities and synaptic vesicle release at axon terminals (Missler et al., 2003). Neuroligins are later discovered as endogenous ligands for neurexins at the postsynaptic sites (Ichtchenko et al., 1995). The expressions of both neurexins and neuroligins are highly restricted in neurons with neurexins mostly on presynaptic axon terminals and neuroligins on postsynaptic membranes. The trans-synaptic interactions between neurexin and neuroligin stabilize synapses and regulate synaptic transmissions (Sudhof, 2008).

There are four neuroligin-encoding genes in rodents, NLGN1/2/3/4 with NLGN-3/4 genes located on the X chromosome. In human, an additional NLGN-5 gene is located on the Y chromosome. Neuroligins are highly conserved from fly to human (Bolliger et al., 2008). In mice, neuroligin-1/2/3 are all up-regulated during early postnatal development and are broadly expressed across the brain, while neuroligin-4 is expressed at very low level and only accounts for 3% of total neuroligin in the adult mice (Varoqueaux et al., 2006). Subcellularly, neuroligin-1 and neuroligin-2 are specifically localized at glutamateergic and GABAergic synapses, respectively (Song et al., 1999; Varoqueaux et al., 2004); neuroligin-3 is present at both types of synapses (Budreck and Scheiffele, 2007).
1.1.1 The structure of neuroligins

Neuroligins are type I transmembrane proteins with a large extracellular domain, a single-pass transmembrane domain, and a short intracellular carboxyl-terminal (Fig. 1-1). The extracellular domain of neuroligin is homologous to acetylcholinesterases but without catalytic activity. It is glycosylated at multiple sites (Hoffman et al., 2004), which accounts for the ~21 kDa size difference between mature and immature form of neuroligins in immunoblot (Comoletti et al., 2003). Alternative splicing of neuroligins involves one insertion A in the esterase-homology domain (an additional insertion B for neuroligin-1), which may contribute to their differential binding affinities to neurexins. It has been proposed that selective binding between different neurexin and neuroligin isoforms may encode the formation of different types of synapses (Sudhof, 2008).

The crystal structures of neurexin-neuroligin complex have been resolved (Arac et al., 2007; Fabrichny et al., 2007). Neuroligins form constitutive dimers in cis through their extracellular domains. Most neuroligin dimers are homodimers, and neuroligin1/3 dimer is the only abundant heterodimer (Poulopoulos et al., 2012). Each neuroligin then binds with one neurexin in trans, forming a trans-synaptic heterotetramer complex. Dimerization of neuroligins has been shown to be critical for clustering presynaptic neurexins and for promoting synapse assembly (Dean et al., 2003). A recent study utilizing chemical inducible neuroligin mutant further support this: while the overexpression of wild type neuroligin-3 increased excitatory synaptic currents, the overexpression of a dimerization-null neuroligin-3 mutant (with inducible dimerization
domain) only increased synaptic currents after the induction of dimerization (Shipman and Nicoll, 2012b).


Figure 1-1. Diagram showing synaptic proteins at glutamatergic synapses.

**a.** Illustration of a glutamatergic synapse with presynaptic axonal terminal and postsynaptic dendritic spine structure. **b.** The trans-synaptic neurexins-neuroligin complex. Each neurexin (NRXN) gene has two major splicing isoforms, a large α-NRXN and a short β-NRXN, and many more alternative splicing sites. L, LNS (laminin, NRXN, sex-hormone-binding globulin) domain. E, epidermal growth factor (EGF)-like domain. Neuroligin (NLGN) has one large esterase-homology domain. Glycosylation of both NRXN and NLGN at carbohydrate attached sequence (CHO) or juxtamembrane stalk region are shown as curved green lines.
The intracellular domains of all neuroligins contain a PDZ-binding motif and a gephyrin-binding motif that mediate their interactions with postsynaptic scaffolding protein PSD-95 (Irie et al., 1997) and gephyrin (Poulopoulos et al., 2009), respectively. A new critical cytoplasmic region without known binding partner has been found in all neuroligins and is required for enhancing excitatory synaptic transmission (Shipman et al., 2011).

Two groups lately reported that neuroligin-1 undergoes activity-dependent cleavage at the juxtamembrane stalk region, which results in the loss of extracellular neurexin-interacting domain and the disassembly of glutamatergic synapses (Peixoto et al., 2012; Suzuki et al., 2012). The two groups identified different enzymes that cleave neuroligin-1, MMP9 (matrix metalloproteinases 9) and ADAM10 (a disintegrin and metalloproteinases 10). ADAM10 is likely more responsible for baseline cleavage level, since ADAM10, but not MMP9, knockout mice showed significant reduction in neuroligin-1 cleavage. While the dynamic cleavage of neuroligin-1 may present a new mechanism in regulating synaptic function, the cleavage of other neuroligins and their functional significance especially on GABAergic synapses are unknown.

1.1.2 The function of neuroligins as cell adhesion molecules

The study of neuroligin and other synaptic cell adhesion molecules was greatly advanced by a seminal work by Scheiffele et al. (Scheiffele et al., 2000): when they co-cultured neuroligin-expressing HEK 293 cells with neurons, extensive axonal
innervations were observed on HEK cells by both immunocytochemistry and electron microscopy. Interestingly, addition of soluble neurexins inhibited the innervations to HEK cells, suggesting that the synaptogenic activity of neuroligins is mediated by their interaction with neurexins on axon terminals. Conversely, following studies showed that neurexin expression in non-neuronal cells is sufficient to induce postsynaptic differentiations (Graf et al., 2004; Nam and Chen, 2005). This kind of heterologous synapse induction assay directly measures the synaptogenic activity of a molecule and has since been broadly applied for studying synaptic cell adhesion molecules and screening for novel proteins or small molecules that promotes synaptogenesis (Linhoff et al., 2009; Shi et al., 2011). By introducing glutamate receptors or GABA receptors together with neuroligins, synaptic transmissions were recorded in non-neuronal cells, indicating the formation of well functional heterologous synapses (Fu et al., 2003; Dong et al., 2007; Wu et al., 2012).

Direct manipulations of neuroligin expression level in cultured neurons by neuronal transfection further demonstrated that neuroligins regulate synaptogenesis (Chih et al., 2004; Graf et al., 2004; Levinson et al., 2005). While neuroligin overexpression increased synapse number, knockdown of neuroligins by RNA interference decreased synapse number. However, the central role of neuroligins in synaptogenesis was challenged by studies in neuroligin knockout mice. Without the three highly expressed neuroligins, neuroligin 1-3, synapses are formed with normal density and ultrastructure as revealed by electron microscopy (Varoqueaux et al., 2006). Nonetheless, NLGN/1/2/3 triple knockout mice die at birth and show significantly decreased synaptic transmissions. Mice with single knockout of NLGN1 or NLGN2 also show relatively normal number of
functional synapse but decreased glutamatergic or GABAergic transmissions, respectively (Chubykin et al., 2007; Blundell et al., 2009; Blundell et al., 2010). This lead to the hypothesis that neuroligins are not required for initial synapse formation but rather required for synaptic maturation.

The absence of changes in synapse number in NLGN knockout mice cannot simply be due to the different conditions between in vitro and in vivo experiments. Transgenic mice overexpressing neuroligin-2 showed specifically increased number of inhibitory synapse in vivo (Hines et al., 2008). A recent study applying in utero electroporation and viral infection approaches clearly demonstrated that altering neuroligin-1 expression in a subset of neurons affect synapse formation in vivo (Kwon et al., 2012). Rather than the absolute neuroligin-1 amount, the transcellular difference in the relative amount of neuroligin-1 was shown to be crucial in regulating excitatory synapse formation in vivo. Conditional knockout of neuroligins in a subtype of neurons will further test this hypothesis. Besides neurexin and neuroligin, there is an increasing list of cell adhesion molecules present at synapses, especially at glutamatergic synapses (Dalva et al., 2007). Compensation from other cell adhesion molecules may also complicate the interpretation of neuroligin manipulations and phenotypes in synaptogenesis.

Besides regulating synapse formation, the trans-synaptic neurexin-neuroligin complex has also been shown to mediate retrograde signal in coordinating presynaptic vesicle release probability and postsynaptic maturation (Futai et al., 2007; Hu et al., 2012). It appears that the extracellular domain of neuroligin is sufficient for inducing presynaptic changes, while the intracellular domain is sufficient for postsynaptic changes
(Wittenmayer et al., 2009). A recent study showed that insertion B-containing neuroligin-1, but not insertion B-lacking neuroligin-3, is required for long-term potentiation specifically in the adult dentate gyrus (Shipman and Nicoll, 2012a). In summary, neuroligins play integral roles in synapse function, from formation to plasticity.

1.1.3 Neuroligin-2 is localized at GABAergic synapses

Among neuroligins, only neuroligin-2 is selectively localized at GABAergic synapses (Graf et al., 2004; Varoqueaux et al., 2004; Hoon et al., 2009). Phylogenetically, neuroligin-2 is also most distant from all other neuroligins (Bolliger et al., 2008). Functionally, neuroligin-2 is highly specific to GABAergic synapses. Knockout of neuroligin-2, but not neuroligin-1 or 3, decreased GABAergic transmissions in vivo (Chubykin et al., 2007; Tabuchi et al., 2007). Transgenic mice overexpressing neuroligin-2 also showed enhanced GABAergic transmission (Hines et al., 2008). In addition, when expressed in non-neuronal cells, neuroligin-2 also displays much stronger potency than neuroligin-1 in inducing heterologous GABAergic synapse formation and in aggregating GABA_A_R (Dong et al., 2007). Triple knockout of NLGN1/2/3 results in more severe defects in GABAergic than glutamatergic transmission (Varoqueaux et al., 2006), suggesting an indispensable role of neuroligin-2 at GABAergic synapses.

While all neuroligins shares PDZ and gephyrin-binding domains and showed some functional overlaps (Chih et al., 2005), there are two motifs that only present in neuroligin-2, which may underlie its functional specificity to GABAergic synapses. First, there is a collybistin-binding site only present in neuroligin-2, which is necessary for
activating collybistin and delivering gephyrin to postsynaptic sites (Poulopoulos et al., 2009). Second, MAM domain containing glycosylphosphatidylinositol anchor proteins (MDGA) have recently been discovered as a novel negative regulator of neuroligin-2 function by two independent groups (Lee et al., 2012; Pettem et al., 2013). MDGA proteins functionally inhibit neuroligin-2, but not neuroligin-1 or 3, possibly by competitively binding neuroligin-2 in cis and preventing neurexin-neuroligin interaction in trans.

The subcellular trafficking and clustering mechanisms of neuroligin-2 to GABAergic synapses are not well understood. The neuroligin-2 intracellular domain 716-782, which includes the gephyrin-binding domain, has been shown to be necessary for the targeting of neuroligin-2 to GABAergic synapses (Levinson et al., 2009). Gephyrin and PSD-95 have been suggested to competitively interact with neuroligin-2 and drive it to glutamatergic or GABAergic synapses, respectively (Levinson et al., 2005; Levinson et al., 2009). When overexpressed, neuroligin-2 may also present at glutamatergic synapses and increase glutamatergic synapse formation through interacting with PSD-95 (Chih et al., 2005; Levinson et al., 2005). Therefore, it is possible that interactions with postsynaptic scaffolding proteins may determine the location of neuroligin-2 on membrane surface.

However, endogenous neuroligin-2 is still correctly targeted to and clustered at GABAergic innervation sites after knockout of GABA_{A}R (Patrizi et al., 2008), gephyrin (O'Sullivan et al., 2009), or collybistin (Poulopoulos et al., 2009). Conversely, deletion of neuroligin disrupts GABA_{A}R clusters (Hoon et al., 2009) and perisomatic gephyrin puncta in hippocampus (Poulopoulos et al., 2009). Therefore, neuroligin-2 is likely
delivered to cell surface independently of other proteins and serve as an initiator of GABAergic synapse formation, wherever it starts clustering and interacting with GABAergic axonal terminals. The reciprocal interactions between neuroligin-2 and gephyrin or GABAARs may later further stabilize neuroligin-2 at GABAergic synapses.
1.2 The GABAergic transmission

1.2.1 The structure of GABA<sub>A</sub> receptors

There are two types of GABA receptors: type A (GABA<sub>A</sub>R) and type B (GABA<sub>B</sub>R). GABA<sub>A</sub>Rs are ligand-gated chloride channels and mediate the fast action of GABA; GABA<sub>B</sub>Rs are G protein-coupled receptors and slowly hyperpolarize neurons by opening potassium channels. Functional ionotropic GABA<sub>A</sub>Rs are composed of 5 subunits. Although the large repertoire of 19 different GABA<sub>A</sub>R subunits could form hundreds of GABA<sub>A</sub>Rs with distinct channel and pharmacological properties, there are far less GABA<sub>A</sub>R subtypes present in the brain. The stoichiometry of GABA<sub>A</sub>R might be restricted by two mechanisms. First, the expressions of GABA<sub>A</sub>R subunits are regulated both spatially and temporally in the brain, which limits the available pool of subunits to assemble. For example, the α subunit is developmentally switched from α3 to α1 in the cerebellum (Ortinski et al., 2004). Second, preferential interactions between different subunits limit the pool of assembled pentamers (Kittler et al., 2002).

