MODELING NEURODEVELOPMENTAL DISORDERS:

EXPRESSION OF NEUROLIGIN ADHESION MOLECULES IN VIVO

by

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ABSTRACT

At post synaptic sites, the neuroligin (NL) family of proteins is thought to play an important role in synapse maturation, and regulation of excitatory and inhibitory synapses. Being selectively enriched at either excitatory (NL1,3) or inhibitory (NL2) synapses, NL’s have been shown to regulate the ratio of excitation to inhibition (E/I ratio), a process critical for normal brain development. In addition, NLs have been linked to neurodevelopmental disorders through genetic studies. To advance our understanding of synaptic regulation by NLs, and their potential role in synaptic dysfunction in neurodevelopmental disorders, we have developed strains of transgenic mice which overexpress either HA tagged-NL1, or -NL2 under control of the Thy1 promoter.

Detailed behavioural analysis of TgNL2 mice revealed anxiety, stereotyped jumping behaviour, and impairments in social approach and reciprocal social interactions. These animals also displayed fronto-parietal seizure activity as shown by chronic in vivo EEG recording. Synapse analysis in TgNL2 frontal cortex revealed changes in the number and morphology of synapses compared to wildtype littermates. A small change in NL2 expression results in enlarged synaptic contact size and vesicle reserve pool and an overall reduction in the E/I ratio. In addition, the frequency of miniature inhibitory synaptic currents was also found to be increased in the frontal cortex of TgNL2 mice.

Behavioural assessment of TgNL1 mice revealed deficits in memory acquisition and retrieval in water maze paradigms. Golgi and electron microscopy analysis revealed changes in synapse
morphology indicative of increased maturation of excitatory synapses. In parallel, electrophysiological examination indicated a shift in the E/I ratio towards increased excitation. Further experiments revealed impairment in the induction of long term potentiation.

These data demonstrate that altered expression of members of the NL family in vivo leads to altered synapse number and morphology, which potentially underlies the profound behavioural changes. We also observed a predominant effect of NL2 expression on inhibitory synapses, with NL1 primarily influencing excitatory synapses, supporting the idea that NL’s may act to regulate the E/I ratio. In addition this data may provide insight into the pathology and symptoms of neurodevelopmental disorders such as autism thought be be caused by synaptic abnormalities.
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ABBREVIATIONS

AMPA - \( \alpha \)-amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid  
AKAP - A-Kinase-Anchoring Protein  
BDNF - Brain-Derived Neurotrophic Factor  
CNS - Central Nervous System  
CNTNAP2 - Contactin Associated Protein-Like  
DLG - Discs Large  
EAAT - Excitatory Amino Acid Transporter  
EEG - Electroencephalography  
E/I - Excitatory/Inhibitory  
EM - Electron Microscopy  
EMG - Electromyography  
EVH1 - Enabled (Ena) / Vasodilator-stimulated phosphoprotein (VASP) Homology 1  
GABA - \( \gamma \)-Aminobutyric Acid  
GABARAP - GABA\( \alpha \) -Receptor-Associated Protein  
GAP - GTPase Activating Protein  
GK - Guanylate Kinase  
GKAP - Guanylate Kinase Associated Protein  
GLR - Glycine Receptor subunit  
GluR - Glutamate Receptor  
GRIP - Glutamate Receptor Interacting Protein  
HA - Heamagglutinin  
IEG - Immediate Early Gene  
IP\(_3\) - Inositol Tri-Phosphate  
KA - Kainic Acid receptor subunit  
KO - Knock-Out  
LNS - Laminin, Neurexin, Sex-hormone-binding protein  
LTD - Long-Term Depression  
LTP - Long-Term Potentiation  
MAGUK - Membrane-Associated Guanylate Kinase  
mGluR - metabotropic Glutamate Receptor  
Munc - Mammalian homologue of Uncoordinated (UNC)  
NARP - Neuronal Activity-Regulated Pentraxin  
NL - Neuroligin  
NMDAR - N-methyl-D-aspartic acid Receptor  
NMJ - Neuromuscular Junction  
NP - Neuronal Pentraxin  
NR - NMDA Receptor subunit  
NRX - Neurexin  
NSF - N-ethylamide Sensitive Factor  
PBH - Piccolo Bassoon Homology domain  
PCR - Polymerase Chain Reaction
PDZ - PSD-95, Dlg and ZO-1
PICK - Protein Interacting with C Kinase
PKA - cAMP-dependent protein kinase
PSD - Postsynaptic Density
RIM - Regulating synaptic Membrane Exocytosis
SALM - Synaptic Adhesion-Like Molecules
SAM - Sterile α Motif
SAP - Synapse-Associated Protein
sER - Smooth Endoplasmic Reticulum
SH3 - Src-Homology-3 domain
siRNA - small interfering RNA
S-SCAM - Synaptic Scaffolding Molecule
SLC - Solute Carrier
SNARE - Soluble NSF Attachment Receptor
STV - Synaptic Transport Vesicles
SYN - Synaptophysin
SynCAM - Synaptic Cell Adhesion Molecule
TARP - Transmembrane AMPA Receptor-binding Protein
TgNL1 - Transgenic mice expressing Neuroligin 1
TgNL2 - Transgenic mice expressing Neuroligin 2
TM – Transmembrane
UNC - Uncoordinated
VAMP - Vesicle-Associated Membrane Protein
VGAT - Vesicular GABA Transporter
VGluT - Vesicular Glutamate Transporter
Wt - Wildtype
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Whether it has been in the lab, in the mountains, or in life,

you have been my constant companion, and often my guide,

in adventure and discovery.
CO-AUTHORSHIP STATEMENT

I was involved in the original conception and planning of all of the experiments involved with this thesis. For the generation of transgenic mice, I was involved in preparation and purification of DNA fragments for pronuclear injection, establishing a working PCR genotyping procedure, and screening of all potential founders. Once founders were identified, I set up the mating pairs, established all of the strains, and characterized the expression of the transgenes using Western blotting and immunohistochemistry in each strain. I then subjected all of the strains to the preliminary behavioural screen, and conducted all behavioural assessments on neuroligin 2 transgenic mice. I conducted immunohistochemistry for HA and synaptic proteins in neuroligin 2 transgenic mice, and assisted with all of the immunohistochemistry on neuroligin 1 transgenic mice. Also, I completed all electron microscopy tissue preparations and imaging for both strains of mice. I was involved in analysis of the data and figure preparation for all of the experiments, except water maze and electrophysiology, as listed below. The neuroligin 2 manuscript was co-authored by Dr. Alaa El-Husseini and myself, while the neuroligin 1 manuscript was written by Dr. Regina Dahlhaus and myself, with assistance from Dr. Brian Christie and Brennan Eadie.

Regina Dahlhaus assisted with Western blotting for neuroligin 2 transgenic mice, and for neuroligin 1 transgenic mice, she conducted Western blotting, golgi staining, and water maze assessment. Longjun Wu and Hendrik Steenland conducted electrophysiological assessments of neuroligin 2 transgenic mice. Brennan Eadie and Timal Kannangara conducted electrophysiological assessment of neuroligin 1 transgenic mice.
1. INTRODUCTION

General Introduction

One of the distinctive features of the central nervous system (CNS) is its ability to transmit signals through specialized contacts called synapses. Estimates state that the human brain is composed of approximately ten billion neurons, each with the capacity to participate in thousands of connections. This means that the nervous system is required to assemble up to $10^{13}$ interconnections based on the genetic programs of CNS cells. Further, following the transcription and translation of proteins in the cell body, complex mechanisms exist to govern the proper trafficking and compartmentalization of these proteins to pre and post synaptic sites independently (Figure 1.1A,B). The formation of these contacts does not occur at random, but instead is governed by precise and tightly regulated mechanisms that dictate the location, number and type of synapses formed (Rao et al., 1998; Sanes and Lichtman, 2001; Li and Sheng, 2003; Kim and Sheng, 2004; Waites et al., 2005). This concerted effort leads to the formation of highly complex, yet highly reproducible synaptic networks.

Individual synapses are composed of complex and highly organized arrays of interacting proteins that facilitate synaptic transmission (Figure 1.1C). The importance of tight regulation of synaptic transmission can be gleaned from the number of CNS disorders arising from alterations to synapses (Holmes and McCabe, 2001; Zoghbi, 2003). Excitatory synaptic transmission is driven mainly by glutamatergic synapses whereas inhibitory synaptic transmission involves $\gamma$-aminobutyric acid (GABA) and glycine signaling. Excitatory and inhibitory synapses are composed of largely distinct collections of scaffolding and signaling molecules, whose recruitment is thought to be governed by distinct mechanisms (Craig et al.,
Figure 1.1. Structural and molecular complexity of synaptic contacts, demonstrating the orchestration required for assembly and function of synaptic contacts. A. Synapse formation requires the transcription and translation of appropriate proteins (1), transport of these proteins to sites of nascent synaptic contact (2), and finally matching, assembly, and maturation into function units competent for signaling (3). B. Electron microscope image illustrating the basic structure of synaptic contacts. The presynaptic compartment contains numerous neurotransmitter vesicles visible via electron microscopy. The postsynaptic compartment of excitatory synaptic contacts also contains a postsynaptic density (PSD) visualized via electron microscopy as a fuzzy thickening of the postsynaptic membrane. C. Assembly of molecules in the PSD of excitatory synaptic contacts, demonstrating the complexity of molecular interactions that support synaptic structure and signalling. Figure from Hines and El-Husseini, Molecular Mechanisms of Synaptogenesis, page 68.
Together, excitatory and inhibitory systems provide the framework for the transmission of signals in the brain. This is conceptualized as a “balance” between the function of excitatory and inhibitory synapses (E/I ratio), and is established during development and maintained throughout life (Turrigiano and Nelson, 2004; Cline, 2005; Levinson and El-Husseini, 2005a). Individual neurons are equipped with mechanisms for the approximation and maintenance of their E/I ratio of synaptic input/output. Perturbations in the E/I ratio have been postulated to underlie a number of nervous system disorders, such as epilepsy, mental retardation and autism (Rubenstein and Merzenich, 2003).

During the last ten years of research, several important discoveries have defined some of the critical processes that govern the development and maturation of excitatory and inhibitory synapses (Sanes and Lichtman, 1999; Friedman et al., 2000; Waites et al., 2005). A great deal of work suggests that families of cell adhesion molecules are key players in the orchestration of numerous molecular and cellular events involved in the maturation of diverse synapse types (Brose, 1999; Rao et al., 2000b; Dean et al., 2003; Missler, 2003; Washbourne et al., 2004; Gerrow and El-Husseini, 2006). However, despite significant progress, several important questions remain. In particular, in vivo experiments will be important to clearly define the roles of different families of proteins thought to be involved in different aspects of synapse development, including contact initiation, target recognition, synapse maturation and plasticity.
Background and Current Knowledge

1.1. Synaptic Structure

Synapses are highly specialized junctions between two cells that provide the structural and functional framework for the transmission of signals in the brain. Under normal conditions, the number, location, and type of synapses formed are well controlled parameters, and consequently reliable circuits are formed. This fact suggests that precise cellular and molecular mechanisms exist to determine the connectivity of networks in the CNS. Within the CNS, several distinct classes or types of synapses exist, which are composed of distinct collections of molecules. Despite these distinct molecular profiles, neurons are capable of properly recruiting molecules and matching pre and post synaptic compartments (O’Brien et al., 1998; Friedman et al., 2000; Aoki et al., 2001; Craig and Boudin, 2001; Ziv, 2001; Nimchinsky et al., 2002; Kim and Sheng, 2004; Craig et al., 2006). The unparalleled complexity of intercellular connections that is seen in the CNS presents a requirement for high levels of both specificity and diversity in molecular constituents. Research into the mechanisms regulating these complex processes will lead to increased understanding of the formation of neural networks, and the function of the brain as a whole. Further, since synapse abnormalities have been implicated in a wide variety of disorders of the CNS, understanding the fundamental principles that govern the formation and maturation of synapses will contribute to our understanding of CNS dysfunction. An immense body of work that has accumulated in recent years has begun to unravel the key players and processes involved in controlling the formation of synapses and the construction of CNS networks.
In terms of both their structure and function, synapses are asymmetric, which distinguishes them from other types of intercellular junctions. The presynaptic side exists as an enlargement of the axon, and is filled with specialized vesicles containing neurotransmitter (Figure 1.1C). These specialized vesicles reside in what are considered to be distinct populations of docked and reserve pools. Docked vesicles sit in the presynaptic active zone, a specialized section of the presynaptic membrane studded with complex matrices of SNARE and related proteins critical for the fusion of vesicles and the release of neurotransmitter. The active zone can be roughly visualized in an electron micrograph as a subtle thickening of the presynaptic membrane (Figure 1.1B). Reserve pool vesicles reside away from the presynaptic membrane, and are considered to replace docked vesicles following release triggered by the arrival of an action potential. The voltage change of an action potential activates voltage-dependent calcium channels and results in an influx of calcium ions. Calcium ions act as the trigger for a biochemical cascade that culminates with SNARE complex-mediated fusion of vesicles with the presynaptic membrane (Rosenmund et al., 2003; Stevens, 2003; Schneggenburger and Neher, 2005). Neurotransmitter that is released spills into the space between the pre and postsynaptic compartments, called the synaptic cleft, and diffuses across the small gap to the membrane of the postsynaptic compartment. Postsynaptic compartments are specialized regions of neuronal cell bodies, dendrites or axon initial segments, and can also be found on muscle cells or cells that form the tissue of a gland. The most common type of synapse in the CNS is formed between a presynaptic axon and a postsynaptic dendrite. Similar to the presynaptic membrane, the postsynaptic membrane is also studded with matrices of interacting proteins; however, the protein complexes are specialized for the binding of neurotransmitter, and the subsequent transmission of the signal. At excitatory synapses, this
network of interacting proteins is termed the postsynaptic density (PSD), and can be observed in electron micrographs as an electron dense thickening of the postsynaptic membrane (Figure 1.1B). Postsynaptic protein complexes are composed of neurotransmitter receptors, and numerous scaffolding, cytoskeletal and signalling proteins (Figure 1.1C; (Kennedy, 1997; Kornau et al., 1997; Boeckers, 2006; Okabe, 2007).

1.2. Comparing Excitatory and Inhibitory Synapses

Whether a synapse is excitatory or inhibitory depends on what types of ion channel conductance the neurotransmitters and receptors activate. In general, synaptic receptors are activated by the binding of neurotransmitter molecules, and respond by opening nearby ion channels in the postsynaptic membrane. This opening of ion channels causes ions to flow down their concentration gradients, and result in changes to the local membrane potential. The resulting change in voltage is called a postsynaptic potential. In general, if the postsynaptic potential is decreased or depolarizing, the result is excitation of the postsynaptic cell; whereas if the potential is increased or hyperpolarizing, the result is inhibition of the postsynaptic cell. Hyperpolarization or inhibition makes it more difficult for the postsynaptic cell to fire an action potential, lowering the firing rate of the neuron. In contrast, depolarization or excitation makes it easier for the postsynaptic cell to fire an action potential. Depolarization is typically mediated by the influx of cations such as sodium and calcium, while hyperpolarization is typically mediated by influx of chloride ions and/or efflux of potassium ions. It is important to note that changes in the postsynaptic potential depend upon the initial concentrations of ions both inside and outside the cell, and thus do not always conform to these typical conditions.
Excitatory and inhibitory synapses can be distinguished both by their typical structures, and also by the complement of proteins that they contain (Figure 1.2). Two distinct types of synapses were originally identified using electron microscopy and were distinguished on the basis of synaptic vesicle and PSD morphology (Uchizono, 1965). Asymmetric or type I synapses, which are typically excitatory, were identified by the presence of a post-synaptic density and round synaptic vesicles within the presynaptic terminal. In contrast, symmetric or type II synapses, which are typically inhibitory, were identified by the presence of both oval or flattened, and round synaptic vesicles and the absence of a pronounced post-synaptic density.

In terms of their molecular content, excitatory and inhibitory synapses differ in terms of the neurotransmitter they contain, the presynaptic machinery for transporting or packing the various neurotransmitters into vesicles, the adhesion systems that link pre and post, the types of postsynaptic receptors they are composed of, and the specific scaffolding proteins that bind to and support the receptors. Despite numerous differences, excitatory and inhibitory synapses do share many common molecular components, such as the machinery required for vesicle fusion and neurotransmitter release, and some common signalling molecules. The major categories of molecules that make up both excitatory and inhibitory synapses will be discussed in detail below.

1.3. The Molecular Composition of Synapses

Synapses are composed of a diverse collection of proteins that interact and participate in concert for the transmission of signals in the CNS. Numerous proteins have been identified at
Figure 1.2. Comparison of key structural and molecular differences between excitatory and inhibitory synaptic contacts. A. Excitatory synaptic contacts, also referred to as asymmetric synapses, are characterized by a pronounced thickening of the postsynaptic membrane known as the PSD, and typically occur onto spines that protrude from the dendrites of the postsynaptic neuron. B. In terms of molecular constituents, excitatory synapses typically contain neurotransmitter vesicles packed with glutamate, via the vesicular glutamate transporter (VGlut), and receptors that are activated by glutamate release from the presynaptic terminal (AMAPR and NMDAR). In addition to these key signalling components, excitatory synapses also specifically contain the postsynaptic scaffolding protein PSD-95, and are enriched with the adhesion molecule NL1 (postsynaptic) typically bound to β-Nrx lacking the insert at splice site 4 (S4; presynaptic). C. Inhibitory synaptic contacts, also called symmetric synaptic contacts are characterized by symmetry in the thicknesses of pre and postsynaptic membranes, and most commonly occur on the dendritic shaft. D. Inhibitory synapses contain GABA packed neurotransmitter vesicles and the vesicular GABA transporter (VGAT), along with GABA receptors (may also contain glycinergic signalling components). In addition, inhibitory synapses are characterized by the presence of the scaffolding molecule gephyrin, and are enriched with NL2 (postsynaptic) adhesion molecules which typically bind α-Nrx and/or β-Nrx with insertion at splice site 4 (S4; presynaptic).
synaptic sites, most of which can be categorized into one of several classes: receptors, scaffolding proteins, signalling molecules, adhesion molecules, or vesicle release/active zone machinery. Insight into the molecular complexity of synaptic specializations can be gleaned from the fact that the presynaptic compartment alone contains greater than 1000 proteins (Olsen et al., 2006). An overview of the major families of proteins located at synapses provides insight into the orchestration required to make and maintain functional synaptic connections. These proteins act in concert, with each family of proteins contributing specialized features to synapse formation and signalling.

**Postsynaptic Components**

1.3.1. **Glutamate Receptors**

Glutamate is the major excitatory neurotransmitter in the brain. There are multiple classes of glutamate receptors, which can be categorized as to whether they contain an ion channel pore or not, or alternatively by the ligand that binds them with highest affinity (Dingledine et al., 1999). In the broadest categorization, glutamate receptors are classified as ionotropic, containing a selective pore that is opened upon ligand binding, or as metabotropic, which do not contain an ion channel pore, but instead activate signal transduction mechanisms often involving G proteins. Ionotropic glutamate receptors can further be subdivided into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), N-methyl D-aspartate (NMDA) or kainate subtypes.
1.3.1.1. AMPA Receptors

AMPA receptors mediate fast synaptic transmission in the CNS. The name for this class of receptors originates from its ability to be activated by the artificial glutamate analog, AMPA. AMPA receptors are found in many parts of the brain and are the most abundant receptor type in the nervous system. AMPA receptors are composed of four types of subunits, designated as GluR1, GluR2, GluR3, and GluR4, which form tetramers of various subunit combinations. Most AMPA receptors are heterotetrameric, composed of a symmetrical dimer of dimers typically consisting of GluR2 and either GluR1, GluR3 or GluR4 (Mayer, 2005). Functional AMPA receptors exist in two basic forms: calcium-impermeable and calcium-permeable channels. The ion permeability is dictated by the presence of the GluR2 subunit, which renders heteromeric AMPA receptor complexes impermeable to calcium.

Each subunit is composed of four transmembrane domains as predicted by the amino acid sequence (Mayer, 2005). However, it was determined that the second transmembrane domain does not fully emerge on the extracellular side, but folds back on itself within the membrane. When the four subunits of the tetramer are assembled together, this second membrane domain forms the ion-permeable pore of the receptor. Each AMPA receptor subunit has one site to which an agonist can bind, creating a total of four binding sites per receptor complex (Mayer, 2005). The binding site is believed to be formed by the N-terminal tail, and the extracellular loop between transmembrane domains three and four (Armstrong et al., 1998; Dingledine et al., 1999; Mayer, 2005). When the agonist binds, the loops move towards each other, leading to opening of the pore. Occupation of two sites is required for channel opening, and as more binding sites are occupied the current increases. Once open, the channel may undergo rapid desensitization by a change in the angle of one portion of the binding site,
closing the pore and stopping the current (Armstrong et al., 2006). AMPA receptor function is modulated by alternative RNA splicing and editing, as well as phosphorylation at multiple sites. Furthermore, AMPA receptor subunits diverge most in the sequences of their C-terminal regions, dictating their interactions with different synaptic scaffolding proteins. Differences in interacting proteins have been shown to dynamically regulate the targeting and trafficking of AMPA receptor subunits at dendritic spines.

1.3.1.2. NMDA Receptors

NMDA-type glutamate receptor number at the synapse is relatively static, in contrast to AMPA-type glutamate receptor cycling, which has been shown to be very dynamic and regulated by synaptic activity. NMDA-type receptor subunits include NR1, NR2A-D, and NR3. Functional NMDA receptors are composed of a heterodimer of NR1 and NR2 subunits, typically two obligatory NR1 subunits and two NR2 subunits A-D depending on the brain region (Dingledine et al., 1999; Stephenson, 2001, 2006). Ligand binding is mediated by the globular extracellular domain which binds the co-agonist glycine in NR1 subunits, while NR2 subunits bind glutamate. The membrane domain, consisting of three trans-membrane segments and a re-entrant loop, makes up the channel pore, and is responsible for the receptor's conductance, calcium permeability, and voltage-dependent block by magnesium (Stephenson, 2001). Similar to AMPA receptor subunits, each NMDA subunit has an extensive cytoplasmic domain that can be modified by phosphorylation, and contains domains for interaction with a large number of adaptor and scaffolding proteins.
1.3.1.3. Kainate Receptors

Kainate receptors are less well understood than AMPA and NMDA receptors. Kainate receptors have been shown to be involved in excitatory neurotransmission by activating postsynaptic receptors, and in inhibitory neurotransmission by presynaptically modulating GABA release. There are five types of kainate receptor subunits, GluR5-7, and KA1 and KA2, which combine to form tetramers of various compositions (Dingledine et al., 1999; Huettner, 2003). GluR5-7 can form homomers and heteromers, however, KA1 and KA2 can only form functional receptors by combining with one of the GluR5-7 subunits. Each subunit is composed of an extracellular N-terminal segment, which forms part of the neurotransmitter binding site. Each subunit also contains three membrane spanning regions, with one segment that does not fully cross, similar to the closely related AMPA receptor subunits, which also determines the calcium permeability of kainate receptors. The ion channel pore of kainate receptors is permeable to sodium and potassium ions, with similar conductance to that of AMPA receptors (Huettner, 2003). However, the duration of pore opening is much shorter compared to AMPA receptors. In addition to sodium and potassium, kainate receptors can be permeable to calcium, depending upon their subunit composition and RNA editing. Unlike AMPA receptors, kainate receptors play only a minor role in signalling at synapses (Song and Huganir, 2002; Huettner, 2003).

1.3.1.4. Metabotropic Glutamate Receptors

In addition to producing excitatory and inhibitory postsynaptic potentials, mGluRs serve to modulate the function of other receptors (such as NMDA receptors), changing the synapse's excitability. The metabotropic glutamate receptor (mGluR) family currently includes eight members, divided into three groups based on their sequence homology and enzyme specificity.
(Ferraguti and Shigemoto, 2006). mGluR₁ and mGluR₅ (group I) activate a G-protein coupled to phospholipase C, which hydrolyzes phospholipids in the plasma membrane. Group I receptors are also associated with sodium and potassium channels. Activation of group I mGluRs can result in increased excitation or increased inhibition, and they can also act to inhibit glutamate release and modulate voltage dependent calcium channels. mGluR₂, ₃, and ₅ receptor (group II) subunits favour activation by trans-1-aminocyclopentane-1,3-dicarboxylate, whereas 1,2-amino-4-phosphonobutyrate is the ideal agonist of mGluR₄, ₆, and ₇ receptor subunits (group III). Group II and III mGluRs prevent the formation of cyclic adenosine monophosphate, (cAMP), by activating a G protein that inhibits the enzyme adenylyl cyclise. These classes of receptors are involved in presynaptic inhibition, and reduce the activity of both excitatory and inhibitory postsynaptic potentials in the cortex. Like other metabotropic receptors, mGluRs are composed of seven transmembrane domains. mGluRs have been shown to modulate NMDA receptor function (Ferraguti and Shigemoto, 2006), and are coupled closely to NMDA receptors via scaffolding proteins (Tu et al., 1999).

1.3.2. γ-aminobutyric Acid and Glycine Receptors

The most common receptor families mediating inhibitory neurotransmission are those that are activated by γ-aminobutyric acid (GABA) and those that are activated by glycine. GABA receptors are divided into three classes, GABAₐ and GABAₐc receptors which are ligand-gated ion channels, and GABAₐ receptors which are G protein-coupled (Bormann, 2000). Both GABA and glycine receptors share structural and functional features.
1.3.2.1. GABA<sub>A</sub> and GABA<sub>C</sub> Receptors

GABA<sub>A,C</sub> receptors are members of family of Cys-loop ligand-gated ion channels characterized by a loop formed by a disulfide bond between two cysteine residues. This family is composed of five subunits arranged around a central chloride permeable pore. There are numerous subunit isoforms for the GABA<sub>A</sub> receptor, which determine the receptor’s properties, including agonist affinity, chance of opening, and conductance. There are six types of α subunits (GABRA1-6), three β's (GABRB1-3), three γ's (GABRG1-3), a δ (GABRD), an ε (GABRE), a π (GABRP), and a θ (GABRQ; Bormann, 2000). Although the subunits arrange in various conformations, the most common type in the brain is a pentamer of two α's, two β's, and a γ (α<sub>2</sub>β<sub>2</sub>γ; Bormann, 2000). The receptor binds two GABA molecules, at the interface between an α and a β subunit. The GABA<sub>A</sub> channel opens quickly and thus contributes to the early part of the inhibitory post-synaptic potential (Couve et al., 2000).

GABA<sub>C</sub> receptors are exclusively composed of ρ subunits (GABRR1-3) that are related to GABA<sub>A</sub> receptor subunits (Bormann, 2000). Some view the GABA<sub>C</sub> receptor as a variant within the GABA<sub>A</sub> family; however, GABA<sub>C</sub> receptors are insensitive to the typical allosteric modulators of GABA<sub>A</sub> receptors including benzodiazepines and barbiturates. In contrast to the rapid GABA<sub>A</sub> receptor, GABA<sub>C</sub> receptors are slow to initiate but sustained in duration.

1.3.2.2. GABA<sub>B</sub> Receptors

GABA<sub>B</sub> receptors are metabotropic transmembrane receptors that are linked to potassium channels via G-proteins. Opening of potassium channels via signalling from GABA<sub>B</sub> brings the neuron closer to the equilibrium potential of potassium, hyperpolarising the neuron. GABA<sub>B</sub>
receptors can also reduce the activity of adenylyl cyclase and decrease the calcium conductance. GABA_8 receptors are in the same receptor family with mGluRs, and share common structural characteristics. There are two subtypes of the receptor, GABA_81 and GABA_82, and these appear to assemble as heterodimers (Bormann, 2000; Couve et al., 2000). GABA_8 receptors mediate a slow response to GABA binding.

1.3.2.3. Glycine Receptors

The glycine receptor is one of the most widely distributed inhibitory receptors in the CNS, and can be activated by a range of simple amino acids including glycine, β-alanine and taurine. Like GABA_A,C receptors, glycine receptors are also members of the Cys-loop family of Ligand-gated ion channels (Kirsch, 2006). Receptors of this family are arranged as five subunits surrounding a central pore, with each subunit composed of four transmembrane segments. There are four known isoforms of the glycine receptor α subunit (GLRA1-4), and one β subunit (GLRB; (Kuhse et al., 1993; Kuhse et al., 1995; Kirsch, 2006). α subunits are essential for ligand binding, and typical receptors are composed of three α_1 subunits and two β subunits, or four α_1 subunits and one β subunit (Kuhse et al., 1993; Kuhse et al., 1995; Kirsch, 2006).

1.3.3. Scaffolding Proteins

Each of the aforementioned receptor types must be properly accumulated within microdomains of the dendritic membrane, and are typically found accumulated in perfect opposition to presynaptic release of neurotransmitter. These microdomains are thought to be supported by accumulations of scaffolding proteins that provide an infrastructure of protein
interactions connecting the postsynaptic membrane and receptors to signalling molecules and the cytoskeleton. Scaffolding molecules are thought to stabilize both excitatory and inhibitory receptors by acting as a selective trap for receptors moving along the membrane (Nusser, 2000; Craig and Boudin, 2001). In addition to their role in diffusion trapping of surface receptors, scaffolding proteins have also been shown to undergo rapid exchange, leading to a dynamic equilibrium of receptor–scaffold complexes.