GABAergic transmission inhibits neurons through activating both synaptic and extrasynaptic GABA<sub>A</sub>Rs, which mediate fast phasic responses and slow tonic responses of GABA, respectively (Farrant and Nusser, 2005). The subunit composition of GABA<sub>A</sub>Rs is crucial in determining their transport to synaptic versus extrasynaptic sites. The prevalent stoichiometry of synaptic GABA<sub>A</sub>R is 2α: 2β: γ, and the inclusion of γ2 subunit is essential for synaptic clustering of GABA<sub>A</sub>Rs (Essrich et al., 1998). Recent
work from our laboratory demonstrated that α subunits also contribute to the synaptic versus extrasynaptic targeting of GABA\(_A\)Rs (Wu et al., 2012).

1.2.2 GABAergic synapses

Glutamatergic and GABAergic synapses are the two major types of chemical synapses in the central nervous system. Besides utilizing different neurotransmitters and receptors, glutamatergic and GABAergic synapses are distinct in many ways. For instance, unlike glutamatergic synapses that are normally formed on dendritic spines, GABAergic synapses are mostly found on somata, dendritic shafts, and axons of neurons. Under electron microscopy, glutamatergic synapses show strong postsynaptic densities and are asymmetric, while GABAergic synapses are ultramorphologically symmetric.

There are several proteins specifically present at most GABAergic synapses, including neuroligin-2 as the major cell adhesion molecule (Fig. 1-2). At presynaptic terminals, glutamic acid decarboxylase 65 (GAD65) is the enzyme that synthesize GABA from glutamate. Vesicular GABA transporter (VGAT) functionally couples with GAD65 on the membrane of synaptic vesicles and load GABA into synaptic vesicles. At postsynaptic site, gephyrins form a hexagonal lattice and stabilize other postsynaptic proteins (Fritschy et al., 2008). Gephyrins directly interact with GABA\(_A\)R α2 subunit (Tretter et al., 2008) and neuroligin-2 (Poulopoulos et al., 2009). The trans-synaptic complex formed between neurexins, neuroligin-2, gephyrin, and GABA\(_A\)R are likely the core components of GABAergic synapses. Their reciprocal interactions may stabilize GABAergic synapses. Supporting this idea, mere addition of neuroligin-2 and GABA\(_A\)R
in HEK cells is sufficient to construct well functional GABAergic synapses between HEK cell and neurons with properties surprisingly similar to conventional synapses and even with short-term plasticity (Dong et al., 2007; Wu et al., 2012).

Figure 1-2. Diagram showing the key components of GABAergic synapses.

VGAT and GAD65 load synaptic vesicles with GABA at presynaptic site. Synaptic GABA$_A$Rs are clustered by interactions with gephyrin and neuroligin-2. Presynaptic neurexin interacts with postsynaptic neuroligin-2. In mature neurons, chloride ions go into neurons through GABA$_A$R upon activation and hyperpolarize neurons.

Unexpectedly, neurexin has been shown to directly interact with GABA$_A$R and decreases the strength of GABAergic transmission (Zhang et al., 2010). However, the underlying mechanism is unclear, and it is unclear whether this interaction is in cis or in trans. Because GABA$_A$R expression alone in HEK cells was incapable of inducing
GABAergic innervations (Dong et al., 2007) and manipulation of neurexins did not change the number of GABAergic synapses (Zhang et al., 2010), the possible interaction between neurexin and GABA\textsubscript{A}R in trans is not synaptogenic. Besides neuroligin-2, dystroglycan is another cell adhesion molecule that is selectively associated with GABAergic synapses and is also a ligand for neurexins (Sugita et al., 2001; Levi et al., 2002). Neuroligin-2 and dystroglycan may both interact with a large scaffolding protein, S-SCAM, and stabilize GABAergic synapses (Sumita et al., 2007). However, the essential function of dystroglycan and S-SCAM for GABAergic synapses has not been demonstrated.

1.2.3 Developmental GABA functional switch

As the primary inhibitory neurotransmitter in the adult brain, GABA depolarizes immature neurons and even evokes action potentials (Ben-Ari, 2002). Using perforated-patch recordings that keep intracellular Cl\textsuperscript{-} intact, Chen et al. revealed a gradual negative shift of GABA reversal potential (E\textsubscript{GABA}) in the first few postnatal weeks (Chen et al., 1996). Young neurons have a more positive E\textsubscript{GABA} than resting membrane potential, and therefore GABA is depolarizing; mature neurons have a more negative E\textsubscript{GABA} than resting membrane potential, and GABA is hyperpolarizing. While the exact effect of GABA\textsubscript{A}R opening on the probability of firing action potential is complicated by the increase in membrane conductance (shunting inhibition) (Gulledge and Stuart, 2003), the shift from depolarization to hyperpolarization normally result in a functional switch of GABA action from excitation to inhibition. This mercurial nature of GABA action may
provide a unique mechanism for assuring developing neurons receive proper depolarizing stimuli without the propensity to excitotoxicity.

1.2.4 The function of KCC2

In retrospect, since GABA_A Rs are Cl^- channels and intracellular Cl^- concentration shows developmental decrease, proteins that regulate neuronal Cl^- homeostasis may serve as switch of GABA functional polarity. NKCC1 and KCC2 are such proteins. They are both cation-chloride co-transporters but of opposite functions in regarding to neuronal Cl^- homeostasis regulations: NKCC1 imports Cl^-, while KCC2 exports Cl^-.

In immature neurons, NKCC1 is highly expressed and is responsible for the initial excitatory action of GABA. In early postnatal development, up-regulation of KCC2 then leads to decreasing intracellular Cl^- concentration and a functional switch of GABA action into inhibition (Fig. 1-3).

Unlike NKCC1 that is expressed in both neurons and glial cells, the expression of KCC2 is highly restricted in neurons (Payne et al., 2003). Previous studies also suggest that developmental KCC2 up-regulation is more important for GABA functional switch than NKCC1 down-regulation. While the up-regulation of KCC2 has been reported in many types of neurons, the down-regulation of NKCC1 is still debatable to some extent (Ben-Ari, 2002). Therefore, it may be reasonable to say that KCC2 is the molecular switch of GABA action polarity.
Adapted from Y. Ben-Ari, Nat Rev Neurosci 3:728-739 (2002)

**Figure 1-3.** Diagram showing GABA functional switch and KCC2 function.

**a,** Immature neurons have high intracellular Cl⁻ concentration ([Cl⁻]ᵢ) due to high expression level of NKCC1. GABA depolarizes neurons and induces calcium influx. **b,** Developmental up-regulation of KCC2 decreases [Cl⁻]ᵢ and renders GABA hyperpolarizing. VDCC, voltage-dependent calcium channels; CLC2, voltage-gated chloride channel 2; E<sub>Cl</sub>, chloride reversal potential; RMP, resting membrane potential.

KCC2 is originally discovered as neuron-specific potassium-chloride co-transporter (Payne et al., 1996). Utilizing the transmembrane K⁺ gradient in neurons, it exports K⁺ and Cl⁻ at 1:1 ratio (Chamma et al., 2012). A seminal work by Rivera et al. first reported developmental KCC2 up-regulation and delayed the appearance of hyperpolarizing GABA action after knockdown of KCC2 expression (Rivera et al., 1999). Further evidence supporting the essential role of KCC2 in GABA functional
switch came from study of KCC2 knockout mice which die at birth. Acute slice recordings showed that KCC2 knockout neurons display GABA-mediated excitation at a stage when GABA is inhibitory in wild type neurons (Hubner et al., 2001). Overexpression of KCC2 is also sufficient to accelerate the GABA functional switch process in developing neurons (Fiumelli et al., 2005; Lee et al., 2005).

KCC2 has been reported to form oligomers, which also showed developmental up-regulation (Blaesse et al., 2006). However, further study is necessary to determine whether oligomerization of KCC2 is necessary for its transporter activity or function at dendritic spines. Detailed morphological study revealed high level of KCC2 at glutamate synapses (Gulyas et al., 2001). Later, it was shown that KCC2 regulates dendritic spine formation possibly by interacting with cytoskeleton through protein 4.1N (Li et al., 2007; Fiumelli et al., 2012). KCC2 may also modulate the surface diffusion of glutamate receptors at dendritic spines (Gauvain et al., 2011). The function of KCC2 at glutamatergic synapses is surprisingly independent of its Cl⁻ transporter activity. Therefore, KCC2 regulates not only neuronal Cl⁻ homeostasis but also glutamatergic synapses. In the Chapter 4 of this thesis, I will present data showing a novel function of neuroligin-2 in regulating KCC2 functions.
1.3 The excitation/inhibition imbalance hypothesis in neuropsychiatric disorders

Since excitatory and inhibitory synaptic transmissions are the two major types of information that neurons receive and transmit, the excitation/inhibition balance is essential for brain function. Structurally, the ratio of glutamatergic versus GABAergic synapses is quite fixed within each individual neuron (Liu, 2004). On the other hand, the disturbance in the excitation/inhibition is an emerging hypothesis underlying many neuropsychiatric disorders. The sheer number of genetic variations associated with neuropsychiatric disorders also suggests that mutations of different genes may impair brain function by tilting the balance between excitation and inhibition in a converging manner (Walsh and Engle, 2010; Gilman et al., 2011).

Recent advances in optogenetics have made it possible to directly test the excitation/inhibition imbalance hypothesis in neuropsychiatric disorders by manipulating neuronal activities in free-moving animals (Tye and Deisseroth, 2012). In an elegant study applying optogenetics, Yizar et al. provide convincing evidence that elevated excitation/inhibition ratio causes autism-related social behavior defects (Yizhar et al., 2011). They expressed opsins specifically in glutamatergic pyramidal neurons or GABAergic neurons in transgenic mice to modulate the strength of excitation or inhibition. When they tested social behavior in mice by a widely used three-chambered social behavior paradigm, transgenic mice preferred novel mice to novel inanimate object without light stimulation. Interestingly, activation of pyramidal neurons by light in the same mice eliminated the preference. Importantly, this reduced social exploration was not
accompanied by defects in motor abnormalities or increased anxiety, and simultaneous activation of GABAergic neurons was able to partially rescue the phenotype.

1.3.1 Neuroligin mutations linked to autism

Considering their roles in regulating synapse functions, it is not surprising that many neuroligin mutations or copy number variations have been linked to neuropsychiatric disorders, especially autism spectrum disorders (ASD). Because autism affects much more males than females, NLGN3/4 genes that are located on the X chromosome have attracted extensive genetic studies of autism. In Table 1.1, I summarized previously identified NLGN3/4 mutations linked to autism or mental retardation and their functional defects. While mutations that lead to truncations of neuroligin mRNA obviously affect neuroligin function in patients, the functional relevance of many point mutations are unclear. Like many other glycoproteins, proper glycosylation of neuroligins is necessary for their cell surface expression and also for binding with neurexins (Comoletti et al., 2003). Therefore, point mutations of neuroligins may either affect the cellular trafficking of neuroligins or impair their interactions with other proteins. On the other hand, copy number variations may generally alter the potency of neuroligin functions.

Neuroligin-3 R451C is one of most studied neuroligin mutations linked to autism. It is first reported in a Swedish family with two affected brothers who inherited the mutation from their unaffected mother (Jamain et al., 2003). Following functional studies revealed that the R451C point mutation severely decreases the cell surface expression of
neuroligin-3 (Chih et al., 2004; Comoletti et al., 2004). However, transgenic mice with R451C mutation by knockin strategy exhibit distinct phenotypes compared with neuroligin-3 knockout mice (Tabuchi et al., 2007; Etherton et al., 2011), suggesting that R451C is a gain-of-function mutation instead of loss-of-function. Behaviorally, R451C knockin mice had defect in social interaction as tested by the three-chambered social behavior task (Tabuchi et al., 2007; Etherton et al., 2011).

Table 1-1. Summary of NLGN3/4 mutations linked to neuropsychiatric disorders.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Disease</th>
<th>Functional defects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNLGN3 R451C</td>
<td>ASD</td>
<td>ER retention, increase IPSC</td>
<td>1, 2, 3</td>
</tr>
<tr>
<td>hNLGN4 D396X</td>
<td>ASD</td>
<td>Truncated protein, ER retention</td>
<td>1, 2</td>
</tr>
<tr>
<td>hNLGN4 R87W</td>
<td>ASD</td>
<td>ER retention</td>
<td>4</td>
</tr>
<tr>
<td>hNLGN4 2bp deletion</td>
<td>ASD, XLMR</td>
<td>Truncated protein</td>
<td>5</td>
</tr>
<tr>
<td>hNLGN4 exon deletion</td>
<td>ASD</td>
<td>Truncated protein</td>
<td>6</td>
</tr>
<tr>
<td>hNLGN4 I679V</td>
<td>ASD</td>
<td>NA</td>
<td>7</td>
</tr>
<tr>
<td>hNLGN4 G99S, K378R</td>
<td>ASD</td>
<td>NA</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V403M, R704C</td>
<td></td>
</tr>
</tbody>
</table>

ASD, autism spectrum disorders; XLMR, X-linked mental retardation; NA, not available

References:

Although a great deal of work has been done to study the molecular mechanism of neuropsychiatric disorders focusing on neuroligins, further research is necessary to propose a causal link between neuroligin mutations and autism. The functional characterization of disease-linked neuroligin mutations will provide insights. In addition, direct demonstration of the essential role of neuroligins in regulating the balance between
excitation and inhibition is necessary. In the Chapter 4 of this thesis, I will present data suggesting neuroligin-2 as a master regulator of excitation/inhibition balance.