1.3.3.1. Membrane-Associated Guanylate Kinases: PSD-95 Family of Proteins

The membrane-associated guanylate kinase (MAGUK) family of proteins is of central importance to protein scaffolding in many cell types (Fanning and Anderson, 1999; Funke et al., 2005). MAGUKs are defined by the presence of a domain homologous to the yeast guanylate kinase (GK) domain, which is catalytically inactive. The GK domain is always preceded by a Src-homology-3 (SH3) domain. In addition, MAGUKs contain PDZ domains, which are named for the original proteins in which the motifs were identified (PSD-95, discs large, and zona occludens 1). PDZ domains bind the carboxyl-terminus of proteins or form dimers with other PDZ domain containing proteins. These domains are often arranged in tandem arrays or associated with other protein-protein interaction domains to form a protein scaffold (Fanning and Anderson, 1999). Protein multimerization may act to enhance clustering of binding proteins into large assemblies confined to specific sites, such as the PSD.

PSD-95 (postsynaptic density protein of 95 kDa) is the prototypical PDZ domain containing protein, and extensive research has been conducted on the structure and function of this
family of proteins (Irie et al., 1997; Migaud et al., 1998; Topinka and Bredt, 1998; Craven et al., 1999; El-Husseini et al., 2000a; El-Husseini A. E. et al., 2002). The PSD-95 family is encoded by four genes – PSD-95 / synapse associated protein 90 (SAP90), postsynaptic density protein of 93 kDa (PSD-93) / chapsyn-110, synapse associated protein 102 (SAP102), and synapse associated protein 97 (SAP97), which are characterized by 3 PDZ domains, in addition to the SH3 and GK domain characteristic of MAGUKs (Funke et al., 2005). PSD-95 family proteins are key players in regulating synaptic organization because of their capacity to assemble highly specific, yet dynamically adaptable protein complexes at synaptic sites.

1.3.3.2. Other Scaffolding Proteins of Glutamatergic Synapses

Several other scaffolding proteins have been identified at excitatory synapse, each with a unique role in maintaining organization of the postsynaptic density. The PDZ-containing glutamate receptor interacting protein GRIP1/ABP binds to the carboxy-terminal PDZ motif of AMPA receptor subunits GluR2 and GluR3 (Dong et al., 1997; Srivastava et al., 1998; Osten et al., 2000). It is thought that GRIP1/ABP may serve to link AMPA receptors as cargoes to the microtubule transport system via binding to liprin-α1 (Wyszynski et al., 2002). In turn, liprin-α1 interacts with a member of the kinesin superfamily KIF1, and GIT1, a GTPase-activating protein for the ADP-ribosylation factor family of small GTPases known to regulate protein trafficking and the actin cytoskeleton. Protein interacting with C kinase (PICK1) is another well characterized PDZ domain-containing protein. PICK1 interacts with the C-termini of AMPA receptors, and is found to be specifically colocalized with AMPA receptors at excitatory synapses (Daw et al., 2000; Perez et al., 2001). In addition to AMPA receptors, PICK has also
been shown to bind cell adhesion molecules including neuroligins (NLs) and nectins (Meyer et al., 2004; Reymond et al., 2005), to be discussed below.

Despite the identification of these proteins as modulators of AMPA receptor localization and function, immunoprecipitation and quantification of the proteins bound to AMPA receptors suggests that transmembrane AMPA receptor regulatory proteins (TARPs) are the major binding partner for AMPA receptors (Fukata et al., 2005). TARPs are transmembrane proteins with four membrane-spanning domains that interact with the transmembrane region of AMPA receptors (Chen et al., 2000; Dakoji et al., 2003; Tomita et al., 2004). This family of proteins includes stargazin, γ-3, γ-4, and γ-8, all of which influence the surface expression of AMPA receptors in different brain regions, and have also been shown to bind MAGUKs to participate in scaffolding of receptors at glutamatergic synapses (Chen et al., 2000; Schnell et al., 2002; Dakoji et al., 2003; Tomita et al., 2004).

The Shank family of proteins are scaffolding molecules that provide a link to the actin cytoskeleton (Sala et al., 2001; Sala et al., 2005). Shank proteins are composed of multiple ankyrin repeats, an SH3 domain, a PDZ domain, a long proline-rich region and a sterile α motif (SAM) domain. Homer proteins are interacting partners of Shank that act as multimeric adaptors (Tu et al., 1999). EVH1 (enabled (Ena) / vasodilator-stimulated phosphoprotein (VASP) Homology 1) domains in Homer interact with proline rich motifs in a variety of proteins. Shank and Homer mediate multiple protein interactions in the PSD providing important links between NMDA receptors and metabotropic glutamate receptors at the synapse, inositol trisphosphate receptors (IP3Rs) in the smooth endoplasmic reticulum (sER) and the actin cytoskeleton via cortactin (Tu et al., 1999; Sala et al., 2001; Sala et al., 2005).
1.3.3.3. Scaffolding Molecules of Inhibitory Synapses

The basic organization of inhibitory synapses is comparable to that of excitatory synapses. Glycine receptors and many, but not all, types of GABA receptors are accumulated at postsynaptic microdomains, where they are stabilized by subsynaptic scaffolding complexes (Moss and Smart, 2001). At inhibitory synapses, gephyrin represents the core protein of the postsynaptic scaffolding complex. Gephyrin is composed of two oligomerization domains (called G and E) connected by a linker region (Meyer et al., 1995; Fritschy et al., 2008). Gephyrin is thought to generate a reversible postsynaptic scaffold for the immobilization of glycine receptors and individual subtypes of GABA<sub>A</sub> receptors (Kneussel and Betz, 2000; Kneussel et al., 2001). Crystal structure generation of gephyrin oligomerization domains and the glycine receptor β subunit binding sites revealed that dimeric gephyrin interacts with glycine receptor β subunits (Meyer et al., 1995). This complex is then thought to multimerize and form a hexagonal lattice (Kneussel and Betz, 2000). In addition to gephyrin, GRIP1 has also been localized to inhibitory synapses, and has also been shown to act as a protein scaffold in both pre and postsynaptic compartments (Li et al., 2005). In contrast to excitatory synapses, somewhat less is known about the full complement of proteins involved in scaffolding of GABA and glycine receptors, and corresponding signalling molecules.

1.3.4. Signalling Molecules

The term signal transduction is used in biology to refer to any process by which a cell converts one kind of signal or stimulus into another. Signal transduction involves ordered sequences of
biochemical reactions carried out by enzymes, typically activated by second messengers, which are diffusible signalling molecules that can be rapidly generated or released and go on to activate effector proteins.

With relevance to synaptic transmission, phosphorylation is a central enzyme signalling system that is dynamically mediated by protein kinases and phosphatases. Phosphorylation is the transfer of phosphate groups from high-energy donor molecules, such as ATP, to specific residues in the amino acid sequences of target substrates. Many proteins contained within synaptic compartments contain sites for dynamic regulation of their structure and function by phosphorylation. Kinases and phosphatases are regulated by synaptic activity and have been shown to regulate numerous synaptic parameters ranging from postsynaptic receptor trafficking and localization (Roche et al., 1994; Strack et al., 2000; Oh et al., 2006), to presynaptic regulation of exocytosis (Foster et al., 1998; Fletcher et al., 1999; Evans and Morgan, 2003; Snyder et al., 2006).

**Presynaptic Components**

**1.3.5. SNARE Proteins and Vesicle Release Machinery**

The major group of proteins involved in vesicle fusion and neurotransmitter release is the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) family of proteins. The SNARE family includes more than 60 members found in yeast and mammalian cells, and is involved in the fusion of cellular vesicles with various membranous targets such as the cell membrane, lysosomes or porosomes (Sorensen, 2005; Rizo and Rosenmund, 2008). SNAREs are generally divided into two categories based on their location, including vesicle or v-SNAREs.
and target or t-SNAREs. Although SNAREs vary greatly in size and structure, all contain a SNARE-motif in their cytosolic domains (Rizo and Rosenmund, 2008). SNARE-motifs are capable of tight assembly into helical bundles, incorporating three SNARE proteins called SNARE complexes, which switch between trans and cis conformations. For example, syntaxin 1 and SNAP-25 resident in cell membrane, and synaptobrevin (vesicle-associated membrane protein or VAMP) anchored in the vesicular membrane form a trans-SNARE complex prior to membrane fusion (Hu et al., 2002; Pobbati et al., 2006). During fusion, the membranes of the vesicle and target merge, converting the complex into a cis-SNARE complex, with all three SNARE proteins residing in the same resultant membrane. Assembly into trans-SNARE complexes brings opposing lipid bilayers in close proximity, priming them for fusion. SNARE complex assembly is completed and neurotransmitter is released following calcium entry into the presynaptic terminal, which activates synaptotagmin, thought to be a calcium sensor. N-ethylmaleimide-sensitive factor (NSF) is involved in the ATP-dependent prefusion priming, and postfusion unzipping of SNARE proteins (Sollner et al., 1993; Banerjee et al., 1996). Dissociated SNARE complex components can then be reused in subsequent vesicle-membrane fusion events.

In addition to SNARE proteins, numerous other families of adaptor proteins are involved with vesicle fusion and neurotransmitter release. One such family are the Munc13s, which are mammalian homologs of the Caenorhabditis elegans UNC13 proteins (Li and Chin, 2003). The Munc13 family is composed of four members, Munc13-1-4, which all share a common structure of their c-terminal domains. Munc13 proteins have been shown to be essential for the priming of synaptic vesicles, converting them to a fusion-ready state, and also for the generation of the readily releasable pool of synaptic vesicles (Betz et al., 1998; Betz et al.,
Specifically, Munc13s are thought to induce the conformational change of syntaxin, thereby promoting the formation of the SNARE complex. Components such as synaptotagmin, complexin, Munc13, and the scaffold RIM, have been shown to participate in exocytosis at synapses specifically, and are thought to be required to meet the special needs of fast calcium-triggered neurotransmitter release (Li and Chin, 2003).

1.3.6. Scaffolding Proteins of the Active Zone

In addition to release machinery, the rapid, high fidelity fusion of vesicles at synaptic sites is also supported by several scaffolding proteins in the presynaptic active zone. Ultrastructural electron microscopy studies of synapses have revealed that the presynaptic active zone is closely and precisely aligned with the PSD, and that the plasma membrane on both sites of the synaptic cleft appears as a thickened or electron-dense structure, illustrating the high concentration of proteins in these regions (Dreyer et al., 1973; Schikorski and Stevens, 1997). A variety of classes of proteins have been identified in association with the presynaptic active zone, including proteins involved in vesicle fusion (SNAREs, Munc13s; discussed above), cytoskeletal proteins, scaffolding proteins (CASK, Mint, SAP97, veils/MALS), voltage gated calcium channels, and cell adhesion molecules (Nrx, cadherins, integrins, sidekicks; discussed above). Since some of these protein groups have been discussed above, this section will focus primarily on scaffolding proteins of the presynaptic active zone.

A number of scaffolding proteins are thought to be involved in maintaining the neurotransmitter release site structure with the postsynaptic reception apparatus. Piccolo (presynaptic cytomatrix protein; (Cases-Langhoff et al., 1996; Wang et al., 1999; Fenster et al.,
2000) and Bassoon (tom Dieck et al., 1998) are the best characterized molecular scaffolds that have been shown to maintain presynaptic active zone structure, and are the largest active zone proteins identified thus far (420 and 530 kDa respectively). Piccolo and Bassoon share ten regions of sequence homology called Piccolo-Bassoon homology domains (PBH). The first two domains contain zinc-finger motifs, while PBH domains 4,6,8 include coiled-coil regions. In addition, Piccolo C-terminus has a PDZ domain and two C2 domains which are structurally related to those of the RIMs, along with a proline rich domain at the N-terminus. Bassoon has been shown to play a structural role in the assembly and function of various types of synapses, including those in the retina and hair cells (Khimich et al., 2005; tom Dieck et al., 2005). Piccolo functions to integrate multiple signals for the regulation of synaptic vesicle endo- and exocytosis. In particular, Piccolo has been shown to act as a calcium sensor with low affinity, which makes it a candidate to act during periods of high activity when calcium levels build-up (Gerber et al., 2001).

A key group of proteins involved in the presynaptic active zone is the regulating synaptic membrane exocytosis (RIM) family of proteins. RIMs are members of the RAS gene superfamily, and function as protein scaffolds that help regulate vesicle exocytosis. RIM1α and RIM2α are composed of the complete set of domains: an N-terminal zinc finger domain, a central PDZ-domain, two C-terminal C2-domains (C2A,B), a short proline-rich SH3 domain-binding motif (Wang et al., 2000; Wang and Sudhof, 2003). RIM2β is identical to RIM2α but lacks the N-terminal sequence, while RIM2γ,3γ,4γ are smaller proteins that contain a unique N-terminal sequence followed by the C2B domain (Wang and Sudhof, 2003). RIM proteins are further diversified by alternative splicing (Wang and Sudhof, 2003). RIMs have been shown to function both during priming and calcium-triggered vesicle fusion (Calakos et al., 2004). Of
particular interest, RIMs have been shown to have different actions at excitatory and inhibitory synapses (Schoch et al., 2002).

RIM binds liprin-α, a presynaptic scaffold that consists of several N-terminal coiled-coil domains, and three C-terminal SAM domains (Schoch et al., 2002). Liprin-α binds to the receptor protein tyrosine phosphatase LAR and together have been shown to be essential for proper active zone formation in *c. elegans* and *drosophila* mutants (Zhen and Jin, 1999; Kaufmann et al., 2002). Liprin-α has recently been shown to bind to a complex containing CASK/Mint/MALS (Olsen et al., 2005; Olsen et al., 2006). CASK is a member of the MAGUK family of PDZ proteins, composed of an N-terminal calcium calmodulin kinase domain, two L27 domains, and a PDZ domain and a SH3/GK domain (Hata et al., 1996). The kinase domain has been shown to bind Mint-1, while the L27 domains bind SAP97 and MALS. The PDZ domain binds the C-terminus of Nrx adhesion molecules, providing a link to the presynaptic membrane (Hata et al., 1996). Essential roles for CASK in the function of the presynaptic terminal remain uncertain, however studies done on the drosophila homolog (CAMGUK) mutant show defects in neurotransmitter release at the neuromuscular junction (Zordan et al., 2005). Mints are composed of two C-terminal PDZ domains that bind N-type calcium channels, and a phosphotyrosine binding domain (Maximov et al., 1999). The Mint family is composed of three proteins (Mint-1-3), which bind different interacting partners via divergent N-termini. One of these divergent sequences enables binding of Mint-1 to CASK, while another enables binding of Mint-1 and -2 to Munc18 (Okamoto and Sudhof, 1997; Borg et al., 1998; Butz et al., 1998). MALS, or mammalian homolog of Lin-7, is a binding partner of CASK that contains a single N-terminal L27 domain, and a C-terminal PDZ domain (Borg et al., 1998). Studies in mice lacking all three MALS family members show a role for these proteins in determining the size of the
releasable pool of vesicles, and further for replenishing this pool from reserve vesicles (Olsen et al., 2005). The complex of CASK, Mint-1, MALs and liprin-α is hypothesised to link the presynaptic release machinery to the active zone, thereby regulating neurotransmitter release (Olsen et al., 2006).

1.3.7. Neurotransmitter Transporters

Neurotransmitter transporters exist in the membranes of neurons and glia to remove excess amounts of the neurotransmitter from the synapse, or in the case of vesicular transporters, to pack neurotransmitter into vesicles for synaptic release. In general, neurotransmitter transporters are membrane-bound pumps that resemble ion channels.

There are two major types of neurotransmitter transporters, the first depends on an electrochemical gradient of sodium ions, and exchange glutamate or GABA with sodium, potassium and hydroxyl ions. Sodium-dependant glutamate transporters are commonly known as excitatory amino acid transporters (EAATs1-5, also known as SLC1A3,2,1,6,7 respectively). Sodium-dependant GABA transporters are referred to simply as GABA transporters (GATs1-3). Vesicular glutamate transporters (VGLutTs1–3, also known as SLC17A7,6,8 respectively) reside in the presynaptic membrane of excitatory synapses, whereas the vesicular GABA transporter (VGAT) resides in the presynaptic membranes of inhibitory synapses exclusively. Vesicular transporters rely on a proton gradient created by the hydrolysis of ATP in order to package neurotransmitter into synaptic vesicles for release (Maycox et al., 1988; Broer et al., 2002). Because of their specificity of localization, VGLutTs and VGAT are commonly used to label presynaptic terminals of excitatory and inhibitory synapses respectively.
Trans-synaptic Components

1.3.8. Synaptic Adhesion Molecules

Adhesion molecules are essential for maintaining the integrity of cell-to-cell junctions, and as such have been shown to have a role in promoting the stability of synapses by linking the pre and postsynaptic membrane. In addition, numerous recent developments have also demonstrated a role for synaptic adhesion molecules in target recognition, differentiation, and regulation of synaptic structure and function (Brose, 1999; Cantallops and Cline, 2000; Zhang and Benson, 2000; Ferreira and Paganoni, 2002; Garner et al., 2002; Missler, 2003; Yamagata et al., 2003; Washbourne et al., 2004; Dean and Dresbach, 2006; Gerrow and El-Husseini, 2006; Craig and Kang, 2007; Lardi-Studler and Fritschy, 2007). One important aspect of adhesion systems is their capacity to facilitate transsynaptic signalling, required for the coordination of pre and postsynaptic function.

Multiple families of adhesion molecules have been identified at the synapse, each with distinct structural features and both independent and intersecting functions in synapse formation and maintenance. The majority of synaptic adhesion molecules are members of the cadherin (Tepass U, Truong K, Godt D, Ikura M, Peifer M, 2000), immunoglobulin (Rougon G, Hobert O, 2003), and integrin (Hynes RO, 2002) superfamilies, with other types of adhesive systems such as the ephrin lignad-Eph receptor system (Kullander K, Klein R; 2002) and NL-Nrx interactions (Yamagata, Sanes, Weiner, 2003).
1.3.8.1. The Neuroligin-Neurexin Trans-Synaptic Adhesion System

One key family of proteins at synaptic sites is the NL family of adhesion molecules. The NL family is comprised of four identified family members in rat and mouse, and five in the human genome (Ichtchenko et al., 1996; Bolliger et al., 2001). NL family members are type I transmembrane proteins, comprised of a cholinesterase-like domain, a cleaved signal peptide, a carbohydrate attachment region, a single transmembrane domain, and a short C-terminal tail that contains a type I PDZ binding motif (Figure 1.3; Ichtchenko et al., 1996; Missler et al., 1998).

All NLs are subject to alternative splicing at two conserved splice sites on loops of the cholinesterase-like domain, referred to as A and B (Figure 1.3; residues 165–184 and residues 298–305; Ichtchenko et al., 1995; Ichtchenko et al., 1996; Chih et al., 2006; Craig and Kang, 2007). Although the position of the spliced sites is conserved, differences in insert sequences have been reported among NL family members. The existence of two alternatively spliced regions allows for the possible generation of up to four different isoforms for each NL gene (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Boucard et al., 2005). Mature NLs are localized to the postsynaptic membrane and interact via a transsynaptic connection with neurexins (Nrx) localized to the presynaptic membrane. This calcium dependent interaction is mediated by the extracellular domain and is regulated by alternative splicing of both NLs and Nrxs (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Boucard et al., 2005). Specifically, a recent study showed that splice site B in NL1 dictates binding to α-Nrx, but has little effect on the binding of β-Nrxs (Boucard et al., 2005). Detailed examination of the crystal structures revealed that NL splice site B is located at the edge of the NL-Nrx binding interface, which could lead to steric hindrance when an insertion is present at this site (Arac et al., 2007; Fabrichny et al., 2007). In
**Figure 1.3. Schematic and crystal structure of neuroligin and neurexin adhesion molecules.** α-Nrx structure is composed of LNS (laminin, neurexin, sex-hormone-binding protein) domains with EGF (epidermal growth-factor)-like domains assembled into three homologous modules, I–III. The positions of the five sites of alternative splicing (SS1–SS5) are indicated. NLs are composed of an extracellular acetylcholinesterase (AChE)-homologous domain that contains either one (NL2,3) or two (NL1) sites of alternative splicing (SSA and SSB). Both NL and Nrx contain a highly glycosylated region (CH) adjacent to the transmembrane domain (TM), and terminate in PDZ-domain-binding domains (PDZ BD) in their intracellular regions. Shown between the Nrx and NL structure schematics are crystal structures of AChE, used as a model for the AChE-homologous domain of NL (yellow), and the Nrx 1β LNS domain (purple). The position of splice sites SS2–SS4 is shown on a single LNS domain for simplicity, although SS2 and SS3 actually occur in different LNS domains of α-Nrxs. Figure from Craig and Kang, 2007. Neurexin–neuroligin signaling in synapse development. Current Opinion in Neurobiology, Volume 17, Issue 1, Pages 43-52.
addition, alternative splicing of NLs influences their targeting and functions to excitatory and inhibitory synapses (Chih et al., 2006), which is also affected by the C-terminal tail of NLs via interaction with scaffolding proteins such as PSD-95 (Levinson and El-Husseini, 2007).

Findings from functional and structural studies have suggested that NLs can form dimers (Dean and Dresbach, 2006; Arac et al., 2007; Budreck and Scheiffele, 2007; Fabrichny et al., 2007). A recent paper demonstrated the presence of NL1–NL3 and NL2–NL3 complexes in brain extracts (Budreck and Scheiffele, 2007). This was confirmed with the recent completion of high resolution crystal structures for the extracellular domains of NL1 and 4, which indicate that NLs exist as constitutive dimers (Arac et al., 2007; Fabrichny et al., 2007). The complexity of features that regulate NL structure and interactions makes them an interesting candidate to regulate the complex molecular signalling that occurs during synapse development.

The family of Nrx proteins is subdivided into α and β forms. α Nrxs are composed of a large extracellular sequence containing six laminin/neurexin/sex-hormone-binding globulin (LNS) domains, with three epidermal growth factor (EGF)-like regions (Rudenko et al., 1999). In contrast, β Nrxs are composed of only the sixth LNS domain of α Nrxs, and contain no EGF-like domains (Missler et al., 1998). Both α and β Nrxs share the same transmembrane sequence and short intracellular C-terminus. α Nrx transcripts are subject to extensive alternative splicing, at five sites referred to as SS1-SS5, with SS4,5 being present in β Nrxs (Ichtchenko et al., 1995; Missler et al., 1998; Missler and Sudhof, 1998; Tabuchi and Sudhof, 2002). Since many of these sites exhibit multiple splice variants, Nrxs may form more than 1000 possible variants (Missler and Sudhof, 1998).
In addition to NL binding, α Nrxs couple to presynaptic calcium channels at both excitatory and inhibitory synapses (O'Connor et al., 1993; Missler et al., 2003). However, detailed studies have demonstrated that α Nrxs specifically induce clustering of NL2 and proteins of inhibitory synaptic contacts, such as gephyrin and GABA receptor subunits (Kang et al., 2008). Accumulation of NL1/3/4 or PSD-95 was not observed in response to α Nrxs expressed in heterologous cells co-cultured with neurons. In addition, this study also showed that inserts at splice site 4 in β Nrxs bias clustering activity toward inhibitory synaptic proteins over excitatory synaptic proteins (Kang et al., 2008). Thus, via selective recruitment and interaction, NLs and Nrxs together may act as a code to specify excitatory and inhibitory synapse fates.

1.3.8.2. Other Trans-Synaptic Adhesion Systems

As mentioned above, representatives of the three major adhesion molecule families are represented at synapses, the cadherin, immunoglobulin, and integrin superfamilies. Perhaps the best characterized examples are the cadherins, which are single transmembrane domain molecules characterized by extracellular ectodomain repeats that mediate homophilic adhesion (Tepass et al., 2000). The cytoplasmic regions bind catenins which are effector molecules providing links to the cytoskeleton and mediating signalling (Yap and Kovacs, 2003). The related family of protocadherins have varying numbers of repeats in their extracellular domains, but do not appear to signal through catenins (Frank and Kemler, 2002). Protocadherins are diverse with individual isoforms being expressed in distinct yet partially overlapping neuronal cell populations, leading to the speculation that protocadherins may
regulate synapse recognition and specificity (Benson et al., 2001; Frank and Kemler, 2002; Wang et al., 2002).

Members of the immunoglobulin superfamily contain extracellular cysteine-looped domains that vary in number (Rougon and Hobert, 2003). The prototypical member of this family at synaptic sites in neural cell adhesion molecule (NCAM), which is composed of five immunoglobulin domains and three fibronectin type III repeats. A role for NCAM has been demonstrated in diverse aspects of synapse formation and function, including plasticity (Ronn et al., 1995; Luthi et al., 1996; Muller et al., 1996; Hartz and Ronn, 2008). In addition to NCAM, several other members of the immunoglobulin family have been identified at synapses, including Synaptic cell adhesion molecules (SynCAMs; (Biederer et al., 2002; Sara et al., 2005), nectins (Mizoguchi et al., 2002), sidekicks (Yamagata et al., 2002). It has been suggested that synapses contain at least one cadherin or cadherin-like adhesion molecule and at least one immunoglobulin superfamily adhesion molecule (Yamagata et al., 2003).

In addition to these large families, many types of synapses also contain integrin family members. Integrins mediate adhesion at synapses outside the CNS, such as the neuromuscular junction (Cohen et al., 2000), and also mediate adhesion at points of cell contact with the extracellular matrix. Integrins have been identified in the developing rodent brain (Einheber et al., 1996), and have been shown to have a role in hippocampal pre and postsynaptic maturation (Chavis and Westbrook, 2001). However, the most common ligands of integrins, including fibronectin and laminin, do not have prominent expression patterns in the CNS, thus it remains to be determined what ligands may be binding laminins at CNS synapses to mediate this adhesion (Yamagata et al., 2003).
The Eph receptor tyrosine kinases and ephrin ligands which have prominent roles in segmentation, angiogenesis and limb development have also been shown to play a role at CNS synapses. At synaptic sites, EphB receptors have been shown to bind NMDA receptors and participate in synapse formation (Dalva et al., 2000). EphB receptors are activated by transmembrane ephrin-B ligands, and activation of Eph receptors results in homodimerization and autophosphorylation. Extensive analysis, using different combinations of double and triple knock-outs of EphB1, EphB2, and EphB3, revealed that these receptors are involved in dendritic spine morphogenesis and synapse formation in the hippocampus (Henkemeyer et al., 2003). Altered spine formation in the EphBR mutants is also associated with reduced excitatory synapse density and clustering of both NMDA and AMPA receptors (Ethell et al., 2001). EphA receptors have also been shown to influence dendritic spine morphology, through expression of these receptors in astrocyte end-feet that tightly surround many synaptic contacts (Murai et al., 2003). The complexities of Eph-ephrin adhesion and repulsion, and the downstream effects on synaptic signalling are yet to be fully understood. Current theories suggest that these multiple systems of adhesion participate together in synapse formation and function, with partially overlapping roles (Yamagata et al., 2003; Washbourne et al., 2004; Gerrow and El-Husseini, 2006).

Of particular interest in this inventory of major synaptic components is the NL-Nrx transsynaptic adhesion system, which is poised to modulate key aspects of synaptic structure and function through its molecular interactions (Figure 1.4). NL-Nrx complexes interact with scaffolding proteins such as PSD-95, indirectly linking them to receptors and signalling proteins.
Figure 1.4. Neuroligins and neurexins as central components of synaptic structure and function. Schematic of NL-Nrx molecular interactions using molecules and interactions identified at excitatory synapses. Via direct and indirect molecular interactions, NL and Nrx link pre and postsynaptic compartments, as well as functionally link molecules involved in calcium-mediated neurotransmitter vesicle fusion in the presynaptic terminal with scaffolding proteins that bind neurotransmitter receptors and signalling molecules in the postsynaptic compartment. As such, the NL-Nrx complex may represent a central core for protein recruitment and complex assembly at synaptic contact sites. Furthermore, NL and Nrx are poised to modulate the assembly and function of several proteins important for synapse function. Figure adapted from Olsen et al., 2006. Synaptic transmission regulated by a presynaptic MALS/Liprin-alpha protein complex. Current Opinion in Cell Biology, Volume 18, Issue 2, Pages 223-227.
in the postsynaptic compartment. In the presynaptic compartment, NL-Nrx complexes bind directly to synaptotagmin, as well as the presynaptic scaffold CASK, which serves to link the complex to Calcium channels and proteins key for synaptic vesicle exocytosis. Thus in addition to the physical link between pre and post, the direct and indirect connections via NL-Nrx serve to functionally link all of the key components required for calcium activated vesicle fusion, neurotransmitter release, receptor activation and downstream signalling.