1.3.2 Behavioral defects of neuroligin transgenic mice

Because neuroligin-1 and neuroligin-2 regulate excitatory and inhibitory synaptic transmissions, respectively, alternations in any of their function may disturb excitation/inhibition balance. Therefore, transgenic mice with altered neuroligin-1/2 expression have been studied extensively as animal models of human neuropsychiatric disorders. Indeed, it turns out that mice either lacking or overexpressing neuroligin-1/2 all exhibited behavioral abnormalities, which further indicates the importance of excitation and inhibition balance. Neuroligin-1 knockout mice have reduction in NMDA receptor-mediated glutamatergic transmission (Chubykin et al., 2007) and showed impaired spatial memory and increased repetitive behavior (Blundell et al., 2010). Increased stereotyped grooming behavior in neuroligin-1 knockout mice is reminiscent of autism and could be partially blocked by NMDA receptor partial agonist (Blundell et al., 2010). In transgenic mice overexpressing exogenous neuroligin-1, the number of glutamatergic synapse is increased and memory acquisition is impaired (Dahlhaus et al., 2010). Neuroligin-2 knockout mice have reduced GABAergic transmissions and controversial anxiety-like behavior (Blundell et al., 2009). Neuroligin-2 overexpressing mice displayed stereotyped jumping and impaired social interactions (Hines et al., 2008).

However, it is necessary to point out that there are sometimes contradictions between different research groups in the behavioral studies of neuroligin transgenic mice,
even using the same experimental paradigms. For instance, using the same three-chambered social behavior test, two groups reported different results regarding the social interaction defects in neuroligin-3 R451C knockin mice (Tabuchi et al., 2007; Chadman et al., 2008). Development of standardized experimental protocol or new experimental paradigms for behavioral study may help establish reliable animal model of neuropsychiatric disorders.

1.3.3 GABAergic deficits in schizophrenia

Schizophrenia is a severe chronic mental disease that affects approximately 1% of the general population worldwide. The onset of schizophrenia usually starts from adolescence and young adulthood, and the symptoms of schizophrenia include both positive symptoms (delusion, hallucination, disorganized thinking, and bizarre behavior) and negative symptoms (poverty of speech, avolition, social withdrawal, and apathy). Genetic epidemiological studies, highlighted by twin studies, have demonstrated that schizophrenia is a complex disorder with high heritability (Cardno and Gottesman, 2000). Specifically, previous studies indicate that synaptic dysfunction is involved in the pathogenesis of schizophrenia and patients with schizophrenia also have synaptic degeneration in the brain (McGlashan and Hoffman, 2000; Lewis and Gonzalez-Burgos, 2006). Therefore, genes involved in the formation and functional integrity of synapses may be potential candidate genes for schizophrenia.

Specifically, emerging evidence suggest that GABAergic deficiency as a pathogenetic mechanism for schizophrenia (Lewis et al., 2005). First, postmortem studies
revealed significant reduction in GABA-related gene expression, including GABA synthesizing enzyme GAD, GABA$_A$R, and KCC2, in dorsolateral prefrontal cortex and hippocampus of schizophrenia patient brains (Hashimoto et al., 2008; Hyde et al., 2011). Dysfunction in dorsolateral prefrontal cortex contributes to working memory deficit, which is a major clinical feature of schizophrenia. Second, human genetic studies implicate many GABA-related gene mutations in schizophrenia (Charych et al., 2009).

There are overlapping symptoms between schizophrenia and autism, especially the negative symptoms (Goldstein et al., 2002; Sheitman et al., 2004), which may suggest that these two diseases share some common biological basis in their pathogenesis. In fact, mutations in autism-linked $NRXN1$ and $NLGN4$ genes have also been found in schizophrenia patients (Sand et al., 2006; Kirov et al., 2008; Walsh et al., 2008; Kirov et al., 2009). Therefore, mutations in neurexin and neuroligin, especially the GABAergic synapse specific neuroligin-2, may be highly associated with schizophrenia as well.

In the Chapter 3 of this thesis, I investigated the functional relevance of $neuroligin$-2 mutations linked to schizophrenia. This work would further test the hypothesis of excitation/inhibition imbalance in neuropsychiatric disorders.
2.1 Cell culture

Primary mouse cortical/hypothalamic/hippocampal neurons neuronal cultures were prepared following similar protocol. Briefly, brains of newborn C57BL/6 mice of either sex were dissociated and meninges were removed. Specific brain regions were dissected out and cut into pieces in Modified Hank’s Balanced Salt Solution (MHBSS) with 10% horse serum: HBSS (Invitrogen), 5 mM HEPES, 20 mM D-glucose. Tissues were then washed with serum free MHBSS and incubated with 0.05% Trypsin for 30 min. Cells were dispersed by trituration with pasteur pipette and harvested by centrifuge (900 rpm, 5 min). Dissociated cells were plated on a monolayer of cortical astrocytes on poly-D-lysine coated glass coverslip at a density of 8,000-12,000 cells/cm² in 24-well plates. Culture medium contained MEM (500 ml, Invitrogen), 5% fetal bovine serum (FBS, HyClone), 10 ml B-27 supplement (Invitrogen), 100 mg NaHCO₃, 20 mM D-glucose, 2 mM Glutamax (Invitrogen), and 25 units/ml penicillin/streptomycin. 4 μM arabinosylcytosine was supplemented in culture medium to stop excessive astrocyte growth. Neurons were maintained at 37°C in a 5% CO₂ humidified incubator. All experiments were repeated in at least three independent cultures.

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. For HEK cell and neuron co-culture experiments, HEK cells were trypsinized 24 hours after transfection and plated on top of hypothalamic cultures (10-12 DIV) and co-cultured for 2-3 days before functional analysis. For GM-6001 treatment experiments, because matrix metalloproteinases are present in serum and
may diminish the inhibitory effect of GM-6001, HEK cells were maintained in DMEM plus penicillin/streptomycin without serum.

2.2 Plasmid constructs and transfection

Calcium-phosphate transfection in cultured neurons was performed similar to a protocol developed in our laboratory (Jiang and Chen, 2006). DNA/Ca\(^{2+}\)-phosphate precipitates were prepared by using the Clontech CalPhos Mammalian Transfection Kit. Neuronal culture on coverslip was transferred into transfection medium containing MEM and 30mM D-glucose and then incubated with DNA/Ca\(^{2+}\)-phosphate precipitates for 30 min and washed with transfection medium three times, before transferred back to the original culture medium. Plasmid at 1 \(\mu\)g each was used for transfection per well in a 24-well plate.

HEK 293T cells in 6-well plates were transfected using polyethylenimine (MW 25,000, Polysciences). 2 \(\mu\)g of plasmid DNA and 8 \(\mu\)g polyethylenimine (1 \(\mu\)g/\(\mu\)l stock) was added in 100 \(\mu\)l OptiMEM (Invitrogen) separately and then mixed together for 10 min. The mixture is added into HEK cell culture dropwise, incubated for 6 hr, and washed by pre-warmed HEK cell culture medium.

Following plasmid constructs were used in this study and all constructs were confirmed by sequencing. The cDNA of WT human NL2 was obtained by PCR amplification of human brain Marathon-Ready cDNA (ClonTech Laboratories, USA) and cloned into pcDNA3.1/CT-GFP-TOPO vector (Invitrogen, USA). This WT cDNA construct was used as a template for the construction of mutant constructs: R215H,
V510M, R621H, and A637T (performed by Min-Chih Cheng); FUGW-GFP, FUGW-NLmiR, pCAG-NLmiR-IRES-GFP, pCAG-NLmiR-IRES-mCherry, HA-tagged shRNA-proof mouse NL1* and rat NL2* with IRES mCherry (gift from Roger Nicoll, University of California at San Francisco, San Francisco, CA) (Shipman et al., 2011); HA-tagged mouse NL1, HA-tagged mouse NL2, and EGFP-NL2shRNA (gift from Peter Scheiffele, University of Basel, Basel, Switzerland) (Chih et al., 2005); a non-tagged NL2shRNA was generated by cutting off EGFP with restriction enzymes; pIRES2-EGFP-rat KCC2 (gift from Yun Wang, Fudan University, Shanghai, China); mCherry (pEGFP-C1) (gift from Yingwei Mao, The Pennsylvania State University, University Park, PA).

2.3 Immunocytochemistry and imaging analysis

Cells were washed with bath solution (as used for electrophysiology) and fixed in 4% paraformaldehyde for 8 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 5% normal donkey/goat serum for 30 min, all at room temperature. Primary antibodies in blocking solution were incubated overnight at 4°C and washed with PBS four times. Secondary antibodies in blocking solution were incubated at room temperature for 45 min and washed with PBS four times. Cultured cells on coverslips were then mounted on glass slides with ProLong Gold antifade reagents (Invitrogen) and sealed with nail polish.

Following primary antibodies were used: KCC2 (07-432, Millipore), HA (sc-7392, Santa Cruz Biotechnology), MAP2 (ab5392, Abcam), NKCC1 (T4, Developmental Studies Hybridoma Bank), vGlut1 (135302, Synaptic Systems), GFP (ab13970, Abcam),
GAD65 (GAD-6, Developmental Studies Hybridoma Bank), calnexin (610523, BD Biosciences Transduction Laboratories). Dylight-conjugated secondary antibodies were bought from Jackson ImmunoResearch.

Fluorescence signals were captured on a Nikon TE-2000-S microscope or an Olympus FV1000 confocal microscope. For the quantification of immunofluorescence intensity, region of interest was selected and the mean intensity (0-255) was analyzed by ImageJ software.

2.4 Immunoblot

For HEK 293T cells, cells in 6-well plates were washed with PBS two days after transfection and total protein lysate was harvested in lysis buffer (20 mM HEPES, 1% Triton X-100, 0.1 mM EDTA, 2 mM CaCl2, 1mM MgCl2 and 50 mM NaCl with PMSF, protease and phosphatase inhibitors, pH 7.3 with NaOH). For cultured neurons, 2 wells from a 24-well plate were washed with PBS and lysated. For mouse cortex protein lysate, cortices were separated and homogenized in lysis buffer. After 2h rotation at 4°C, supernatants were harvested by centrifugation (12,000g, 30 min). Protein concentration was measured by Bradford Protein Assay Kit (Thermo Scientific Pierce Protein Biology Products). Samples were incubated with NuPAGE LDS Sample Buffer (Invitrogen) and 1% β-mercaptoethanol at 95°C (for NL2) or 50°C (for KCC2) for 15 min before resolved in 10% SDS-PAGE and transferred to PVDF membrane. KCC2 (07-432, Millipore), NL2 (129202, Synaptic Systems), HA (sc-7392, Santa Cruz Biotechnology), and actin (612656, BD Transduction Laboratories) primary antibodies and HRP conjugated
secondary antibodies (Abcam) were used. Immunoblot band intensities were measured with ImageJ software.

2.5 Calcium imaging

Cells were incubated in 2.5 μM Fura-2 AM (Invitrogen) for 45 min at 37°C and washed for 15 min in bath solution at room temperature. Coverslips were transferred to a perfusion chamber mounted on a Nikon TE-2000-S inverted microscope with a 20× objective and imaged with a 340/380 nm transmittance filter set (Chroma Technology). SimplePCI software (HCImage, Hamamatsu) was used to measure the ratio of 340/380 fluorescence signal in neuronal soma. Sister coverslips from 3-4 independent cultures were imaged at 3-4 time points. Because GFP interferes with Fura-2 signal, mCherry was coexpressed to identify transfected cells in all calcium imaging experiments. All recordings were done in the presence of DNQX (10 μM) to block AMPA receptor and indirect activation of neurons. The threshold of a significant $Ca^{2+}$ response was set as 10 times of baseline standard deviation.

2.6 Electrophysiology

Briefly, Multiclamp 700A amplifier and pClamp software (Molecular Devices) were used for acquiring data (sampling at 10 kHz and filtered at 1 kHz). Neurons were continually perfused with bath solution (in mM): 128 NaCl, 30 glucose, 25 HEPES, 5 KCl, 2 CaCl$_2$ and 1 MgCl$_2$ (320 mOsm, adjusted to pH 7.3 with NaOH). Pipette solution
contained (in mM): 147 KCl, 5 Na₂-phosphocreatine, 2 EGTA, 10 HEPES, 2 MgATP, 0.3 Na₂GTP (300 mOsm adjusted to pH 7.3 with KOH). Gramicidin (40 μg/ml, Sigma) was included in the pipette solution for perforated patch recording (Chen et al., 1996). A Picospritzer (Parker Instrumentation) was used to eject GABA directly to neuronal soma through a fine pipette (~2 μm tip). In whole-cell patch clamp mode (holding at -70 mV), mEPSCs were recorded in the presence of TTX (0.5 μM) and BIC (20 μM); mIPSCs were recorded in the presence of TTX (0.5 μM) and DNQX (10 μM).