1.4. Steps of Synapse Formation

The number of different families of proteins assembled at synapses illustrates the complex task that must be performed in order to make a signalling competent connection. Not only do the appropriate proteins need to be synthesised and transported, they need to be arranged into interacting arrays forming the functional units for presynaptic vesicle release and postsynaptic receptor signalling. In addition, the task is further complicated, since multiple systems of neurotransmitters, receptors, and their interacting proteins exist, a problem resides in the matching of appropriate molecules at pre and post synaptic compartments. Under normal conditions, mismatching is not found to occur (Rao et al., 2000a; Lardi-Studler and Fritschy, 2007); therefore a form of trans-synaptic communication must exist for recognition of appropriate partnerships. Because neurons both produce and receive millions of signals at millions of synaptic sites, they must be programmed to deal with the major task of sorting, assembling and targeting pre- and postsynaptic proteins to appropriate sites to ensure correct formation of synaptic contacts and neuronal circuits in the brain (Lardi-Studler and Fritschy, 2007; McAllister, 2007).
The formation of synaptic contacts is generally divided into four stages including contact initiation, recruitment of presynaptic and postsynaptic proteins, maturation and elimination (Figure 1.5; Brose, 1999; Sanes and Lichtman, 1999; Ziv and Garner, 2001; Ahmari and Smith, 2002; Garner et al., 2002; Waites et al., 2005; Gerrow and El-Husseini, 2006; McAllister, 2007). Within the brain, multiple types of synapses can be distinguished based on their molecular constituents and the neurotransmitters that they release. As touched on above, the major classes of synapses in the CNS are coarsely divided into excitatory, mediated by glutamate release; inhibitory, mediated by GABA and glycine; and modulatory, mediated by aminergic signalling including dopamine, serotonin, acetylcholine and other neurotransmitter systems. Because excitatory synapses are the most abundant type, the majority of our knowledge on synapse formation is based on this system. Although the identity of the specific molecules involved may differ, it is anticipated that the major steps mediating the formation of different classes of synapses are similar, including the basic stages of contact initiation, protein recruitment, maturation and elimination.

The various stages of synapse formation are thought to principally involve two major classes of proteins, cell adhesion molecules and scaffolding molecules. Cell adhesion molecules are transmembrane proteins involved in the junctions between two different cells, at points of cell-to-cell contact. Adhesion molecules are a likely candidate for synapse formation, since a synapse is simply a specialized junction between two cells (Shapiro et al., 2007). A large body of work has implicated cell adhesion molecules in triggering synapse formation (Scheiffele et al., 2000; Biederer et al., 2002; Fu et al., 2003), target recognition (Yamagata et al., 2002; Shen and Bargmann, 2003), and maturation (Chavis and Westbrook, 2001; Togashi et al., 2002). Adhesion molecules are often anchored at synaptic sites by scaffolding proteins that contain
Figure 1.5. Key stages thought to be involved in the formation of synaptic contacts. Synapse formation is divided into characteristic stages typically including axon and dendrite growth and guidance (1) followed by the formation of initial contact between axon and dendrite (2). Following contact initiation, recruitment of appropriate synaptic proteins to nascent sites of contact (3) and maturation and stabilization of these synaptic contacts (4) result in functional synapses. Additionally, the stage of synapse elimination is often included in the process of synapse formation, due to its important role in the assembly of functional synaptic networks. Figure from Gerrow and El-Husseini. 2008. Cell adhesion molecules at the synapse. Frontiers in Bioscience. 11:2400-19.
1. Axon Guidance and Dendritic Growth

2. Initial Contact and Target Selection

3. Recruitment of Presynaptic and Postsynaptic Proteins

4. Spine Morphogenesis and Maturation
multiple protein-protein interaction domains required for the assembly of large protein complexes. Scaffolding proteins have been shown to be important for active zone assembly and vesicle recruitment in presynaptic terminals (Srivastava and Ziff, 1999; Ahmari et al., 2000; El-Husseini et al., 2000a; El-Husseini et al., 2000b; Zhai et al., 2001; Bamji et al., 2003; Bresler et al., 2004), and receptor recruitment on the postsynaptic side (Srivastava et al., 1998; El-Husseini et al., 2000a; El-Husseini et al., 2000b; Kneussel et al., 2001; Sala et al., 2001; Ehrlich and Malinow, 2004). It is clear that the full complement of molecules involved in synapse formation remains to be determined, however a number of adhesion molecules and scaffolding proteins have been identified to be key players in the stages of this important process, and will be discussed in detail below.

1.4.1. Contact Initiation

In the first stage, a contact is made between an axon and the target postsynaptic cell. The detailed workings of this stage were originally described in the neuromuscular junction synapse, and have been described as the extensions of axonal growth cone filopodia in search of postsynaptic partners. However, in the case of the neuromuscular junction, the target muscle cell is stationary relative to the dynamic dendritic processes of neuronal cells (Sanes and Lichtman, 1999). Dendrites of neurons also extend growth cones and are lined with filopodia. These dendritic filopodia have been shown to rapidly extend and retract, which is consistent with an ability to initiate contact formation (Ziv and Smith, 1996; Fiala et al., 1998). A debate still exists as to whether contact formation is initiated by the axon, the dendrite or
both, however despite this debate it is clear that this process is tightly governed to ensure appropriate connectivity.

In the process of contact initiation axons often travel long distances before reaching the site of appropriate contact. On this journey, axons pass by a huge number of potential partners, yet do not establish synapses on unmatched targets. Target specification is thought to involve multiple systems including target-derived factors and adhesion molecules (Waites et al., 2005; Gerrow and El-Husseini, 2006). It has long been recognised that because there are multiple synapse types and numerous potential targets, the systems mediating initial contact and recognition are predicted to be complex and polymorphic in order to provide the permutations and combinations necessary (Sperry, 1963; Shapiro et al., 2007).

With the diversity possible via multiple family members and sites of alternative splicing, NL/Nrx complexes have been suggested as possible mediators of initial contact formation at synaptic sites (Boucard et al., 2005). To study the minimum molecular requirements for synapse induction, a clever assay has been developed using co-culture of transfected heterologous cells with neurons. In a series of experiments by Scheiffele’s group, HEK cells were transfected with DNA encoding the adhesion molecules NL1 or NL2 and co-cultured with developing neurons (Scheiffele et al., 2000). Remarkably, the expression of these postsynaptic adhesion molecules in heterologous cells resulted in the differentiation of presynaptic terminals at sites of axon-HEK cell contact. Furthermore, these contacts were found to have not only morphological but functional characteristics of actual presynaptic contacts, with the accumulation of synaptic vesicles (Scheiffele et al., 2000). The addition of soluble β-Nrx resulted in inhibition of contact formation between neurons and transfected HEK cells (Scheiffele et al., 2000). The induction
of synapse-like contacts onto heterologous cells by Nrxs and NLs supported a role in early stages of synapse formation. However, subsequent studies in the triple knock-out mouse, lacking NLs1-3, demonstrated that synaptic contacts were still formed, suggesting the NL/Nrxs are not necessary for synapse formation (Varoqueaux et al., 2006). These mice demonstrated a reduction in some NL-associated synaptic proteins, and demonstrated functional changes in GABA/glycine and glutamatergic transmission in brain regions controlling respiration, however no changes were detected in synapse density. These results led to the suggestion that NLs may instead be involved in later stages of synapse formation including protein recruitment (to be discussed below in section 1.4.2) influencing the function of synapses formed (Varoqueaux et al., 2006).

One family of proteins that have been shown to mediate contact initiation is the cadherin family. A role for cadherins is suggested by their localization, which is diffuse within dendritic filopodia early in development and once contacted by an axon, becomes clustered at the point of contact (Togashi et al., 2002; Jontes et al., 2004). In addition, dominant negative studies have demonstrated that blocking cadherin function results in a loss of mature spines in exchange for thin filopodia-like spines (Togashi et al., 2002). However, it was also shown that neurons transfected with dominant negative cadherins were still capable of making synapses, albeit at a delayed rate (Inoue and Sanes, 1997; Bozdagi et al., 2004). This suggests that cadherins are most likely to function in target specificity rather than being integral for the formation of synaptic contacts.
1.4.2. Synaptic Protein Recruitment

The second step in synaptogenesis centers on the recruitment of the molecular components of pre- and postsynaptic compartments. The major constituents of presynaptic terminals are neurotransmitter vesicles and the machinery for vesicle release, whereas postsynaptic constituents include neurotransmitter receptors, interacting scaffolding proteins and associated signalling molecules. The recruitment of different proteins to pre versus postsynaptic sites suggests the involvement of trans-synaptic signalling to ensure matching of appropriate neurotransmitter vesicles on the presynaptic paired with the cognate receptors on the postsynaptic side.

The transport of proteins to synaptic compartments has been shown to occur via a number of different mechanisms including gradual accumulation from diffuse cytoplasmic pools (Bresler et al., 2001; Bresler et al., 2004), lateral diffusion along the membrane from extrasynaptic sites (Dahan et al., 2003; Tardin et al., 2003), as well as trafficking of pre-assembled transport packets (Ahmari et al., 2000; Prange and Murphy, 2001; Washbourne et al., 2002; Bamji et al., 2003; Gerrow, 2005). Whether proteins are recruited as preassembled complexes or individual molecules, some debate still exists about the temporal order of protein recruitment (Waites et al., 2005; McAllister, 2007).

Multiple cell adhesion complexes have been suggested as inducers of synaptic recruitment and differentiation. Both NL/Nrx and SynCAM have been shown to induce active zone assembly in heterologous cell-neuron co-culture experiments (Scheiffele et al., 2000; Biederer et al., 2002; Dean et al., 2003). Out of the numerous molecules tested to date, only Nrxs, NLs and SynCAM are capable of promoting corresponding synaptic protein clustering at sites of contact between
neuronal axons and transfected heterologous cells. These data suggest that these adhesion systems may be early players at new sites of synaptic contact, however a direct link between synaptic adhesion and recruitment has not been made. In addition to the link to adhesion molecules, scaffolding proteins have also been implicated in protein recruitment, which is discussed in detail below.

1.4.2.1. Postsynaptic Recruitment

Scaffolding proteins of the PSD-95 family are thought to be one of the earliest molecules recruited to nascent synaptic contacts. PSD-95 has been detected within 20 minutes following contact between an axon and a dendrite in cultured neurons (Friedman et al., 2000; Bresler et al., 2001; Okabe et al., 2001), and is present in synapses as early as two days postnatal in the immature hippocampus (Sans et al., 2000). PSD-95 has been observed to accumulate via both bulk transport of PSD-95 clusters (Prange and Murphy, 2001; Gerrow, 2005), and gradual accumulation (Bresler et al., 2001; Marrs et al., 2001; Bresler et al., 2004).

Recruitment of NMDA and AMPA glutamate receptor types closely follows PSD-95. NMDA and AMPA receptors have been shown to be recruited via distinct mechanisms, likely related to the differences in their biosynthetic pathways and interacting partners. Transport of NMDA receptors during synapse formation has been shown to occur gradually (Bresler et al., 2004), as well as via the transport of receptor clusters (Washbourne et al., 2002). Transport vesicles containing AMPA receptors have been shown to be inserted locally at sites near nascent synapses, as well as bulk insertion throughout the dendritic plasma membrane followed by diffusion and trapping by scaffolding proteins at synaptic sites (Passafaro et al., 2001;
Borgdorff and Choquet, 2002; Bats et al., 2007). A large body of research has focused on how interacting proteins influence the delivery of AMPA and NMDA receptors to sites of synaptic contact (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Wenthold et al., 2003). NMDA receptors have been shown to interact directly with PSD-95 (Kornau et al., 1995; Niethammer et al., 1996), whereas AMPA receptors interact with PSD-95 indirectly via binding to transmembrane AMPA receptor associated proteins (TARPs; (Chen et al., 2000; El-Husseini et al., 2000a; Chen et al., 2003a).

Although substantial evidence exists for an interaction between PSD-95 and NMDA receptors, it remains unclear how PSD-95 modulates NMDA receptor trafficking or function. Insertion of NMDA receptors at synaptic contact sites is not altered by mutations of the cytoplasmic tails of NMDA receptor subunits, by genetic disruption of PSD-95 or by interfering peptides that disperse synaptic clusters of PSD-95 (Lim et al., 2003). Despite this, a recent study shows that expression of PSD-95 and NR1/NR2A subunits in heterologous cells increases the number of functional channels at the cell surface and the channel opening rate of NMDA receptors (Rutter and Stephenson, 2000; Rutter et al., 2002; Cousins et al., 2008), suggesting that PSD-95 may regulate NMDA trafficking and recruitment of specific NMDA receptor subunits, with other family members playing a role.

In contrast to NMDA receptors, mounting evidence indicates that the clustering of AMPA receptors is highly regulated by PSD-95. This effect seems paradoxical as PSD-95 does not directly bind AMPA receptors. Current research suggests that PSD-95 and AMPA receptors interact via stargazin, a member of the TARP family (Chen et al., 2000). Stargazin, the archetypal TARP, is critical for AMPA receptor targeting to synapses in cerebellar granule cells.
Stargazin contains a carboxy-terminal PDZ binding region that interacts with PSD-95. Stargazin overexpression dramatically increases the number of extra-synaptic AMPA receptors (Schnell et al., 2002). Conversely, expression of a mutant stargazin lacking the PDZ-binding domain disrupts synaptic clustering of AMPA receptors in hippocampal cells, demonstrating the importance of this domain in synaptic targeting. Further, TARPs have been shown to modulate the function of AMPA receptors (Menuz et al., 2007; Soto et al., 2007), which has established them as an important auxiliary subunit of AMPA receptors.

Another mode of regulation of AMPA clustering involves neuronal activity-regulated pentraxin (NARP) protein, a secreted immediate-early gene (IEG). NARP is part of a pentraxin complex that also includes neuronal pentraxin-1 (NP1; O'Brien et al., 1999; O'Brien et al., 2002; Xu et al., 2003). These proteins combine to form highly organized complexes, where the relative ratio of each protein in the complex is dependent upon the neuron's prior activity and developmental stage. These proteins interact via N-terminal coiled-coil domains, and contain C-terminal pentraxin domains which mediate association with AMPA receptors (O'Brien et al., 1999). Neurons overexpressing wild-type NARP show increased clustering of AMPA receptors. Transfection with dominant-negative NARP mutants results in selective binding of endogenous NARP and prevention of its synaptic accumulation, ultimately leading to decreased GluR1 subunit clustering (O'Brien et al., 2002). Huganir and colleagues identified the minimum number of proteins sufficient for AMPA receptor recruitment to excitatory contact sites using a reconstituted synapse system. These experiments identified an adhesion complex containing NL, and the secreted factor NP-1 and its receptor as being the minimum requirements for AMPA clustering (Sia et al., 2007).
The clustering of glutamate receptors at synaptic sites has also been shown to be influenced by adhesion complexes. For example, EphrinB activation of EphB was shown to promote an association of EphB with NMDA receptors (Dalva et al., 2000). In addition, EphB has been shown to control AMPA-type glutamate receptor localization through PDZ domain interactions (Kayser et al., 2006). Both of these studies also showed a role for the ephrin binding domain in regulating presynaptic differentiation (Dalva et al., 2000; Kayser et al., 2006). The cadherins have also been shown to interact with and regulate the localization of AMPA glutamate receptors. The N-terminal domain of GluR2 can interact directly with N-cadherin, resulting in immobilization and decreases in GluR2 lateral diffusion on the neuronal surface (Saglietti et al., 2007). This interaction was also shown to stimulate presynaptic development and function as well as promoting dendritic spine formation. These studies also show that transsynaptic signalling through adhesion complexes can mediate development of both pre and post synaptic compartments, which may be important for recruitment of the appropriate complement of proteins and matching of pre and postsynaptic.

1.4.2.2. Presynaptic Recruitment

Formation of the protein complexes of the active zone is a major step in the differentiation of the presynaptic terminal at sites of new contact. The presynaptic active zone has been shown to be composed of numerous proteins including Bassoon, Piccolo, RIMs, Muncs and CASTs. The rapid construction of the active zone following new synaptic contact prompted theories that the molecular constituents may be delivered in pre assembled packets via transport vesicles. Indeed, axonal growth cones have been shown to contain numerous large dense-core vesicles that contain Piccolo and Bassoon, as well as RIMs, Muncs, Syntaxins, SNAP-25 and calcium...
channel subunits (Mason, 1985; Matteoli et al., 1992; Hannah et al., 1999; Zhai et al., 2001; Ohtsuka et al., 2002; Shapira et al., 2003). The fusion of these dense-core vesicles has been observed shortly after initial contact formation between and axonal growth cone and target dendrite (Ziv, 2001; Ziv and Garner, 2004). An additional population of vesicles containing proteins important for the release of neurotransmitter, such as vesicle associated membrane proteins (VAMPs) and synaptophysin has also been shown to fuse shortly after contact formation (Ahmari et al., 2000). Despite our knowledge of the existence of transport vesicles in presynaptic compartment differentiation, little is known about the specific molecular cascades responsible for their recruitment.

1.4.3. Maturation

The maturation of newly formed synaptic connections can be a prolonged phase characterized by increasing numbers of synaptic vesicles in the presynaptic compartment, as well as increases in the protein content and size of the postsynaptic density (Vaughn, 1989; Pierce and Mendell, 1993). Many studies have shown that maturation of synapses is regulated by activity, such that synapses that are used are matured and maintained, while those that are not active are pruned or eliminated. Maturation is easily evidenced in the transition from a newly formed site of contact onto a dendritic filopodia, to a glutamatergic synapse onto a mature spine. Mature spines appear as bulbous protrusions from the dendrite, characterized by a narrow neck and enlarged head, enriched with actin. The narrow neck of the spine is thought to aide in the spatial and biochemical compartmentalization of the postsynaptic compartment. For example, spine morphology has been shown to regulate calcium handling, with spines with
longer or narrower necks retaining more calcium in their heads following activation (Rusakov et al., 1996; Majewska et al., 2000b; Holthoff et al., 2002; Noguchi et al., 2005). In addition, proteins such as inositol 1,4,5-triphosphate (IP₃) (Santamaria et al., 2006) and PSD-95 (Gray et al., 2006) can be more effectively trapped in spines with narrower or longer necks, further impacting synaptic efficacy (Bloodgood and Sabatini, 2005).

Scaffolding proteins that link to the cytoskeleton have been shown to regulate spine morphology. For instance, overexpression of Shank results in enlargement and maturation of dendritic spines and enhancement of presynaptic function in cultured hippocampal neurons (Sala et al., 2001; Sala et al., 2005). Experiments employing a series of deletion constructs demonstrated that synaptic targeting and cooperation with Homer are necessary for Shank to exert its effects on spine morphology. In addition to morphological effects, the Shank / Homer complex influences the recruitment of related proteins to synaptic sites. For example, Shank and Homer mediate the recruitment of IP₃ and smooth endoplasmic reticulum into dendritic spines (Sala et al., 2001). Dominant-negative mutants of Shank cause reductions in spine density, which may result from alteration in spine stability or from inhibition of spine formation. Shank can thus influence spine morphology, mediated by the link between receptor activation and actin-based spine remodelling (Tu et al., 1999; Sala et al., 2001).

Numerous cell adhesion molecules also interact with proteins that modulate the actin cytoskeleton, and thus may also influence the structural maturation of the synapse (Hering and Sheng, 2001; Carlisle and Kennedy, 2005; Ethell and Pasquale, 2005). In particular, nectins, members of the immunoglobulin superfamily of molecules, function with cadherins to activate Rac1 and Cdc42 and can lead to the formation of filopodia and lamellipodia (Kawakatsu et al.,
2002; Mizoguchi et al., 2002). Modulation of actin structure seems to be a common factor in molecules that promote spine maturation, in order to provide structural support for recruitment of additional proteins and increases in size.

1.4.4. Elimination

Although counter-intuitive, synapse elimination is an integral step in the appropriate formation of synapses in the formation of networks in the brain (Rao and Craig, 1997). This final stage, where unused synapses are pruned away, depends upon external stimuli and synaptic activity. The role of elimination is highlighted by the fact that the initial number of synapses in development is far greater than the number retained into adulthood (Lichtman and Colman, 2000; Hashimoto and Kano, 2003). The role of activity in synapse pruning is exemplified in the rodent barrel cortex, where trimming of whiskers causing sensory deprivation results in a decrease in stable synaptic contact (Trachtenberg et al., 2002). In contrast, increased whisker stimulation has been shown to increase the number of synapses (Knott et al., 2002).

Two mechanisms have been demonstrated by which synapses are removed. The first is called input elimination, where the presynaptic cell loses all synaptic contacts with its postsynaptic target, thereby functionally and anatomically uncoupling the cells although contact with other targets persist (Jackson and Parks, 1982; Sretavan and Shatz, 1986; Sanes and Lichtman, 1999; Keller-Peck et al., 2001; Hashimoto and Kano, 2003). The second, called synapse disassembly refers to the deconstruction of individual synaptic contacts, which represents a mechanism for subtle modulation of the strength of connectivity between two cells (Goda and Davis, 2003). The molecular mechanisms that drive synapse disassembly and input elimination remain
unclear; however the rate of synapse removal can be substantially faster than the rate of protein turnover (Huh and Wenthold, 1999), suggesting a specialized mechanism for destabilization that is distinct from a simple cessation of new protein synthesis.

Investigation into the temporal regulation of synapse disassembly has shown that vesicle associated proteins are removed prior to the clearance of postsynaptic receptors (Eaton et al., 2002; Hopf et al., 2002). At the neuromuscular junction, one of the first steps in contact disassembly is the removal of agrin adhesion and acetylcholine receptors by matrix metalloproteases (Tyc and Vrbova, 1995; VanSaun et al., 2003). It is unclear whether protease cleavage plays a role at disassembling synapses in the CNS, however preliminary evidence has been derived from cleavage of the γ-protocadherin ectodomain at the cell surface when expressed in heterologous cells (Haas et al., 2005; Hambsch et al., 2005).

In addition it has been shown that low frequency stimulation, resulting in a form of plasticity termed long-term depression (LTD), can result in synapse separation or elimination (Zhou et al., 2004; Kamikubo et al., 2006; Bastrikova et al., 2008). Using simultaneous imaging and electrophysiological recording, it was observed that LTD inducing stimuli lead to a reduction in closely opposing (colocalizing) pre and postsynaptic terminals (Bastrikova et al., 2008). Further, in many cases, complete separation between the presynaptic compartment and dendritic spine was observed, however spine loss was not observed. Other studies have noted shrinking of dendritic spines following LTD (Zhou et al., 2004) as well as complete elimination of synapses after repeated induction of LTD (Kamikubo et al., 2006), demonstrating how activity patterns can shape neural connectivity.
1.5. The Ratio of Excitation to Inhibition

Glutamate is the most prevalent excitatory neurotransmitter, and as such plays a critical role in the formation of brain architecture, and normal brain function (Carlsson, 1998; Purcell et al., 2001; Jamain et al., 2002; Owens and Kriegstein, 2002a). Conversely, GABA is the major inhibitory neurotransmitter, and provides an important control of the excitatory tone in the brain (Moss and Smart, 2001; Owens and Kriegstein, 2002b). A precise ratio of excitation and inhibition must exist in the brain in order for the transmission of signalling and normal function. The E/I ratio could possibly be modulated by factors governing the formation of excitatory and inhibitory synaptic contacts, including stages of contact initiation between matched partners, recruitment of appropriate pre and postsynaptic machinery, synapse maturation and activity dependent elimination. Further, once the appropriate contacts are formed, the E/I ratio can be altered by factors that regulate the number and/or activity of glutamate/GABA receptors, and the amount of glutamate/GABA in the synapse. Dysregulation of the E/I ratio has been implicated in a number of CNS disorders including epilepsy and seizure disorders (Ben-Ari and Holmes, 2005; Mann and Mody, 2008), and schizophrenia (Deutsch et al., 2001). Of particular interest, a recent theory has suggested that autism, a neurodevelopmental disorder characterized by repetitive and stereotyped behaviour, and varying degrees of abnormality in communication ability, social interactions, as well as a high incidence of seizure (Tuchman and Rapin, 2002; Christ et al., 2007; Dover and Le Couteur, 2007; Garber, 2007; Scambler et al., 2007), may be caused by alterations in the excitatory to inhibitory ratio (Rubenstein and Merzenich, 2003).
1.6. Neuroligin-Neurexin and the Ratio of Excitation to Inhibition

Different members of the NL family are enriched at either excitatory (NL1, NL3) or inhibitory (NL2, NL3) synapses (Song et al., 1999; Graf et al., 2004; Varoqueaux et al., 2004; Chih et al., 2005; Levinson et al., 2005a; Budreck and Scheiffele, 2007; Chubykin et al., 2007). Despite their specific localization, in vitro gain of function studies indicate that NLs can induce excitatory and inhibitory presynaptic specializations (Song et al., 1999; Scheiffele et al., 2000; Graf et al., 2004; Prange et al., 2004b; Chih et al., 2005; Levinson et al., 2005a). The specificity of influence on synapses is regulated in part by the extracellular splice insertions, which influence binding to different Nrx isoforms and act to bias the function of NL1 and NL2 to glutamatergic and GABAergic contacts respectively (Boucard et al., 2005; Chih et al., 2006; Kang et al., 2008). In addition to the selective extracellular interactions, specificity of NL function is likely to be further regulated by cytoplasmic interaction with different scaffolding proteins such as PSD-95 (Prange et al., 2004b; Levinson et al., 2005a).

The ability of NLs and Nrxs to regulate both excitatory and inhibitory synapse function led to the proposal that NLs may comprise the molecular machinery that maintains the ratio of excitation to inhibition (E/I ratio), fundamental for normal brain function (Figure 1.6; (Cline, 2005; Levinson and El-Husseini, 2005a). Work on cultured neurons shows that abnormal targeting of NLs to particular synapse types alters E/I ratio (Levinson et al., 2005a). In further support of a role for these proteins in controlling E/I ratio, analysis of acute slices from NL knock-out mice shows that specific NLs differentially affect excitatory and inhibitory synapse function (Chubykin et al., 2007). In addition, mice lacking NLs 1-3 die shortly after birth due to
Figure 1.6. Control of the ratio of excitatory to inhibitory synaptic contacts by neuroligins and related molecules. This series of diagrams depicts the hypothesis of how changes in the amounts of specific proteins enriched at either excitatory or inhibitory synaptic contacts could shift the E/I ratio. A. Under normal conditions, NL1 is enriched at excitatory synaptic sites, whereas NL2 is concentrated at inhibitory synaptic sites. NLs associate with the scaffolding proteins, including PSD-95, which is exclusively localized at excitatory synaptic sites. Interaction with PSD-95 enhances NL1 accumulation at excitatory synapses, while other unidentified scaffolding proteins sequester NL2 at inhibitory synapses. The relative levels of these proteins ensure an appropriate ratio of excitatory to inhibitory synaptic input received by individual neurons. B. Loss of NLs hinders maturation of both excitatory and inhibitory synapses, as suggested by findings from NL1-3 triple knock-out mice. Total synapse loss may be accounted for by redundancy of cell adhesion molecules present at excitatory synaptic sites compared to inhibitory synapses, causing a shift in the E/I ratio. C. In this model, increased PSD-95 enhances clustering of NL1 at excitatory synapses and redistributes NL2 to excitatory sites, resulting in an enhancement of excitatory presynaptic terminals at the expense of inhibitory contacts, thus shifting the E/I ratio toward increased excitation. D. This model depicts a situation where loss of PSD-95 could result in redistribution of NLs from excitatory synapses to inhibitory sites, leading to a reduction in excitatory synaptic transmission, and a shift in the ratio toward inhibition. Levinson and El-Husseini. 2005. Building excitatory and inhibitory synapses: balancing neuroligin partnerships. Neuron 48(2):171-4.
alterations in the balance between excitation and inhibition in brainstem regions that regulate respiration (Varoqueaux et al., 2006). In keeping with a fundamental role for NLs in synapse maturation, a reduction in presynaptic terminal content, but not number, has been observed in NL1-3 triple knock-out mice (Varoqueaux et al., 2006). Thus, disrupting the function of NLs and Nrxs leads to altered neuronal excitability through altered synapse maturation and E/I ratio. Such observations are surprising in light of the homeostatic mechanisms that exist to maintain the proper E/I ratio.

1.7. The Case for Autism as an E/I Ratio Disorder

It was recently hypothesised that the neurodevelopmental disorder Autism may result from an alteration in the E/I ratio (Rubenstein and Merzenich, 2003). The central role for alterations in the E/I ratio of autism is supported by three central pieces of information: 1. Studies have shown abnormalities in glutamate and GABA in blood and post-mortem brain tissue of autistic subjects (Hussman, 2001). Post-mortem analysis revealed significant reductions in different GABA receptor subunits in brain regions implicated in autism (Fatemi et al., 2002; Fatemi et al., 2008). 3. Alleviation of autism symptoms with pharmacological agents that reduce neural excitation such as anticonvulsants (Belsito et al., 2001; Di Martino and Tuchman, 2001; Rugino and Samsock, 2002). These observations have led to the hypothesis that autism arises from increased ratios of excitation to inhibition in key neural circuits underlying sensation, communication and social interaction behaviours (Rubenstein and Merzenich, 2003; Cline, 2005). In addition it has been suggested that autism may arise from a hypoglumaturnergic state, or a decreased E/I ratio (Carlsson, 1998; Purcell et al., 2001). In support of the shift in autism
favouring inhibition, Rett syndrome, a related neurodevelopmental disorder, has been linked to a decreased E/I ratio (Dani et al., 2005). Although evidence supports a role for dysregulation of the excitatory to inhibitory ratio in autism, it remains unclear whether circuits are biased in favour of excitation or inhibition.