2.7 Cell aggregation assay

To investigate the interaction between NLGN2 mutants and NRXN, I expressed each protein separately in different HEK 293T cells and then mixed the cells together to perform cell aggregation assay. All NLGN2 plasmids were GFP-tagged. DsRed was co-transfected to visualize NRXN1β (Gift from Dr. Ann Marie Craig) expressing cells. One day after transfection, the same amount of NRXN1β transfected cells were trypsinized and mixed with GFP, GFP-NLGN2 or GFP-NLGN2 mutant transfected HEK 293T cells. As a control, mixtures were imaged immediately after mixing (0 min). Mixtures were then incubated at 37°C with gentle rotation for 90 min and plated in 24-well plate for aggregation assay. Fluorescent images of 3-5 fields were chosen randomly from each well. The number of cell clusters (particles) was counted by ImageJ software. The percentage of aggregation was determined by $A = (N_{GFP} + N_{RFP} - N_{mix}) / (N_{GFP} + N_{RFP})$. $A$, degree of aggregation; $N_{GFP}$, number of GFP-labeled particles; $N_{RFP}$, number of RFP-labeled particles; $N_{mix}$, total number of particles in the GFP and RFP merged images. If no aggregation, $N_{mix} = N_{GFP} + N_{RFP}$, and $A = 0$. If all cells aggregate together, $A = 1$. The final aggregation index was normalized by $A_{90min} / A_{0min}$. Data from three independent experiments were presented as average ± standard error.
2.8  Statistical analysis

Unpaired student’s $t$-test was used for comparisons between two groups. One-way ANOVA with Bonferroni multiple comparisons was used for comparisons between multiple groups. Two-way ANOVA with Bonferroni multiple comparisons was used for comparisons between multiple time points and groups. GraphPad Prism (GraphPad Software) was used for all statistical analysis. Data are shown as mean ± standard error of the mean in all bar graphs and numbers inside bars indicate the total number of cells quantified.
Chapter 3

Functional Study of *neuroligin-2* Mutations Linked to Schizophrenia

In this study, our laboratory collaborated with Dr. Min-Chih Cheng in the laboratory of Dr. Chia-Hsiang Chen at National Health Research Institutes of Taiwan. They performed sequencing experiments and the clinical evaluation of schizophrenia patients. I performed all functional analysis. Part of this work has been published in *Human Molecular Genetics* (Sun et al., 2011).
3.1 Results

3.1.1 Identification of 6 rare missense mutations of NLGN2 gene

Systemically sequencing of all the exons and the promoter region of NLGN2 gene in 584 schizophrenia patients and 549 control subjects, we totally identified 19 genetic variants in this sample. The positions of these variants are illustrated in Fig. 3-1A. Notably, among these 19 variants, 6 were novel missense mutations that were not reported before in the literature, including a G-to-A substitution (c.644G>A) at codon 215 (R215H) in one schizophrenia patient; a G-to-A substitution (c.1528G>A) at codon 510 (V510M) in two patients; a G-to-A substitution (c.1862G>A) at codon 621 (R621H) in one patient; a G-to-A substitution (c.1909G>A) at codon 637 (A637T) in two patients; a C-to-T substitution (c.2399) at codon 800 (P800L) in one patient and one control subject, and a G-to-T substitution at codon 819 (A819S) in one patient and one control subject. The sequencing data are shown in Fig. 3-1B. Two missense mutations (R215H and V510M) were further verified using restriction fragment length polymorphism (RFLP) as illustrated in Fig. 3-1C. Protein sequence alignments of all human NLGNs surrounding mutant sites are shown in Fig. 3-1D. Amino acids R215, V510, R621, and A637 are located at the extracellular domain of NLGN2, while P800 and A819 are at the intracellular terminal. Because mutants P800L and A819S also appeared in control subjects, I focused on schizophrenia patient-specific mutants, R215H, V510M, R621H, and A637T, in my functional characterization studies. WT and mutant human NLGN2 were cloned into plasmid constructs with a GFP tag at their intracellular carboxyl-terminus to facilitate study.
Figure 3-1. Genetic variants of the NLGN2 gene identified in this study.

A, Distributions of the 19 variants of the NLGN2 gene found in this study, including 6 missense mutations. B, Sequence electropherograms of 6 missense mutations of the NLGN2 gene identified in this study. C, PCR-based RFLP analysis for the genotyping of R215H and V510M. D, Protein sequence alignments of all the human NLGNs surrounding mutant sites (generated by Clustal X2 software, http://www.clustal.org/). Red asterisks indicate missense mutations identified in this study. Data contributed by Min-Chih Cheng (National Health Research Institutes, Taiwan).
3.1.2 The R215H mutant was impaired in mediating cell adhesion

I first tested the ability of NLGN2 mutants in mediating cell adhesion through binding with their receptor NRXN1β. In cell aggregation assay as shown in Fig. 3-2, NRXN1β-expressing HEK 293T cells (red) formed large cell aggregates with wild type NLGN2-expressing cells (green) after 90 min incubation (Fig. 3-2A). In contrast, NRXN1β-expressing cells did not aggregate with GFP-expressing cells (Fig. 3-2B). Therefore, the observed cell aggregation after brief incubation in suspension was mediated by specific interaction between NLGN2 and NRXN1β, not by other endogenous cell adhesion molecules expressed in HEK 293T cells. In the assay of four NLGN2 mutants in forming cell aggregates with NRXN1β-expressing cells, R215H-expressing cells formed significantly reduced aggregates (Fig. 3-2C), while the other three mutants performed similarly to wild type NLGN2 (Fig. 3-2D-F). Quantitative analysis confirmed that the R215H mutant, but not the other three mutants, has significantly reduced aggregation index as compared with the wild type NLGN2 ($p < 0.01$, Fig. 3-2G). Together, these data suggest that the R215H mutant has severe impairment in mediating heterophilic cell adhesion, a primary function of neuroligins.
Figure 3-2. R215H cannot mediate cell adhesion with neurexins.

A. Cells expressing the wild type NLGN2 (green) were mixed with cells expressing NRXN1β (Red), and merged image showed the aggregation of NLGN2-expressing and NRXN1β-expressing HEK 293T cells. B, GFP-expressing and NRXN-expressing HEK 293T cells didn’t aggregate. C-F, The aggregation ability of each mutant was assessed and representative merged images were showed for each mutant, including R215H (C), V510M (D), R621H (E), and A637T (F). Scale bar, 60 μm. G, Quantification of the aggregation assay shows that R215H mutation had significantly impaired aggregation compared with the wild type, while V510M, R621H, and A637T mutants did not show significant differences. *p < 0.05, **p < 0.01.
3.1.3 R215H expression in HEK cells failed to attract GABAergic innervations

While the R215H mutant cannot interact with NRXNs to mediate cell adhesion in HEK cells, it is possible that it interact with endogenous NRXNs present on nerve terminals. NLGN2 expression in HEK cells has been shown previously to promote the formation of heterologous GABAergic synapses between HEK cells and neurons (Dong et al., 2007). Using this model system, I directly examined the synaptogenic potency of NLGN2 mutants by co-culturing NLGN2-expressing HEK cells with neurons. Immunostaining of glutamic acid decarboxylase (GAD), a presynaptic marker for GABAergic axon terminals, was conducted to assess the extent of GABAergic innervation onto NLGN2-expressing HEK cells. While GFP-expressing HEK cells showed no GAD immunofluorescent signals (Fig. 3-3A), WT NLGN2-expressing HEK cells showed strong GAD signals (Fig. 3-3B), indicating that WT NLGN2 was able to attract GABAergic innervations. Interestingly, the R215H mutant-expressing HEK cells performed poorly like the negative GFP control in this assay (Fig. 3-3C). Quantification of the percentage of GAD positive cells, at least two-fold increase of the GAD intensity compared with non-transfected HEK cells, further confirmed that the R215H mutant-expressing HEK cells had significant less GABAergic innervations (Fig. 3-3G). The other three mutants showed comparable percentage of GAD positive cells with WT NLGN2. Therefore, the R215H mutant is deficient in promoting GABAergic synapse formation.
Figure 3-3. Heterologous synapse formation assay of NLGN2 mutants.

A-F, Representative images showing the transfected HEK 293T cells (green) and immunostaining of GAD to visualize GABAergic terminals (Red). Cell transfected with GFP alone did not have GAD positive staining (A), while the wild type NLGN2-expressing cell had positive GAD staining (B). Among four NLGN2 mutants (C-F), the R215H mutant did not have GAD positive staining. Scale bar, 10 μm. G, Quantification confirms that the R215H mutant has no detectable GAD positive cells, while the other three mutants showed no differences in the counts of GAD positive cells compared the wild type (GFP, 0 out of total 16 cells examined; wild type NLGN2, 18/21; R215H, 0/27; V510M, 14/15; R621H, 14/17; A637T, 12/15). Arrows point to transfected HEK 293T cells in merged images. Data contributed by Rosie Qin (Department of Biology, The Pennsylvania State University)
3.1.4 R215H mutant cannot induce GABAergic transmissions in HEK cells

To corroborate the immunocytochemistry analysis of GABAergic innervations, I employed whole-cell patch clamp recordings to detect spontaneous inhibitory postsynaptic currents (IPSCs) in HEK cells co-expressing NLGN2 and GABA\(_A\)Rs (\(\alpha_2\beta_3\gamma_2\)). Wild type NLGN2 induced robust IPSCs in cocultured HEK cells, which were completely blocked by GABA\(_A\)R antagonist bicuculline (Fig. 3-4A). Brief application of GABA (100 \(\mu\)M) resulted in significant whole-cell GABA currents in transfected HEK cells, indicating the surface expression of functional GABA\(_A\)Rs (Fig. 3-4A). Consistent with the GAD staining data, the R215H mutant was significantly less potent than the wild type NLGN2 in inducing GABAergic events in co-cultured HEK cells (Fig. 3-4B). As the wild type NLGN2 induced IPSCs in 64 ± 8% of transfected HEK cells (n = 33), the R215H mutant only induced IPSCs in 3 ± 3% (n = 24) (Fig. 3-4C).

On the other hand, there was no significant difference in the amplitudes of whole-cell GABA currents among all tested NLGN2 groups, suggesting that all four mutants did not affect the surface expression of GABA\(_A\)Rs in HEK cells (Fig. 3-4D). Kinetic analysis of the IPSCs recorded in HEK 293T cells found no differences between wild type and mutant NLGN2-expressing cells and they are similar to synaptic events recorded from neurons (data not shown). In summary, the R215H mutation abolished the synaptogenic activity of NLGN2 in promoting GABAergic synapse formation.
Figure 3-4. Electrophysiological activity assay of NLGN2 mutants.

A, HEK 293T cells-expressing GABA_A R and NLGN2 showed spontaneous activity after co-culturing with neurons, which was blocked by bicuculline (BIC, 20 μM). The right panel shows the whole cell GABA current after the application of 100 μM GABA. B, Representative traces of whole-cell recording in HEK 293T cells expressing NLGN2 mutants. C, Comparison of the percentages of HEK 293T cells showed spontaneous activity after co-transfection of GABA_A R with each NLGN2 mutant. The R215H mutant had significant reduced percentage compared with the wild type, while the other three mutants did not show significant differences from the wild type (GFP, 10 ± 10%; NLGN2, 64 ± 8%; R215H, 3 ± 3%; V510M, 84 ± 14%; R621H, 59 ± 9%; A637T, 47 ± 4%) (n = 12, 33, 24, 9, 9, 9, respectively). D, Quantification of whole cell GABA currents showed no significant differences between each group. **p < 0.01.
3.1.5 The R215H mutant showed trafficking defect HEK 293T cells

To understand the molecular mechanism underlying the incompetence of the R215H mutant in mediating cell adhesion and promoting GABAergic synaptogenesis, I examined the sub-cellular localization of the wild type and the mutant NLGN2 proteins. HEK 293T cells were transfected with GFP or GFP tagged wild type and mutant NLGN2. After 2-day expression, immunostaining against GFP (to enhance the signal of GFP tag) and the endoplasmic reticulum (ER) marker calnexin revealed that the R215H mutant was largely retained in the ER and showed decreased surface expression (Fig. 3-5C). In contrast, wild type NLGN2 and the other three NLGN2 mutants showed high expression level on plasma membranes and less co-localization with calnexin (Fig. 3-5B, Fig. 3-6).

Because NLGNs are highly glycosylated at the extracellular domain and glycosylation is essential for protein maturation (Hoffman et al., 2004; Hebert et al., 2005), the ER retention of R215H suggested a possible defect in glycosylation and protein maturation. Indeed, immunoblotting against NLGN2 revealed that the wild type NLGN2, mutants V510M, R621H, and A637T all had two bands presumably corresponding to mature glycosylated protein and immature non-glycosylated protein. However, the mutant R215H only showed one band of lower molecular weight, likely representing the immature non-glycosylated protein (Fig. 3-5D). Previous studies have shown that the difference in molecular weight between mature and immature NLGNs is caused by the addition of glycans (De Jaco et al., 2006; Zhang et al., 2009; De Jaco et al., 2010). Therefore, the NLGN2 R215H mutant is a loss-of-function mutant that is largely
retained in the ER and is deficient in post-translational modification and surface expression.

![Image](image.png)

**Figure 3-5. The ER retention of the R215H mutant.**

A-C, Representative DIC and fluorescent images showing the expressions of GFP (A), GFP-tagged WT NLGN2 (B) or the R215H mutant (C) in HEK 293T cells. The ER was labeled by calnexin immunostaining (red). Scale bar, 10 μm. D, Immunoblot revealed that the R215H mutant showed only one band with low molecular weight, while WT NLGN2 and the other three mutants all showed two bands.
Figure 3-6. Expression pattern of wild type and NLGN2 mutants in HEK cells.

GFP-tagged WT NLGN2 and mutants were expressed in HEK 293T cells and their subcellular distributions were revealed by immunostaining against GFP (green) and calnexin (red). Scale bar, 10 μm.

3.1.6 Decreased surface expression of the R215H mutant in neurons

To further investigate the protein trafficking defects of the R215H mutant directly in neurons, I expressed GFP-tagged wild type human NLGN2 and R215H mutant in cultured mouse cortical neurons. While the expression of wild type human NLGN2 showed broad distribution across the dendritic tree, R215H was mostly expressed near the soma. Since neuroligins form dimers and dimerization is required for the full-function of neuroligins, I next studied whether the trafficking-defective R215H impaired the trafficking of wild type NLGN2 as a dominant negative mutant. To do this, I coexpressed HA-tagged wild type mouse NL2 with GFP-tagged WT NLGN2 or R215H mutant (Fig.
While R215H was rarely expressed in dendrites, HA-tagged WT NLGN2 shows broad distribution in the same transfected neurons. Therefore, the R15H did not affect the trafficking of co-expressed WT NLGN2.

Furthermore, when the R215H mutant was overexpressed in neurons, GABAergic synaptic transmission was comparable to that of control neurons, suggesting exogenous R215H did not alter the function of endogenous NLGN2 (Fig. 3-7C). As expected, overexpression of WT NLGN2 or knockdown of endogenous NLGN2 by NL2shRNA slightly increased or significantly decreased GABAergic synaptic transmissions, correspondingly (Fig. 3-7C). The peak amplitude of whole-cell GABA (100 μM) evoked current was comparable between all groups, suggesting the amount of surface functional GABA\(_{\lambda}\)Rs was not altered in all transfection groups (Fig. 3-7D). Together, these results suggest that the R215H mutant is defective in dendritic targeting but is not a dominant negative mutant.
Figure 3-7. Decreased surface expression of R215H mutant in neurons.

A-B, Wild type human NLGN2 (A) and R215H mutant (B) was co-transfected with HA-tagged wild type mouse NLGN2 in cultured mouse cortical neurons at 9 DIV and their expressions were revealed by GFP (green) and HA (red) immunostaining at 11 DIV. Scale bar, 20 µm for whole image and 5 µm for inlets. C, Quantification of mIPSC frequency in control neurons and neurons transfected with WT NLGN2, R215H mutant, or NL2shRNA (n = 35, 20, 18, 13). D, Quantification of the peak amplitude of whole-cell GABA current (n = 17, 11, 8, 6).
3.2 Discussion

3.2.1 The association between NLGN2 mutations and schizophrenia

In this study, we identified 6 missense rare point mutations of the NLGN2 gene in a sample of 584 schizophrenia patients and 549 control subjects from Taiwan after resequencing all the exonic and promoter regions. Among these 6 mutations, 4 (R215H, V510M, R612H, and A637T) were detected in patient group only, while 2 (P800L and A819S) were detected in both patient and control group. We found a tendency of the missense mutations over-represented in patient group compared with control group (8/584 versus 2/549), but the difference did not reach statistical significance ($p = 0.07$, Fisher’s exact test), which might be due to the limited sample size of this study. Replicate studies with large sample size in different populations and in related disorders may further investigate the significance of the association between NLGN2 mutations and neuropsychiatric disorders.

In the genetic study of mutation, we found one of his elder brothers also carried this mutation, indicating that this mutation might inherit from one of their parents who had passed away. The mutation carrier brother has neither past history of psychiatric disorders nor present mental illness in this study, indicating that the R215H mutation is not fully penetrant. In view of genetic underpinnings of psychiatric disorders are complex and usually involve multiple genes and interactions with environmental factors, there might be the presence of modifying factors that determine the threshold of the clinical presentation in carriers of this mutation. In addition, since we were not able to collect DNA samples and evaluate the clinical phenotypes of all the family members, the

[47]
penetrance and full picture of clinical presentations of this mutation are unclear. Hence, the clinical relevance of this mutation should be interpreted with caution.

3.2.2 R215H is a loss-of-function mutant

In the functional characterization of these four patient specific missense mutations, I identified the R215H mutant as a loss-of-function mutant based on three lines of evidence: first, when expressed in HEK cells, the R215H mutant cannot mediate cell aggregates with NRXN1β-expressing cells; second, the R215H mutant failed in inducing GABAergic synapse formation between HEK cells and neurons as demonstrated by both immunocytochemistry and electrophysiological assays; third, the expression of the R215H mutant in neurons did not alter the surface expression of co-expressed WT NLGN2. Moreover, I demonstrated that the defect of R215H is caused by deficiencies in post-translational modification and cell surface expression. The other three mutations, V510M, R621H, and A637T, behaved similarly to the wild type NLGN2 in this study, suggesting that their functional implications might be subtle. Further study is necessary to determine they affect binding affinity with neurexins or other possible functional relevance.

3.2.3 The prevalence of ER retention phenotype of NLGN mutations

Arginine 215 is highly conserved among all human NLGNs and across species. My imaging and biochemical data suggest that the R215H substitution undermines the
surface expression and the maturation of NLGN2. Recently, De Jaco et al. have shown that disease-related mutations in the α/β-hydrolase fold superfamily of proteins, including NLGNs, share common trafficking deficiencies (De Jaco et al., 2010). The prevalence of NLGN mutants with ER retention phenotype (including R451C of NLGN3, R87W of NLGN4, and R215H of NLGN2) raises the possibility of applying pharmacological chaperone to restore the function of NLGNs (Morello et al., 2000; Chih et al., 2004), since some mutant NLGNs, such as NLGN4 R87W, still binds NRXN and may promote synaptogenesis after reaching plasma membrane (Zhang et al., 2009). Based on recently resolved structure of NLGN-NRXN complex and systematically mutational analysis study (Arac et al., 2007; Fabrichny et al., 2007; Reissner et al., 2008), Arginine 215 is located away from either NLGN dimerization or NLGN-NRXN binding interfaces. However, it is possible that the R215H substitution affects the NRXN-binding affinity of NLGN2 because the mutation of a distant aspartate (NLGN1 D271), not mutations of residues near interfaces, completely abolished the binding of NLGN1 to NRXN1β (Reissner et al., 2008).

3.2.4 The implication of NLGN mutations in neuropsychiatric disorders

Although many mutations in the NLGN and NRXN gene families have been identified in patients with psychiatric disorders, few of them have been functionally characterized. Considering the importance of NLGN2 at GABAergic synapses, my functional study of R215H suggest that impaired inhibitory circuit formation contributes to the onset of schizophrenia. Together with previously reported R451C of NLGN3,
R87W of *NLGN4*, and D396X of *NLGN4* (Jamain et al., 2003; Zhang et al., 2009), we propose that mutations in the *NLGN* gene family are critically associated with neuropsychiatric disorders, including schizophrenia, autism, and mental retardation. Our system of molecularly engineered GABAergic synapse has the advantage of blank background and easy manipulation, which could be broadly applied in characterizing human disease related mutants.

Following genetic association studies, several lines of *NLGN* transgenic mice have been generated in an effort to pinpoint the functional consequences of human *NLGN* mutations and establish animal models for human psychiatric disorders. Notably, the R451C mutant of NLGN3 was originally discovered in two Swedish brothers, one with autism and the other with Asperger syndrome (Jamain et al., 2003). Knock-in of the R451C mutant in mice resulted in autism-related impaired social interaction behaviors (Tabuchi et al., 2007). Mice over-expressing NLGN1 or NLGN2 also had abnormal behaviors including anxiety-like behavior, learning and memory deficits, and impaired social interactions (Hines et al., 2008; Dahlhaus et al., 2010). As a loss-of-function mutation, the NLGN2 R215H mutation could decrease the formation or maturation of GABAergic synapses and alter the balance between neuronal excitation and inhibition, which is essential for the normal cognitive function of the brain.

Acknowledging the complicated nature of psychiatric disorders including schizophrenia and autism, our discovery of schizophrenia-associated NLGN2 mutants supports the notion that mutations in synaptic proteins are highly associated with psychiatric disorders. As mutations of *NLGN* family genes were originally reported to be associated with autism in the literature, we evaluated carefully the psychiatric diagnosis
of this patient. After reviewing his medical records and examining his mental status during this study, we did not find evidence to indicate that the patient co-morbid with autism spectrum disorder or mental insufficiency. Future study using transgenic mouse models carrying these *NLGN* mutations is necessary to understand the functional consequence of these NLGN mutants *in vivo* and, most importantly, to elucidate whether and how they contribute to the etiology of psychiatric disorders.
Chapter 4

Neuroligin-2 Regulates KCC2 and GABA Functional Switch
4.1 Results

4.1.1 KCC2 expression is unexpectedly decreased after knockdown of neuroligin-2

During my continued study of NL2 in synapse formation and plasticity, I made an unexpected finding that NL2 regulates KCC2, a K⁺-Cl⁻ cotransporter that is critical in controlling intracellular Cl⁻ concentration and hence the polarity of GABA action. I investigated the function of NL2 by using small-hairpin RNA (shRNA) mediated knockdown in cultured mouse cortical neurons. Two previously characterized shRNAs were used to knockdown NL2 expression level: a chained shRNA targeting all NL1-3 (NLmiR) (Shipman et al., 2011) and a NL2 specific shRNA (NL2shRNA) (Chih et al., 2005). When HA-tagged wild type (WT) NL2 was co-transfected with mCherry and NL2shRNA or NLmiR, the expression level of HA-NL2 was reduced by more than 80% compared to mCherry control (Fig. 4-1A-B). However, the expression of a shRNA-proof mutant version of HA-NL2* was not affected by NL2shRNA or NLmiR (Fig. 4-1A-B).

Surprisingly, after knockdown NL1-3 with NLmiR, I observed a significant reduction in the KCC2 expression level compared to non-transfected (nonTF) or mCherry-transfected control neurons (Fig. 4-1C-D). To find out the relative contribution of NL1 versus NL2 to the reduced KCC2 expression, shRNA-proof HA-NL1* or HA-NL2* was coexpressed with NLmiR for a rescue. HA immunostaining confirmed the expression of HA-NL1* and HA-NL2* in the presence of NLmiR (Fig. 4-1C inlets). Coexpression of HA-NL2*, but not HA-NL1*, with NLmiR significantly rescued the KCC2 expression level (Fig. 4-1C-D), suggesting that NL2 as the main regulator of KCC2. In accordance with this rescue experiment, knockdown of NL2 specifically by
NL2shRNA showed similar reduction of KCC2 expression as that induced by NLmiR (Fig. 4-1C-D). To test whether NL2shRNA has off-target effects on KCC2, I co-transfected NL2shRNA and KCC2 in HEK 293T cells. Immunoblot demonstrated that, while WT NL2 expression was significantly reduced by NL2shRNA, KCC2 expression was not affected by NL2shRNA at all (Fig. 4-1E). Therefore, NL2shRNA did not have an off-target effect on KCC2, but rather acted through knockdown of NL2 in decreasing KCC2 expression in neurons.

While KCC2 extrudes Cl⁻, NKCC1 imports Cl⁻ and is mainly responsible for the excitatory action of GABA during early brain development (Blaesse et al., 2009). I wondered whether NKCC1 expression might also be altered after NL2 knockdown. Immunostaining with antibodies specific for NKCC1 showed that NL2 knockdown did not change NKCC1 expression level (Fig. 4-1F). Together, my data demonstrated a novel function of NL2 in regulating KCC2, with both proteins play critical roles in the GABAergic system.
Figure 4-1. Knockdown of neuroligin-2 decreases KCC2 expression.