1.8. Autism Spectrum Disorders

1.8.1. Symptoms and diagnosis

The term autism was introduced by Leo Kanner in 1943 to describe a syndrome characterized by significant impairments in social, communicative, cognitive, and behavioural function (Kanner and Eisenberg, 1957). By current standards, autistic disorders are typified by three classes of features, including: (1) impairments in social interactions, (2) impairments in both verbal and nonverbal communication, and (3) repetitive or stereotyped patterns of behaviour (American Psychiatric Association, 2000). Behaviourally, individuals with autism often display social withdrawal, insistence on routine or sameness, tantrums and difficulties with communicating, and emotional-social behaviours (Kanner, 1949; Frith et al., 1991). Social impairments are the key aspect that differentiates autism from other developmental disorders. These impairments effect reciprocal social interaction, non-verbal social communication such as eye contact, and emotional content of speech (Joseph et al., 2002; Baron-Cohen and Belmonte, 2005; Grice et al., 2005; Johnson et al., 2005; Dapretto et al., 2006). Autism may develop gradually between birth and three years of age or as a rapid regression during early development (Rogers, 2004).
In addition to the core symptoms, several additional symptoms are associated with autism with variable expression in individuals. For example, autism is typically associated with mental retardation as assessed using standard IQ testing (Folstein and Rosen-Sheidley, 2001; Muhle et al., 2004; Veenstra-Vanderweele et al., 2004). An additional common accessory symptom in autistic individuals is anxiety (Edelson et al., 1999; Tsai, 1999; Gillott et al., 2001). Anxiety in autistic individuals has been related to their insistence on sameness and need for routine, such that minor changes in the environment or even the fear of possible change can lead to distress and anxiety (Groden et al., 2001). The strongest association in autistic individuals is found with seizure disorders (Deykin and MacMahon, 1979; Volkmar and Nelson, 1990; Fombonne, 2002; Hughes and Melyn, 2005). Estimates of the mean rate of epilepsy in autistic individuals range from 16.8% (Fombonne, 2002) to 30% (Gillberg and Billstedt, 2000), and reach as high as 46% (Hughes and Melyn, 2005). In addition it is estimated that 50–70% of autistic individuals display ongoing “sharp-spike” activity during sleep (Lewine et al., 1999; Wheless et al., 2002).

Since the severity and behavioural manifestation of the three core features and associated symptoms is highly variable among individuals, the term autism spectrum disorder is now commonly used. For instance, at the “high functioning” end of the autism spectrum lies Asperger’s syndrome1, which shares the core features of autism, but is characterized by IQ in the normal to high range, and intact language ability (Frith et al., 1991; Woodbury-Smith and Volkmar, 2008). It has been suggested that a portion of the heterogeneity of autism features may be explained by the heterogeneity of genetic factors (described below) that underlie it (Baird and August, 1985). Autism also bears symptomatic similarities with other pervasive

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1 Asperger’s syndrome was originally described by the German scientist, Hans Asperger, around the same time as Kanner’s description of autism.
developmental disorders such as Angelman’s and Rett syndromes, tuberous sclerosis, and fragile X syndrome (Williams et al., 2001; Zoghbi, 2005; Jedele, 2007; Zafeiriou et al., 2007). Due to the heterogeneity of symptoms and inclusion criteria, estimates on the prevalence of autism spectrum disorders range from as high as 73 per 10,000 (Kadesjo et al., 1999; Fombonne, 2005) to as low as 10 per 10,000 (Fombonne, 2003b). Additionally, studies have noted an increase in the prevalence of autism from 4.4 per 10,000 between 1966 and 1991, to 12.7 per 10,000 between 1992 and 2001 (Fombonne, 2003a, 2008). This increase in rate however, must be carefully interpreted, since several changes in diagnosis, and tools available, as well as increased awareness of the disorder complicate the issue (Fombonne, 2003a, 2008).

1.8.2. Genetic Basis of Autism

It has been postulated that one factor contributing to the behavioural heterogeneity of autism, is the heterogeneity genetic factors that have been identified. The importance of genetic factors in autism has been highlighted by epidemiological studies showing that the risk of first relatives is about 100-fold higher than the risk in the normal population and the concordance reaches >90% for identical twins, compared to <10% for fraternal twins and siblings (Folstein and Rosen-Sheidley, 2001; Muhle et al., 2004; Veenstra-Vanderweele et al., 2004; Veenstra-VanderWeele and Cook, 2004; Geschwind, 2007; Geschwind and Levitt, 2007; Zhao et al., 2007).

Genetic models most congruent with current inheritance data suggest many loci (>10) of weak to moderate effect, with three to six epistatic loci being the most simple possibility (Risch et al., 1999; Folstein and Rosen-Sheidley, 2001; Bonora et al., 2005; Santangelo and Tsatsanis,
In conflict with this, evidence from eight independent full-genome scans demonstrated the heterogeneity of autism loci, with multiple intersecting sets of alleles at different loci (Jiang et al., 2004). The genetic component of autism is consequently best described by a multilocus model that takes into account epistatic interactions between several susceptibility genes.

The chromosomal regions most consistently linked to autism include 2q32, 7q21–q22, 7q32 and 15q11–q13 (Ashley-Koch et al., 1999; Barrett et al., 1999; Philippe et al., 1999; 2001a; 2001b; Bradford et al., 2001; Buxbaum et al., 2001; Auranen et al., 2002; Nurmi et al., 2003; Alarcon et al., 2005; Cantor et al., 2005; Lamb et al., 2005; Vorstman et al., 2006).

1.8.2.1. Neuroligins and Related Proteins in Autism

Important insight into autism neuropathology was recently revealed when mutations in synaptic proteins were found to be associated with autism (Table 1.1). In particular, recent studies have drawn a link between the NL family of adhesion molecules and autism. Multiple studies have demonstrated rearrangements of chromosomal regions harboring NL1 and -2 (Mariner et al., 1986; Konstantareas and Homatidis, 1999; Risch et al., 1999; Auranen et al., 2002; Auranen et al., 2003). In addition, a point mutation in NL3 was identified in two autism/Asperger affected brothers (Jamain et al., 2003b). This mutation was identified as a C to T transition, which resulted in substitution of a cysteine residue in the place of a highly conserved arginine residue (R451C). R451 is located in a predicted EF-hand domain conserved in all known NLs known to confer structural integrity and functional properties (Tsigelny et al., 2000). A large amount of evidence has also accumulated for alterations in NL4 in autism from multiple independent studies. Alterations in NL4 have been shown to include de novo
Table 1.1. Summary of the genetic mutations in neuroligin-neurexin adhesion molecules and related synaptic proteins implicated in populations with autism.

<table>
<thead>
<tr>
<th>NL/Nrx Protein</th>
<th>Established Function</th>
<th>Chromosome</th>
<th>Linkage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroligin 1</td>
<td>Postsynaptic adhesion molecule of excitatory synapses</td>
<td>3q26.3</td>
<td>• Chromosomal abnormality • Linkage in family members</td>
<td>Konstantareas and Homatidis, 1999; Auranen et al., 2002; 2003</td>
</tr>
<tr>
<td>Neuroligin 2</td>
<td>Postsynaptic adhesion molecule of inhibitory synapses</td>
<td>17p13.2</td>
<td>• Chromosomal abnormalities • Linkage in affected siblings</td>
<td>Mariner et al., 1986; Risch et al., 1999</td>
</tr>
<tr>
<td>Neuroligin 3</td>
<td>Postsynaptic adhesion molecule of excitatory and inhibitory synapses</td>
<td>Xq13.1</td>
<td>• Point mutation (R451C)</td>
<td>Jamain et al., 2003</td>
</tr>
<tr>
<td>Neuroligin 4</td>
<td>Reduced expression in the CNS relative to other Neuroligin family members</td>
<td>Xp22.33</td>
<td>• De novo deletions in 3♀ with autism • Frame shift mutation (1186insT) • 2-base-pair deletion (1253delAG) • 4 miss-sense mutations in unrelated patients(G99S,F378R,V403M,R704C).</td>
<td>Thomas et al., 1999; Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2008; Marshall et al., 2008</td>
</tr>
<tr>
<td>Neurexin</td>
<td>Presynaptic adhesion molecule of excitatory &amp; inhibitory</td>
<td>2p16.3</td>
<td>• Alpha and beta affected • incomplete penetrance</td>
<td>Feng et al., 2006; Kim et al., 2008; Marshall et al., 2008; Yan et al., 2008</td>
</tr>
<tr>
<td>Related Protein</td>
<td>Established Function</td>
<td>Chromosome</td>
<td>Linkage</td>
<td>References</td>
</tr>
<tr>
<td>Shank 3</td>
<td>Postsynaptic scaffolding protein of excitatory synapses; spine morphology</td>
<td>22q13.3</td>
<td>• Linkage in siblings affected with autism</td>
<td>Durand et al., 2007; Moessner et al., 2007; Marshall et al., 2008</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic scaffolding protein of excitatory synapses; clusters glutamate receptors</td>
<td>17p13.2</td>
<td>• Linkage in siblings affected with autism</td>
<td>Risch et al., 1999</td>
</tr>
<tr>
<td>GABRB3</td>
<td>GABA receptor subunit</td>
<td>15q12</td>
<td></td>
<td>Cook et al., 1998; Buxbaum et al., 2002; Samaco et al., 2005</td>
</tr>
<tr>
<td>GABARAP</td>
<td>Postsynaptic scaffolding protein of inhibitory synapses; clusters GABA receptors</td>
<td>17p13.2</td>
<td>• Linkage in siblings affected with autism</td>
<td>Risch et al., 1999</td>
</tr>
<tr>
<td>Related Protein</td>
<td>Established Function</td>
<td>Chromosome</td>
<td>Linkage</td>
<td>References</td>
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<tr>
<td>CNTNAP2</td>
<td>Member of the neurexin superfamily, localized to juxtaparanodes of myelinated axons, thought to be involved in axon differentiation</td>
<td>7q35</td>
<td>• loss of function truncation • de novo 7q35 inversion • Single nucleotide polymorphisms rs7794745, rs2710102</td>
<td>Strauss et al., 2006; Abrahams et al., 2007; Bakkaloglu et al., 2008; Arking et al., 2008; Alarcon et al., 2008</td>
</tr>
</tbody>
</table>

deletions in three females with autism (Thomas et al., 1999), a frame shift mutation in two autism-affected brothers (1186insT; (Jamain et al., 2003b), a two-base-pair deletion in affected males of a French family with mental retardation/autism/pervasive developmental disorder (1253delAG; (Laumonnier et al., 2004), and four miss-sense mutations in unrelated patients (G99S,F378R,V403M,R704C; (Yan et al., 2004; Yan et al., 2008a).

In addition to alterations in NLs, single copy chromosomal deletion of a region containing α and β-Nrxs has also been identified in families with autism (Szatmari et al., 2007; Kim et al., 2008; Yan et al., 2008b). The association of Nrx with autism indicates that changes in the expression of NL related proteins can confer the behavioural changes associated with autism. A recent genome-wide assessment for structural abnormalities in 427 unrelated autism cases has also shown that autistic patients have novel deletions and duplications in their genomes, some of which affected NL-Nrx genes (Sebat et al., 2007; Marshall et al., 2008). Again this result highlights the importance of dose, and suggests that both reduced and enhanced expression of affected genes could possibly contribute to the manifestation of autism.

Subsequent mapping of specific mutations associated with autism onto the crystal structure of NLs revealed how these mutations may exert their effects (Figure 1.7; (Arac et al., 2007; Fabrichny et al., 2007). The majority of autism related NL mutations are found between the NL
Figure 1.7. Schematic structure of neuroligin and neurexin interaction at synaptic contacts, and influence of autism related mutations in neuroligins on their interaction. A. Dimerization of NLs (blue) through interaction of their four-helix bundles allows two Nrx molecules (orange) to bind the dimer. Interaction of NL and Nrx forms an intact transsynaptic connection that links presynaptic vesicle fusion with receptor localization and signalling in the postsynaptic compartment. B. Mutations in NLs associated with autism are predicted to disrupt NL dimerization and thus reduce the affinity of NLs for Nrxs. Loss of the NL-Nrx interaction would lead to alterations in synaptic adhesion, as well as impaired trans-synaptic signalling through this complex. Levinson and El-Husseini. 2007. A crystal-clear interaction: relating neuroligin/neurexin complex structure to function at the synapse. Neuron 56(6):937-9.
A Coupling of neuroligins and neurexins at the synapse

B Autism-associated mutations disrupt neuroligin dimerization and binding to neurexins

presynaptic terminal

postsynaptic plasma membrane

trans-synaptic signaling required for synapse maturation and function

altered trans-synaptic signaling leads to defects in synapse maturation and function
dimerization interface and the site corresponding to the AChE active site. Therefore, these mutations are unlikely to directly interfere with binding of NLs to Nrxs, but more likely cause defects in NL folding and dimerization, thus indirectly altering Nrx binding (Levinson and El-Husseini, 2007). Alternatively, it has also been proposed that altered folding of NLs could lead to defects in protein processing in the ER and subsequent trafficking (Dean and Dresbach, 2006). From these studies it is clear that further in vivo studies of the NL-Nrx families of adhesion molecules are warranted to understand both their basic function at the synapse, as well as to define what roles they may have in neurodevelopmental disorders.

1.8.3. Neuropathology and Brain Systems and Circuits Implicated in Autism

One common theory of autism neuropathology is that of excessive brain growth early in postnatal development, followed by subsequent impairments in brain growth and development (Courchesne et al., 2003; Amaral et al., 2008). This theory is of interest since it parallels the symptoms in children with autism which often appear to be developing normally in early postnatal stages, followed by a halting or regression. Evidence to support brain overgrowth comes from studies of head circumference (Courchesne et al., 2003; Dementieva et al., 2005; Dawson et al., 2007), as well as MRI analysis (Courchesne et al., 2001; Sparks et al., 2002; Hazlett et al., 2005). Although these studies are intriguing, they provide little specific information regarding candidate brain region or circuits involved in autism neuropathology.

Based on the symptoms that characterize autism, several neural systems appear to be likely candidates for involvement in the underlying neuropathology (Figure 1.8). For example, systems that have been shown to regulate social behaviours, language and communication,
Figure 1.8. Brain regions predicted to be involved in the behavioural expression of autism spectrum disorders. Visualization of human brain regions implicated in controlling the three core behaviours associated with autism, including impairments in social behaviour, language and communication, as well as the incidence of repetitive and stereotyped behaviours. Brain regions implicated in each of the three core behaviour are summarized in the table. Amaral DG, Schumann CM, Nordahl CW. 2008. Neuroanatomy of Autism. Trends in Neuroscience 31(3):137-45.
<table>
<thead>
<tr>
<th>Social impairment</th>
<th>Communication deficits</th>
<th>Repetitive behaviors</th>
</tr>
</thead>
<tbody>
<tr>
<td>OFC – Orbitofrontal cortex</td>
<td>IFG - Inferior frontal gyrus (Broca’s area)</td>
<td>OFC – Orbitofrontal cortex</td>
</tr>
<tr>
<td>ACC – Anterior cingulate cortex</td>
<td>STS – Superior temporal sulcus</td>
<td>ACC – Anterior cingulate cortex</td>
</tr>
<tr>
<td>FG – Fusiform gyrus</td>
<td>SMA – Supplementary motor area</td>
<td>BG – Basal ganglia</td>
</tr>
<tr>
<td>STS – Superior temporal sulcus</td>
<td>BG – Basal ganglia</td>
<td>Th – Thalamus</td>
</tr>
<tr>
<td>A – Amygdala mirror neuron regions</td>
<td>SN – Substantia nigra</td>
<td>PN – Pontine nuclei cerebellum</td>
</tr>
<tr>
<td>IFG – Inferior frontal gyrus</td>
<td>Th – Thalamus</td>
<td></td>
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<tr>
<td>PPC – Posterior parietal cortex</td>
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cognition, attention and fear or anxiety represent good targets for the study of autism, and include systems that integrate cortical centres with the hippocampus, amygdala, basal ganglia, thalamus, hypothalamus and cerebellum. Indeed, post-mortem and magnetic resonance imaging (MRI) in autistic individuals have revealed abnormalities in several of these candidate cortical and subcortical regions; however no consensus has been reached on the neural systems involved and their specific pathologies (Amaral et al., 2008). Below, some of the key findings on the most commonly implicated brain regions, frontal cortex, amygdala and cerebellum, will be discussed in more detail.