A. WT HA-NL2 or shRNA-proof HA-NL2* was co-transfected with mCherry, mCherry + NL2shRNA or mCherry + NLmiR in mouse cortical neurons at 2 DIV. Exogenous NL2 expression was revealed by HA immunostaining at 8 DIV. Scale bar, 10 µm. B, Bar graph showing quantified somatic HA immunostaining intensity (normalized by mCherry controls). ***p < 0.001 (vs. mCherry, one-way ANOVA). C, Representative images showing KCC2 immunostaining (green) in non-transfected neurons (nonTF, arrows, 12 DIV) and neurons transfected (red, arrowheads) with mCherry, NLmiR, NLmiR + NL1*, NLmiR + NL2*, or NL2shRNA. Inlets showed HA immunostaining (gray) to confirm the expression of NL1* and NL2*. Scale bar, 20 µm. D, Bar graph showing quantified somatic KCC2 immunofluorescence intensity in nonTF neurons (173 ± 4 a.u.) and neurons transfected with mCherry (172 ± 10), NLmiR (73 ± 8), NLmiR + NL1* (93 ± 9),
NLmiR + NL2* (120 ± 12), or NL2shRNA (80 ± 11). a.u., arbitrary unit. **p < 0.01, ***p < 0.001, n.s., not significant, one-way ANOVA. E, HEK cells were transfected with NL2 + GFP, NL2 + NL2shRNA, KCC2 + GFP, KCC2 + NL2shRNA. Total protein lysate were analyzed by immunoblot. Actin was used as loading control. F, Representative images showing NKCC1 immunostaining (red) in 9 DIV nonTF (arrows) and NL2shRNA-transfected neurons (green, arrowheads). Scale bar, 20 μm. Bar graph shows quantified somatic NKCC1 signal intensity (p > 0.7, unpaired student’s t-test). Data in panel F contributed by Lei Zhang (Department of Biology, The Pennsylvania State University)

4.1.2 Neuroligin-2 controls GABA excitation-inhibition switch

KCC2 is the key player in controlling intracellular Cl- concentration and driving GABA functional switch from excitation to inhibition during early development (Rivera et al., 1999). Therefore, I hypothesized that, if KCC2 expression is decreased after NL2 knockdown, GABA functional switch might be affected accordingly. To test this idea, cortical neurons were transfected at 2 DIV with mCherry and NLmiR or NL2ShRNA. Fura-2 Ca²⁺ ratio imaging was employed to monitor GABA-evoked Ca²⁺ influx to determine whether GABA action is excitatory or inhibitory (van den Pol et al., 1996). Fig. 4-2A-C illustrates that when neurons were analyzed at 8 DIV, application of GABA (100 μM) evoked small Ca²⁺ responses in less than 50% of nonTF control neurons but large Ca²⁺ responses in over 90% of adjacent NLmiR-transfected neurons. All control and NLmiR-transfected neurons showed robust Ca²⁺ responses to high potassium (90 mM) induced depolarization (Fig. 4-2C). GABA-evoked Ca²⁺ increase was completely blocked by GABAA receptor antagonist bicuculline (BIC, 20 μM) (Fig. 4-2B), suggesting that Ca²⁺ influx was mediated by GABAA receptor activations.
Using Ca$^{2+}$ imaging approach, I further delineated the time course of GABA functional switch by monitoring the gradual decrease of GABA-evoked Ca$^{2+}$ responses in developing neurons. I found that cortical neurons complete their GABA functional switch around two weeks in dissociated rodent neuronal culture (Fig. 4-2D), similar to previous reports (Chen et al., 1996). Specifically, for control neurons transfected with mCherry, GABA evoked Ca$^{2+}$ responses in 80 ± 5% (n = 96) neurons at 4 DIV, but only 8 ± 3% (n = 61) at 12 DIV (Fig. 4-2D), suggesting that the majority of neurons have finished the GABA excitation-inhibition transition by this time. However, after knockdown of NLs, even at 12 DIV, GABA still evoked Ca$^{2+}$ responses in more than 80% of transfected neurons (Fig. 4-2D; NLmiR, 86 ± 5%, n = 86; NL2shRNA, 86 ± 8%, n = 41; mCherry vs. NLmiR or NL2shRNA, p < 0.001 for transfection, two-way ANOVA). Importantly, coexpression of NL2*, but not NL1*, with NLmiR partially rescued GABA functional switch by 12 DIV (Fig. 4-2D; NLmiR vs. NLmiR+NL2*, p < 0.001, one-way ANOVA at 12 DIV), suggesting again that NL2 as the key regulator of GABA excitation-inhibition switch.

In addition to quantifying the percentage of neurons responding to GABA, I also compared the amplitude of GABA-evoked Ca$^{2+}$ responses in each individual neuron at 4 and 12 DIV. While all neurons in different groups showed significant GABA-evoked Ca$^{2+}$ responses at 4 DIV, control neurons transfected with mCherry showed greatly diminished Ca$^{2+}$ responses at 12 DIV due to normal functional switch (Fig. 4-2E). However, neurons transfected with NLmiR or NL2shRNA still showed large GABA-evoked Ca$^{2+}$ responses at 12 DIV with the amplitudes similar to those at 4 DIV (Fig. 4-2E), suggesting no GABA functional switch occurred even at this late stage.
Coexpression of NL2*, but not NL1*, with NLmiR resulted in significantly smaller GABA-evoked Ca$^{2+}$ response amplitude at 12 DIV (Fig. 4-2E). Collectively, my Ca$^{2+}$ imaging data suggest that NL2 plays a critical role in regulating GABA functional switch during early development.

Next, I investigated whether NL2 is also required for maintaining GABAergic inhibition in mature neurons after the completion of GABA functional switch. To address this question, I transfected mature cortical neurons at 12-14 DIV with NLmiR or NL2shRNA and analyzed GABA-evoked Ca$^{2+}$ responses at 16 and 21 DIV. Mature neurons in control group rarely showed any GABA-evoked Ca$^{2+}$ response (Fig. 4-2F; nonTF, 4 out 264 neurons; mCherry-transfected, 0/7), but more than 50% mature neurons transfected with NLmiR (n = 59) or NL2shRNA (n = 12) showed significant GABA-evoked Ca$^{2+}$ responses (Fig. 4-2F). Therefore, NL2 is not only required for GABA functional switch in immature neurons, but also required for the maintenance of GABA inhibition in mature neurons.
Figure 4-2. Knockdown of neuroligin-2 abolishes GABA functional switch.

A. Representative images showing 8 DIV NLmiR-transfected neurons loaded with Fura-2. Somata of both nonTF and transfected neurons were selected (Fura-2 panel, white circles) to measure 340/380 ratio signal. Scale bar, 40 μm. B, Averaged sample traces
showing that BIC (20 μM) blocked GABA-evoked Ca2+ responses in both nonTF (gray, n = 6) and NLmiR-transfected neurons (black, n = 17). C, Sample traces showing somatic Ca2+ responses after stimulation with 100 μM GABA and 90 mM KCl in nonTF neurons (gray, 8 DIV) and NLmiR-transfected neurons (black). D, The time courses of GABA functional switch in nonTF neurons and neurons transfected with mCherry, NLmiR, NLmiR + NL1*, NLmiR + NL2*, or NL2shRNA (3-4 independent cultures; n = 2291, 272, 433, 350, 322, 152 neurons, respectively). E, Bar graph showing the amplitude of GABA-evoked Ca2+ increases in neurons transfected with mCherry, NLmiR, NLmiR + NL1*, NLmiR + NL2*, or NL2shRNA at 4 and 12 DIV. There was no difference between all groups at 4 DIV (p > 0.6). ***p < 0.001, one-way ANOVA. F, Percentage of mature neurons showing GABA-evoked Ca2+ increase after NLmiR or NL2shRNA transfection. Arrows in D and F indicate the time of transfection (TF).

4.1.3 Neuroligin-2 regulates GABA equilibrium potential

The observed KCC2 reduction and large GABA-evoked Ca2+ responses after NL2 knockdown suggest a depolarized GABAA receptor reversal potential (EGABA). To directly examine EGABA, I performed gramicidin-perforated patch clamp recordings to keep intracellular Cl− intact (Chen et al., 1996). In control neurons at 10-13 DIV, GABA application (40 μM, 50 ms) typically evoked small depolarizing or hyperpolarizing membrane potential changes (Fig. 4-3A, top traces). In contrast, in neurons transfected with NL2shRNA, GABA reliably evoked action potentials on top of large depolarizing responses (Fig. 4-3A, bottom traces), confirming that GABA function remains excitatory after NL2 knockdown. Changing holding membrane potentials under voltage-clamp condition revealed a significant depolarizing shift in EGABA after NL2 knockdown (Fig. 4-3B). Quantitatively, knockdown of NL2 alone resulted in a depolarizing shift of 16 mV in EGABA and knockdown of NL1-3 induced a shift of 22 mV (Fig. 4-3C-D; EGABA: Control, -56 ± 3 mV; NL2shRNA, -40 ± 2 mV; NLmiR, -34 ± 2 mV). Interestingly,
overexpression of NL2 caused an opposite change: a significant hyperpolarizing shift of 12 mV in $E_{\text{GABA}}$ (Fig. 4-3C-D; NL2, -68 ± 3 mV). On the other hand, the resting membrane potential was not significantly altered by NL2 manipulations (Control, -60 ± 2 mV; NLmiR, -58 ± 4 mV; NL2shRNA, -63 ± 2 mV; NL2, -67 ± 2 mV; $p > 0.08$). These results suggest that NL2 plays an active role in controlling the functional polarity of GABA action.
Figure 4-3. Neuroligin-2 regulates GABA equilibrium potential.

A. Representative current clamp recordings showing differential changes in membrane potentials evoked by GABA in 10-13 DIV control neurons (gray) and NL2shRNA-transfected neurons (black). Note GABA evoked action potentials in most NL2shRNA-transfected neurons (4 out of 5 neurons) but rarely in control neurons (1/6). B, Representative voltage-clamp recordings showing GABA-evoked currents at different holding potentials, in the presence of TTX (0.5 μM) and DNQX (10 μM). C, I-V plot of the mean GABA-evoked peak currents in control neurons and neurons transfected with NLmiR, NL2shRNA, or NL2. Note the opposite shift in EGABA between NL2 overexpression and NL2 knockdown groups. D, Bar graphs showing mean EGABA of control neurons and neurons transfected with NLmiR, NL2shRNA, or NL2. Dashed line indicates the mean EGABA of control neurons. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.
4.1.4 The regulation of KCC2 by neuroligin-2 is independent of GABA\(_A\) receptor activation and neuronal firing

Although controversial (Ludwig et al., 2003), GABAergic transmission itself has been suggested to regulate GABA functional switch (Ganguly et al., 2001). Since NL2 regulates GABAergic synaptogenesis, I wondered whether alterations in GABA\(_A\) receptor activations and neuronal activity are involved in the NL2 regulation of KCC2. To test this idea, cortical neurons were transfected with NL2shRNA at 2 DIV and chronically treated (2-12 DIV) with BIC (20 \(\mu\)M) to block GABA\(_A\) receptors or tetrodotoxin (TTX, 1 \(\mu\)M) to block action potential firing. As illustrated in Fig. 4A-B, quantification of the KCC2 immunofluorescence intensity showed that NL2 knockdown reduced KCC2 expression level to about one third of the control level regardless of drug treatments. Therefore, decreased KCC2 expression after NL2 knockdown is not mediated by GABAA receptors or neuronal activity.

4.1.5 Developmental up-regulation of NL2 is independent of neuronal activity

I then investigated whether developmental expressions of NL2 and KCC2 rely upon GABA\(_A\) receptor activation or neuronal activity. As shown in Fig. 4-5A-B, KCC2 expression showed a significant increase from 4 to 12 DIV as revealed by immunostaining but no differences in KCC2 expression were found between control and BIC- or TTX-treated neurons (\(n = 12\) neurons per group; \(p < 0.001\) for time, \(p > 0.5\) for drug treatment, Two-way ANOVA). Similar to KCC2, western blot analysis also found that NL2 expression increased significantly from 4 to 12 DIV, but not affected by BIC or TTX treatment (Fig. 4-5C-D; \(n = 3\); \(p < 0.05\) for time, \(p > 0.3\) for drug treatment, Two-way ANOVA).
Figure 4-4. Neuroligin-2 regulates KCC2 expression independent of GABAergic transmission or network activity.

A. Representative images showing KCC2 immunostaining (red) in 11 DIV nonTF (arrows) and NL2shRNA transfected neurons (green, arrowheads). DMSO (0.1%, Control), BIC (20 μM) or TTX (1 μM) was added into culture medium after transfection at 2 DIV and replenished every two days. Scale bar, 20 μm. B. Bar graph showing somatic KCC2 immunofluorescence intensity in nonTF and NL2shRNA-transfected neurons treated with DMSO, BIC or TTX (n = 11-12 neurons per group). ***p < 0.001, unpaired student’s t-test.

Functionally, Ca\(^{2+}\) imaging experiments showed comparable developmental time courses of GABA-evoked Ca\(^{2+}\) responses among control and BIC- or TTX-treated neurons (Fig. 4-5E; \(p > 0.7\) for drug treatment, Two-way ANOVA). Together, my data suggest that the developmental up-regulations of NL2 and KCC2 as well as GABA functional switch are likely regulated by cell-intrinsic mechanisms and independent of GABA\(_A\) receptor activation or neuronal activity.
Figure 4-5. The up-regulation of neuroligin-2 is independent of neuronal activity.

A, Representative images showing MAP2 (green) and KCC2 (red) immunostaining at 4 and 12 DIV in neurons treated with DMSO, BIC or TTX starting at 2 DIV. Scale bar, 20 μm. B, Bar graph showing somatic KCC2 immunofluorescence intensity (n = 12 neurons per group). a.u., arbitrary unit. C, Representative immunoblot showing NL2 expression in 4 and 12 DIV cultured neurons treated with DMSO, BIC or TTX starting at 2 DIV. Actin was used as loading control. D, Quantification of NL2 expression level as measured by immunoblot (n = 3 cultures). NL2 expression was normalized to expression of 4 DIV control. E, Calcium imaging showing time courses of GABA switch in control neurons and neurons treated with BIC or TTX (3 cultures; n = 1141, 717, 737 neurons, respectively).
4.1.6 The expression of neuroligin-2 precedes that of KCC2

If NL2 regulates KCC2 expression, the onset of NL2 expression should precede that of KCC2 during development. This was indeed what I found in the mouse brain in vivo when I analyzed the temporal expression profile of both NL2 and KCC2 from postnatal day 1 to day 20 (Fig. 4-6A-B). In neonatal mouse brain (P1-P4), NL2 was already expressed in a significant amount whereas the expression of KCC2 was minimal, consistent with a delayed KCC2 expression that correlates with GABA functional switch (Rivera et al., 1999). Quantitatively, if the expression level at P20 was as 100%, NL2 expression reached 50% at about P4, whereas KCC2 did not reach 50% even at P11 (Fig. 4-6B). Therefore, the in vivo sequential expression of NL2 and KCC2 makes it possible that NL2 plays a regulatory role in controlling the KCC2 function.

Figure 4-6. Neuroligin-2 expression precedes KCC2 in vivo.

A. Representative NL2 and KCC2 immunoblot of mouse cortex total protein lysate at postnatal day 1 (P1), P4, P8, P11, P15, and P20. Actin was used as loading control. B. Quantification of NL2 and KCC2 expression level as measured by immunoblot (n = 3). Protein expression was normalized to expression level at P20.
4.1.7 Neuroligin-2 regulates both GABAergic and glutamatergic synapse formation

Previous study reported that NL2shRNA not only decreased GABAergic synapses but also affected glutamatergic synapses (Chih et al., 2005), but the underlying mechanism is not well understood. I transfected cortical neurons at 2 DIV with NL2shRNA and employed patch clamp recordings to analyze synaptic events at 10-12 DIV. As expected, the miniature inhibitory postsynaptic currents (mIPSCs) were largely abolished in neurons transfected with NL2shRNA (Fig. 4-7A and C; Control, 0.67 ± 0.25 Hz, n = 10; NL2shRNA, 0.002 ± 0.002 Hz, n = 10; p < 0.05, unpaired student’s t-test). Interestingly, the frequency of miniature excitatory postsynaptic currents (mEPSCs) were also decreased (Fig. 4-7B-C; Control, 1.2 ± 0.3 Hz, n = 10; NL2shRNA, 0.01 ± 0.01 Hz, n = 10; p < 0.01), confirming that NL2 not only regulates GABAergic synapses, but also affects glutamatergic synapses. I then investigated whether knockdown NL2 modulates neuronal intrinsic excitability. As shown in Fig. 4-7D-E, the voltage-dependent sodium and potassium currents were similar in control and NL2shRNA-transfected neurons. Quantitatively, the I-V curves of both Na⁺ and K⁺ currents showed no significant difference between control and NL2shRNA-transfected neurons (Fig. 4-7F; I_{Na⁺}, p > 0.9 for transfection; I_{K⁺}, p > 0.2 for transfection, Two-way ANOVA). Therefore, NL2 is required for both GABAergic and glutamatergic synapse formation.
Figure 4-7. Neuroligin-2 regulates both GABAergic and glutamatergic synapse formation.

A-B. Sample traces showing mIPSCs (A) and mEPSCs (B) recordings from 10-12 DIV nonTF and NL2shRNA-transfected neurons. C, Bar graph showing the frequency of mIPSCs (n = 10 neurons per group; left y-axis) and mEPSCs (n = 10 per group; right y-axis). *p < 0.05, **p < 0.01, unpaired student’s t-test. D-E, Sample traces showing whole-cell sodium currents (D) and potassium currents (E) in response to voltage step depolarizations. F, I-V plot of the mean amplitude of sodium and potassium currents in control (n = 7 neurons) and NL2shRNA-transfected neurons (n = 9).

4.1.8 The effect of neuroligin-2 on glutamatergic synapses is mediated by KCC2

The reduction in mEPSC frequency after NL2 knockdown is not easy to interpret considering the specific targeting of NL2 to GABAergic synapses. Since NL2 is not directly localized at glutamatergic synapses, a mediator is necessary to mediate the effect of NL2shRNA. My results suggest that KCC2 may be such a downstream effector of NL2. Interestingly, KCC2 has recently been shown to modulate dendritic spines and
AMPA receptor diffusion through interactions with cytoskeleton proteins, which is independent of its transporter activity (Li et al., 2007; Gauvain et al., 2011; Fiumelli et al., 2012). I therefore hypothesized that NL2 may indirectly regulate glutamatergic synapses through the mediation of KCC2. To test this hypothesis, KCC2 were co-transfected with GFP, GFP-NL2shRNA, or GFP-NL2shRNA, in more mature neurons (10 DIV) to examine dendritic spine morphogenesis. By 15 DIV (cultured on astrocytes), GFP-transfected neurons developed extensive dendritic spines (Fig. 4-8A), which were apposed to glutamatergic presynaptic terminals (labeled by vGlut1, Fig. 4-8D). In contrast, GFP-NL2shRNA-transfected neurons showed significantly reduced spine density (Fig. 4-8B). Interestingly, co-transfection KCC2 with GFP-NL2shRNA restored normal spine density (Fig. 4-8C and E; GFP, 10 ± 1 per 20 μm; GFP-NL2shRNA, 4 ± 1; GFP-NL2shRNA + KCC2, 9 ± 1; n = 11 per group), suggesting that NL2 regulates spine density through KCC2. Similar to knockdown results in immature neurons, knockdown of NL2shRNA significantly decreased KCC2 expression in more mature neurons, which was rescued by KCC2 co-transfection with NL2shRNA (Fig. 4-8F).

In addition, electrophysiological recordings showed that KCC2 co-transfection with GFP-NL2shRNA partially rescued the decrease of the frequency of mEPSCs, but not mIPSCs (Fig. 4-8G). Therefore, in consistent with the morphological of dendritic spines, KCC2 co-expression is able to rescue NL2shRNA-induced loss of glutamatergic synapses. Together, these data suggest that NL2 indirectly regulates glutamatergic synapse formation through KCC2.
Figure 4-7. Neuroligin-2 regulates glutamatergic synapses through KCC2.

A-C, Representative images showing 15 DIV neurons transfected with GFP (A), GFP-NL2shRNA (B), or GFP-NL2shRNA + KCC2 (C). Scale bar, 20 µm. D, Enlarged inlets in A-C showing dendritic spines labeled by GFP (green) and glutamatergic presynaptic terminals labeled by vGlut1 (red). Scale bar, 5 µm. E, Quantification of dendritic spine numbers per 20 µm in neurons expressing GFP, GFP-NL2shRNA, or GFP-NL2shRNA + KCC2. Two secondary dendritic segments of 20 µm were counted per neuron. F, Bar graph showing somatic KCC2 immunofluorescence intensity: control neurons, 153 ± 9, n = 17; GFP-NL2shRNA transfected neurons, 88 ± 14, n = 11; GFP-NL2shRNA + KCC2 transfected neurons, 168 ± 13, n = 9. a.u., arbitrary unit. G, Bar graph showing the frequency of mIPSCs (n = 9 per group; left y-axis) and mIPSCs (n = 11 per group; right y-axis). mIPSCs: Control, 0.58 ± 0.14 Hz; GFP-NL2shRNA, 0.02 ± 0.01 Hz; GFP-NL2shRNA + KCC2, 0.03 ± 0.02 Hz. mEPSCs: Control, 2.55 ± 0.42 Hz; GFP-NL2shRNA, 0.12 ± 0.03 Hz; GFP-NL2shRNA + KCC2, 1.07 ± 0.22 Hz. *p < 0.05, ***p < 0.001, n.s., not significant, one-way ANOVA. Data in panel A-E contributed by Lei Zhang (Department of Biology, The Pennsylvania State University).
4.1.9 Neuroligin-2 is cleaved by matrix metalloproteinase

Most recent studies have revealed that NL1 is cleaved by matrix metalloproteinases (MMPs) after experimental seizure induction, which lead to decreased glutamatergic transmission and loss of dendritic spines (Peixoto et al., 2012; Suzuki et al., 2012). However, the phenotype of NL1 cleavage is more likely a consequence than a cause of epileptic activity. Since down-regulation of KCC2 has been widely reported in epilepsy (Blaesse et al., 2009), we hypothesized that NL2 is also cleaved under pathological conditions, and loss of NL2 contributes to KCC2 down-regulation. If so, drugs inhibiting NL2 cleavage may provide new therapeutic mechanisms of epilepsy.

To test this idea, I first examined the cleavage state of NL2 in HEK 293T cells. The predicted cleavage site of NL2 is located at the juxtamembrane stalk region (amino acid 655-676) (Suzuki et al., 2012). I used two NL2 constructs with different size of predicted C-terminal fragments (Fig. 4-8A). Interestingly, I detected endogenous cleavage product with predicted size when I expressed NL2 in HEK 293T cells (Fig. 4-8B). In addition, treatment of HEK cells immediately after transfection with a general MMP inhibitor, GM-6001, blocked NL2 cleavage in a dosage-dependent manner (Fig. 4-8C). Since I used serum-free medium for this experiments, NL2 is likely cleaved by HEK cells endogenously expressed MMPs. Together, my data demonstrated the presence of endogenous NL2 cleavage and provide preliminary data supporting a role of NL2 cleavage in KCC2 down-regulation under pathological conditions.
Figure 4-8. Cleavage of neuroligin-2 by matrix metalloproteinases in HEK cells.

A, Schematic depiction of two NL2 constructs used: a C-terminal GFP-tagged human NL2 and an N-terminal HA-tagged rat NL2. “X” indicates the predicted cleavage site and “TM” indicates the transmembrane domain. The predicted sizes of the cleaved C-terminal fragments are approximately 45 kD and 18 kD for GFP-NL2 and HA-NL2, respectively. NL2 antibody recognizes both full-length and C-terminal NL2, while HA antibody only recognizes full-length NL2 and N-terminal fragment. B, Immunoblot of HEK cells overexpressing GFP-NL2 or HA-NL2 with NL2 antibody. Arrow indicates full-length NL2 band and asterisks indicate C-terminal fragments. C, Immunoblot of HA-NL2 overexpressed in HEK cells with NL2 (left) and HA antibody (right). DMSO or GM-6001 was added into medium after transfection and total protein were harvested 1 day after. Note the C-terminal fragments were detected by NL2, but not HA, antibody.
4.2 Discussion

In this study, I report a novel function of NL2 in regulating KCC2 and GABA functional switch by several lines of evidences: first, knockdown of NL2 induced a significant decrease of the expression level of KCC2; second, overexpressing and knockdown of NL2 caused negative and positive shift of $E_{\text{GABA}}$, respectively; third, after knockdown of NL2, GABA evoked large $\text{Ca}^{2+}$ influx even in mature neurons; fourth, the decrease of KCC2 expression and GABA functional changes after knockdown of NL1-3 were rescued only by shRNA-proof NL2*, but not NL1*. Therefore, KCC2 is specifically regulated by NL2. Interestingly, besides the regulation of GABAergic functions, NL2 may also regulate glutamatergic transmission synapses through KCC2. In summary, my work suggests that NL2 play multiple roles of in regulating GABAergic synapse formation, GABA excitation-inhibition switch, and glutamatergic synapse formation.

4.2.1 Classical function of neuroligin-2 in GABAergic synaptogenesis

Neuroligins and their presynaptic binding partners neurexins are important trans-synaptic cell adhesion molecules playing a critical role in inducing synaptogenesis in the central nervous system (Craig and Kang, 2007; Huang and Scheiffele, 2008; Sudhof, 2008). The synaptogenic effect of neuroligins was demonstrated by their potent induction of presynaptic differentiation when overexpressing neuroligins in non-neuronal cells such as HEK or COS cells (Scheiffele et al., 2000; Dean et al., 2003; Fu et al., 2003; Graf et al., 2004; Nam and Chen, 2005; Dong et al., 2007). The NL1-3 triple KO and NL1-3 knockdown with shRNAs all showed significant deficits in glutamatergic and
GABAergic synapse formation and synaptic transmission (Chih et al., 2005; Varoqueaux et al., 2006). It was later found that neuroligins may also play a role in synaptic validation and plasticity (Chubykin et al., 2007; Shipman and Nicoll, 2012a), possibly through a trans-synaptic feedback (Futai et al., 2007; Hu et al., 2012). Most recent studies have revealed that NL1 may be cleaved by matrix metalloproteinases in an activity-dependent manner and regulates glutamatergic synaptic transmission (Peixoto et al., 2012; Suzuki et al., 2012). Our laboratory has previously demonstrated that overexpression of NL2 with GABA receptors in HEK cells is capable of inducing fully functional GABAergic innervations from surrounding neurons (Dong et al., 2007). I also identified a novel loss-of-function mutation of NL2 (R215H) from human schizophrenic patients, with the mutant NL2 incapable of inducing GABAergic synapse formation (Sun et al., 2011). In the current study, I further demonstrate that knockdown NL2 with NL2shRNAs significantly reduced GABAergic synaptogenesis, consistent with previous studies (Chih et al., 2005; Fu and Vicini, 2009). The function of NL2 in regulating GABAergic synapse formation is likely mediated by interactions with scaffolding protein gephyrin and collybistin (Poulopoulos et al., 2009). All these studies are consistent with a centric role of neuroligins as cell adhesion molecules to regulate synapse formation and synaptic plasticity.

However, the interpretation of NL2 manipulations and phenotypes in synaptogenesis could be complicated by the effect of NL2 on the GABA functional switch as reported here. Developmental GABA switch and synaptogenesis both peak in the first two postnatal weeks in rodents. It has been proposed that depolarizing GABA action plays a permissive role in promoting both excitatory and inhibitory synaptogenesis
(Akerman and Cline, 2007). Disruption of GABA excitation prematurely by NKCC1 knockdown or KCC2 overexpression decreased glutamatergic synapse formation but increased GABAergic synapse formation (Chudotvorova et al., 2005; Akerman and Cline, 2006; Wang and Kriegstein, 2008). In vivo, KCC2 knockout reduces the number of mature glutamatergic synapses (Li et al., 2007); NKCC1 knockout decreases both glutamatergic and GABAergic transmission in newborn neurons in the adult brain (Ge et al., 2006). Therefore, the timing of GABA switch is important for proper synaptogenesis.

4.2.2 Novel function of neuroligin-2 in regulating KCC2 and GABA functional switch

The most surprising finding of this work is the regulation of KCC2 by NL2. I initially intended to investigate GABAergic synaptic changes after knockdown of NL2, but unexpectedly observed a significant decrease of the KCC2 expression level. I tested whether this might be caused by off-target effects of the shRNAs, but coexpression of NL2shRNA with KCC2 had no effect on the expression of KCC2 at all. Moreover, the shRNA-proof NL2*, but not NL1*, rescued the KCC2 expression, further suggesting that this is a NL2-specific regulation of KCC2. Similar to previously reported knockdown of KCC2 (Rivera et al., 1999), I discovered that after NL2 knockdown the GABA reversal potential was shifted to more depolarized state. Moreover, after NL2 knockdown bath application of GABA induced large Ca\(^{2+}\) influx and even triggered action potentials in relatively mature neurons, which is also similar to that reported in KCC2 knockout mice (Hubner et al., 2001). Together, NL2 not only regulates GABAergic synapse formation like previously demonstrated, but also regulates GABA excitation-inhibition functional
switch that has been the main function of KCC2. KCC2 is a Cl⁻ transporter with a major function in controlling intracellular Cl⁻ concentration and therefore playing an important role in determining whether GABA function is excitatory or inhibitory (Ben-Ari, 2002). Thus, my work greatly expands the function of NL2 beyond its classical role as a cell adhesion molecule.

Our pharmacological experiments with BIC and TTX, to block GABA$_A$R action and action potential firing respectively, demonstrated that developmental KCC2 up-regulation and GABA functional switch are independent of GABA$_A$R activation and neuronal activity, which is in agreement with previous studies (Ludwig et al., 2003; Titz et al., 2003). Then, what is responsible for KCC2 regulation during neuronal development? Our new findings suggest that KCC2 up-regulation requires NL2, a cell adhesion molecule that also undergoes developmental up-regulation independent of GABA$_A$R and neuronal activity. In vivo, NL2 and KCC2 expression are up-regulated significantly during early postnatal development, and NL2 expression precedes that of KCC2. Previous studies also reported that NL2 expression is first detected at embryonic day 16 (Varoqueaux et al., 2004) while KCC2 is first detected at postnatal day 1 (Rivera et al., 1999). It is likely that immature neurons, which have not received many innervations yet, up-regulate NL2 and KCC2 through cell-intrinsic mechanisms. However, neuronal activity may regulate NL2 and KCC2 expressions in mature neural network. For instance, hyper-excitation induced by 0 Mg$^{2+}$, 4-AP (a voltage-dependent K$^+$ channel blocker), or glutamate exposure downregulates KCC2 and induces a positive shift in $E_{\text{GABA}}$ (Rivera et al., 2004; Lee et al., 2011). It may provide new therapeutic
targets by investigating how NL2 expression is altered and whether NL2 regulates KCC2 under pathologic conditions.

4.2.3 Neuroligin-2 regulates glutamatergic synapses through KCC2

Although NL2 is mainly localized at GABAergic synapses (Varoqueaux et al., 2004), NL2 overexpression increased both glutamatergic and GABAergic synapse formations (Chih et al., 2005; Levinson et al., 2005). This was explained by that NL2 is mislocated to glutamatergic synapses after NL2 overexpression or the overexpression of glutamatergic postsynaptic protein PSD-95 (Prange et al., 2004; Levinson et al., 2005). However, it was quite puzzling to understand how knockdown of NL2 significantly reduced GABAergic synapses as well as glutamatergic synapses, which was also reported in the original study using the same NL2shRNA (Chih et al., 2005). On the other hand, KCC2 has been found by different groups to regulate glutamatergic events and dendritic spinogenesis independent of its transporter activity (Li et al., 2007). Specifically, KCC2 interact with actin cytoskeleton through protein 4.1N to regulate spine morphology and also AMPA receptor surface diffusion (Li et al., 2007; Gauvain et al., 2011; Fiumelli et al., 2012). In light of our current finding, the effect of NL2shRNAs glutamatergic synapses is likely mediated by KCC2.
4.2.4 Functional separation of Neuroligin-2 in regulating glutamatergic versus GABAergic synapse formation

Apparently, neuroligin-2 regulates GABAergic and glutamatergic synapse formation through two distinct and separate pathways: neuroligin-2 directly regulates GABAergic synapse formation by cell adhesion; neuroligin-2 indirectly regulates glutamatergic synapse formation through KCC2. Supporting this idea, KCC2 overexpression partially rescued decreased glutamatergic transmission and reduced dendritic spine number, but not decreased GABAergic transmission, induced by NL2 knockdown; knockdown of KCC2 in developing neurons decreases the frequency of glutamatergic transmission but does not change the frequency of GABAergic transmission (Gauvain et al., 2011; Succol et al., 2012).

The reduction in both glutamatergic and GABAergic transmission after NL2 knockdown are seemingly in conflict with previous in vivo data showing that GABAergic, but not glutamatergic, transmission was decreased in NL2 knockout mice (Poulopoulos et al., 2009). However, the discrepancy could well be explained by the different scenarios in global genomic deletion and acute shRNA-mediated knockdown. A very recent study clearly demonstrated that the transcellular differences in the relative amounts of NL1, rather than the absolute NL1 amount, governs the number of excitatory synapses in vivo (Kwon et al., 2012), which may also be the case for NL2. Strategies that manipulate NL2 level in a subset of neurons in vivo, such as in utero electroporation, will further elucidate the role of NL2 in both glutamatergic and GABAergic synaptogenesis.
4.2.5 The significance of KCC2 regulation by NL2

My discovery of the NL2 regulation of KCC2 potentially connects some interesting previous findings regarding NL2 and KCC2 functions, which were either seemingly unrelated or sometimes difficult to comprehend. For example, overexpression of NL2 in cerebellar granule cells has been shown to accelerate GABAergic synapse maturation by promoting the switch of GABA\(_A\)R \(\alpha\) subunits from \(\alpha3\) to \(\alpha1\) during early development (Fu and Vicini, 2009). Interestingly enough, overexpression of KCC2 in cerebellar granule cells was also found to accelerate the switch from \(\alpha3\) to \(\alpha1\) subunit (Succol et al., 2012). With my finding that NL2 may directly regulate KCC2, the similar function of NL2 and KCC2 in promoting \(\alpha\) subunit switch is expected to some extent, although we do not exclude other possible links. The regulation of KCC2 and therefore intracellular Cl\(^-\) concentration by NL2 may constitute a new mechanism in regulating the strength of GABAergic transmission. Therefore, NL2 orchestrates many steps in the establishment of the GABAergic system: initial synapse formation, recruitment of postsynaptic proteins, differential expression of GABA\(_A\)R subunits, and the regulation of Cl\(^-\) driving force.

As discussed earlier, the regulation of KCC2 by NL2 may underlie the effect of NL2shRNA on glutamatergic synapses. Therefore, NL2 may regulate both GABAergic and glutamatergic synapse formation. The molecular mechanism of the regulation of KCC2 by NL2, however, is currently unknown. Future study investigating the mRNA level, phosphorylation, and oligomerization state of KCC2 after knockdown of NL2 or in NL2 knockout mice will be necessary.
To conclude, my study supports a central role of NL2 in regulating the GABAergic system from initial synapse formation to functional maturation. With their unique functions in cell adhesion, Cl− extrusion, and spine morphogenesis, NL2 and KCC2 are suitable candidate molecules in coordinating GABA functional switch and synaptogenesis in developing neurons and dynamically balance excitatory and inhibitory neurotransmission in the adult brain.
Chapter 5

Concluding Remarks
While there are much more glutamatergic neurons than GABAergic neurons in the brain, the small population of GABAergic neurons (~20%) may play a determinant role in the regulation of network activities. A subpopulation of GABAergic neurons has been proposed as hub neurons in developing hippocampal networks: GABAergic hub neurons formed extensive connections with other neurons and the activity of a single GABAergic hub neuron greatly influenced the entire network (Bonifazi et al., 2009). In neocortex, somatostatin-positive GABAergic interneurons innervated almost all glutamatergic pyramidal neurons in the neighborhood (Fino and Yuste, 2011). Notably, loss of MECP2, which mutations cause Rett syndrome (a monogenic autism), in all or a subset of GABAergic neurons reduced GABAergic transmission and was able to recapitulate many symptoms of Rett syndrome (Chao et al., 2010). Therefore, dysfunction of even a small percentage of GABAergic neurons or GABAergic synapses may severely affect brain function, which may underlie neuropsychiatric disorders that are accompanied with rather subtle neuronal or synaptic loss.

As the major cell adhesion molecule at GABAergic synapses, neurexin-2 plays a central role in regulating GABAergic synapse formation. In Chapter 3, my work demonstrated the functional defects of novel schizophrenia-linked neurexin-2 mutations, suggesting that deficits in GABAergic synapse formation confer risk for neuropsychiatric disorders.

Transgenic mouse line carrying the R215H point mutation has been generated in our laboratory to investigate its potential as animal model of schizophrenia. Preliminary data revealed a significant reduced protein level of mutant neurexin-2 in the R215H knockin mice, suggesting that neurexin-2 R215H mutant is unstable and degraded in
In vivo. In combination with neuroligin-2 knockout mice, behavioral studies of R215H knockin mice may provide new insights in the etiology of schizophrenia and related disorders.

In Chapter 4, my discovery of a novel role of neuroligin-2 in regulating KCC2 and GABA functional switch greatly extends the function of neuroligin-2 in maintaining excitation/inhibition balance, beyond its classical role as a cell adhesion molecule. Neuroligin-2 not only regulates the development of the GABAergic system, from synapse formation to functional maturation, but also orchestrates GABAergic versus glutamatergic synapse formation (Fig. 5-1A). As a result, neuroligin-2 plays a central role in balancing excitation and inhibition in neural networks (Fig. 5-1B).

Given the present new findings, it may be postulated that the regulation of KCC2 by neuroligin-2 may add another layer of the link between neuroligin-2 and schizophrenia. KCC2 expression has been shown to be differentially expressed in schizophrenia patients (Hyde et al., 2011; Tao et al., 2012). DISC-1 (Disrupted-in-Schizophrenia 1), a well-known risk gene for schizophrenia, has also been shown to interact with GABA functional switch and contribute to schizophrenia susceptibility (Kim et al., 2012).
Figure 5-1. Schemes illustrating NL2 regulates excitation and inhibition balance.

A. A working model showing NL2 coordinates GABA functional switch and synaptogenesis. Immature neurons up-regulate NL2 and KCC2 through cell autonomous mechanisms and NL2 is required for KCC2 up-regulation. NL2 promotes GABAergic synapse formation by cell adhesion; KCC2 decreases intracellular Cl\(^-\) concentration, renders GABA inhibitory, and promotes dendritic spine formation. With knockdown of NL2, KCC2 up-regulation is abolished and GABA stays excitatory. Both glutamatergic and GABAergic synapse formation are reduced. Inlet traces indicate differential GABA evoked membrane potential changes in neurons with or without sufficient NL2 expression. B. Scheme illustrating NL2 regulates excitation and inhibition (E / I) balance in the brain.
The implication of neuroligin-2 dysfunction is likely not limited to schizophrenia. The reduction of KCC2 expression and the reappearance of GABA excitation in the adult brain have been reported by many groups under epileptiform activity. Transgenic mice with reduced KCC2 expression also display propensity to epilepsy (Blaesse et al., 2009). Intriguingly, epileptiform activity in mice has recently been shown to induce neuroligin-1 cleavage. My data showing the cleavage of neuroligin-2 by matrix metalloproteinase indicate that down-regulation of KCC2 in epilepsy maybe mediated by neuroligin-2 cleavage. The cleavage level of neuroligin-2 in animal model of epilepsy, such as pilocarpine model of epilepsy, would be of great interest to study. In addition, application of drugs inhibiting neuroligin-2 cleavage, will address whether targeting neuroligin-2 cleavage has therapeutic benefits in treating epilepsy.
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