1.8.3.1. Frontal Cortex

The frontal cortex is considered to be the centre of information integration required for social interaction and emotional behaviour, placing it at the centre of targets for study in autism (Courchesne and Pierce, 2005; Amodio and Frith, 2006). Despite the strong link to symptoms, little data has been collected on specific alterations in the frontal cortex of patients with autism.

Evidence for a role of the frontal cortex in autism has been provided by MRI studies. In a comparison between groups of individual with autism and low intellect, autism and normal intellect and control subjects, bilateral abnormality was observed in the frontal cortex common to the two autistic groups (Salmond et al., 2007). Abnormalities were also found in the dorsolateral prefrontal cortex when comparing normal and low intellect groups with autism.

Post-mortem studies have also provided evidence of changes in the cyto-architecture of cortical regions (Casanova et al., 2002; Casanova et al., 2006). In general, cortical minicolumns
have been shown to be more narrow, with more dispersed cell bodies, in both frontal and temporal cortices of persons with autism. It has been suggested that this type of neuropathology could arise from altered inhibitory activity at the boundaries of each column, leading to noisy or unsynchronized cortical activity. It has also been suggested that this finding supports an additional theory of autism that posits enhanced local connectivity at the expense of long range connectivity (Courchesne and Pierce, 2005; Casanova, 2006). In particular, it has been suggested that excessive and unselective connectivity in local frontal cortex circuitry, paired with impoverished long-range connectivity to limbic and sensory systems could explain the neuropathology and symptom range of autistic individuals (Courchesne and Pierce, 2005).

1.8.3.2. Amygdala

Like the frontal cortex, the amygdala is thought to be a likely candidate for autism neuropathology due to its connection with the symptoms of autism. The amygdala has been associated with social behaviour, and also has been shown to have a prominent role in anxiety (Amaral and Corbett, 2003; LeDoux, 2003, 2007). MRI study has suggested that the amygdala displays altered developmental growth patterns in individuals with autism. Studies have shown a 13-16% enlargement of the amygdala in both young children with autism (Sparks et al., 2002) and boys aged 8-12, but did not differ in 13-18 year old boys (Schumann et al., 2004). Further this study by Schumann et al. (2004) showed that the typical development of the amygdala from age 8 to 18 is paralleled by 40% increase in size, whereas this developmental growth pattern did not occur in boys with autism. These data suggest that the amygdala is larger in autistic children, but then does not undergo the typical pattern of growth into adolescence. In parallel with this, studies in older adolescents or adults have shown no difference or decreased
amygdala size in autistic patients (Aylward et al., 1999; Haznedar et al., 2000; Nacewicz et al., 2006).

Post-mortem studies in the amygdala of autistic subjects have also revealed abnormalities. Specifically, it has been shown that neurons in amygdala subnuclei appear more densely packed and unusually small in size (Kemper and Bauman, 1993). A subsequent study revealed differences in the number of neurons in amygdala subnuclei, but did not find decreased neuron size, or alterations in neuron packing density (Schumann and Amaral, 2006). Post-mortem and MRI findings taken together support the theory of normal to excessive early development, followed by slowing or subsequent elimination at adolescence into adulthood, however more detailed examination of amygdala tissue and longitudinal MRI studies will be needed to confirm this possibility (Amaral et al., 2008).

1.8.3.3. Cerebellum

Although the cerebellum has traditionally been regarded as a regulator of motor behaviour, accumulating evidence suggests that the cerebellum is involved in a wider range of functions, including learning, planning, temporal estimation, and cognitive processing of words (Ramnani, 2006). In particular, cerebellar connectivity with limbic and cortical systems could possibly underlie language, socioemotional, motor and sensory abnormalities observed in autistic individuals (Dum et al., 2002; Gowen and Miall, 2007).

Several MRI studies have examined cerebellar volume in autistic subjects with varying results (Hardan et al., 2001; Cleavinger et al., 2008; Hallahan et al., 2008). The most consistently reported finding is cerebellar enlargement, however the increases found were generally
proportional to total brain volume (Hrdlicka, 2008). In contrast to the total cerebellum, assessment of the size of the vermis is smaller in individuals with autism (Courchesne et al., 1994; Kaufmann et al., 2003). Post-mortem studies of the cerebellum have shown a decreased density of purkinje cells, particularly in the cerebellar hemispheres (Ritvo et al., 1986; Kemper and Bauman, 1993; Bailey et al., 1998; Palmen et al., 2004). Further studies will be needed to rationalize MRI findings with more thorough post-mortem assessment to determine the precise changes that occur in the cerebellum of autistic individuals.

The heterogeneity of both core and associated symptoms in autism is suggestive of a heterogeneous pattern of underlying neuropathology. Our understanding of autism is currently limited because of the lack of defined pathophysiological mechanisms, or clearly defined risk genes, highlighting the importance of generating a relevant model to promote our understanding of this complex disorder.

1.9. Animal Models of Autism

Current animal models of autism include amygdala (Wolterink et al., 2001) or medial cerebellar (Bobee et al., 2000) lesions, valproic acid (Ingram et al., 2000) or oxytocin and vasopressin (Insel et al., 1999) treatments, as well as genetic models in mice such as knock-out of the genes encoding homeobox 1.6 (Chisaka et al., 1992) or oxytocin (Young et al., 1997; Ferguson et al., 2001), or inactivation of Disheveled II (Lijam et al., 1997). A series of recent mouse models for autism were generated using the R451C-substitution in NL3 (Crawley, 2007; Tabuchi et al., 2007; Chadman, 2008) and the loss of function mutation in NL4 (Jamain et al., 2008). In the
first set of studies, the NL3R451C model showed enhanced inhibitory synaptic activity and impairments on some but not all tests of social interaction (Crawley, 2007; Tabuchi et al., 2007). In contrast, a second group that generated and tested mice with the R451C-substitution, detected no differences between NL3R451C and their wildtype littermate controls on any autism related behaviours tested, including multiple measure of social interaction, cognitive ability, and resistance to change (Chadman, 2008). Mice with NL4 loss of function mutation exhibited deficits in reciprocal social interactions and communication (Jamain et al., 2008). Despite this work, it remains unclear whether altering NLs in vivo can recapitulate multiple aspects of autism, and provide a useful tool for investigation of neuropathology and therapeutics.

While the currently available models all resemble certain aspects of autistic disorders, most models have not yet been systematically used to explore the complex multiple facets of autistic-like behaviours or their underlying neuropathology (Andres, 2002; Tordjman et al., 2007; Chadman et al., 2008). The value of animal models can be assessed in terms of their similarity to aspects of autism. These assessments of validity have been divided into three major categories including behavioural similarity to the core symptoms of autism, which is referred to as face validity; similarity to the underlying biological mechanisms of autism, referred to as construct validity; and pharmacological similarity or the model’s ability to identify correctly drugs with potential therapeutic value in humans, which is referred to as predictive validity (Robbins and Sahakian, 1979).

The main limitations with animal models of autism concern their validity, as well as the ability to assess their validity due to the complex cognitive nature of the disorder (Andres, 2002;
Tordjman et al., 2007). In particular, the relevance of animal models to neuropsychiatric disorders such as autism depends upon the assumption that humans and animals have in common the basic neurobiological mechanisms that underlie complex behaviours such as sociability and communication. Given this limitation it is important to develop multi-trait models, as it is likely that models with a large number of similar features will provide researches with a closer approximation to autism (Chadman et al., 2008).

1.10. Rationale and Hypotheses

The rationale for the experiments performed in this thesis is based on multiple key findings in the fields of synaptogeneis and neurodevelopment. Firstly, several studies have indicated that the NL/Nrx transsynaptic adhesion system is a major factor in regulating synapse maturation and a key determinant for specifying the E/I synaptic ratio (Prange et al., 2004b; Chih et al., 2005; Cline, 2005; Levinson et al., 2005a; Levinson and El-Husseini, 2005b, a; Chubykin et al., 2007; Craig and Kang, 2007; Kang et al., 2008). In parallel, genetic studies in populations with autism have demonstrated a link between synaptic proteins, specifically NL and Nrx proteins, and autistic disorders (Mariner et al., 1986; Konstantareas and Homatidis, 1999; Risch et al., 1999; Auranen et al., 2002; Auranen et al., 2003). Based on the recent genetic findings as well as the symptoms of autism, a hypothesis on the pathogenesis of autism was proposed by Rubenstein and Merzenich (2003) suggesting that autistic disorders may arise from an imbalance in the E/I ratio.

Despite these advances, it remains unclear whether changes in the level of expression of a single NL isoform in vivo will disturb the molecular composition, structure, and function of
excitatory and inhibitory synapses, and thereby contribute to the pathogenesis of developmental brain disorders such as autism. We hypothesize that autism or other neurodevelopmental disorders result from changes in NL expression and can be related to shifts in the E/I ratio. To test this hypothesis, we will examine mouse models expressing either NL1 or NL2 for changes in the E/I ratio, as well as for behavioural deficits manifested in neurodevelopmental disorders.

1.11. Objectives

In order to determine the impact of altered expression of NLs, we will examine the distribution of excitatory and inhibitory synaptic proteins in NL1 and NL2 transgenic mouse strains using Western blotting and immunohistochemistry, and examine synapse structure and number using electron microscopy. We will also examine synapse function using electrophysiological recordings of spontaneous and evoked activity. Further, we will characterize how differential effects on excitatory and inhibitory synapse types in NL1 versus NL2 mice can differentially influence behaviour. The primary goal of this thesis is to test the hypotheses outlined above with the experiments described in the following major aims.

**Aim 1: Generate strains of mice expressing either NL1 or NL2.** We will use the the Thy1.2 promoter to drive neuron specific expression of either NL1 or NL2, tagged with hemaglutinin (HA) for easier detection of the transgene. Founder mice created from pronuclear injection of the transgenes will be backcrossed with C57 Bl/6 mice to generate stable strains that will be used in the following aims to test our specific hypotheses. (For the description of transgenic
Aim 2: Determine the influence that expression of NL1 or NL2 in vivo will have on related proteins. We hypothesize that enhanced expression of different NLs will exert differential effects on their various binding partners, and related proteins. To assess this hypothesis we will use Western blotting and immunohistochemistry to examine the expression and distribution of excitatory and inhibitory synaptic proteins in strains of mice expressing NL1 and NL2. Specific proteins to be assessed include the proteins VGlut/PSD-95 and VGAT/gephyrin which are markers of pre-/postsynaptic excitatory and inhibitory compartments respectively.

Aim 3: Determine the influence that expression of NL1 or NL2 in vivo will have on excitatory and inhibitory synapse number, structure and function, and the E/I ratio. We hypothesize that expression of different NLs will exert differential effects on excitatory and inhibitory synapses. Specifically, enhanced expression of NL1 will lead to a shift toward excitation by increasing the maturation and/or number of excitatory synapses. In contrast, expression of NL2 will lead to a shift toward inhibition, as evidenced by increased maturation and/or number of inhibitory synapses. We will use electron microscopy to analyse the density and morphology of excitatory and inhibitory synaptic contacts in TgNL1 and TgNL2 strains. Morphological parameters to be assessed include PSD and synaptic contact length, pre- and postsynaptic compartment area, and vesicle numbers. In addition, we will collaborate with laboratories
specializing in electrophysiology to functionally assess alterations in the E/I ratio, using spontaneous and evoked recordings.

**Aim 4: Examine the result of in vivo alterations to NL1 and NL2 expression levels on mouse behaviour.** We hypothesize that TgNL1 and TgNL2 mice will display different types of dysfunction arising from their differential shifts in the E/I ratio. To examine the influence of differential shifts in the E/I ratio on behaviour we will examine and compare TgNL1 and TgNL2 mice on tasks assessing a variety of behavioural outputs. Specific tasks to be used will assess phenotypes relevant to autism, including assessments of social behaviour, anxiety, and learning.

**1.12. Significance**

This work is expected to clarify the molecular mechanisms that dictate synapse morphology and the specificity for excitatory versus inhibitory synapse formation. With expression of NLs in vivo, we will also be able to evaluate what implications altering stages of synaptogenesis will have on central nervous system function, and behaviour. The proposed experiments will shed light on how enhancing the amounts of synaptic adhesion molecules will influence synapse function. Ultimately, these experiments will help us to understand how alterations in synaptic proteins and synaptic dysfunction can contribute to complex disorders such as autism.
1.13. Bibliography


Daw MI, Chittajallu R, Bortolotto ZA, Dev KK, Duprat F, Henley JM, Collingridge GL, Isaac JT (2000) PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-


2. SYNAPTIC IMBALANCE, STEREOTYPIES, AND IMPAIRED SOCIAL INTERACTIONS IN MICE WITH ALTERED NEUROLIGIN 2 EXPRESSION

2.1. Introduction

Synapse maturation is a critical step in the generation of the complex neuronal networks of the CNS (Waites et al., 2005). Homeostatic control has been suggested to regulate neuronal function through synaptic efficacy, strength, and membrane excitability (Turrigiano and Nelson, 2004). The importance of tight regulation of synapses can be gleaned from the number of disorders arising from alterations to synapses (Holmes and McCabe, 2001; Zoghbi, 2003).

Neuroligin (NL) adhesion molecules and their presynaptic binding partners, neurexins (Nrxs), are involved in regulating excitatory and inhibitory synapse function. NL function at the synapse is modulated by alternative splicing and association with binding partners (Ichtchenko et al., 1995; Irie et al., 1997; Prange et al., 2004; Boucard et al., 2005; Levinson et al., 2005; Chih et al., 2006; Graf et al., 2006). Consistent with a fundamental role for NLs in synapse maturation, a reduction in presynaptic terminal content, but not number, has been observed in NL1–3 triple knock-out mice (Varoqueaux et al., 2006). NLs are enriched at either excitatory (NL1,3) or inhibitory (NL2) synapses (Song et al., 1999; Graf et al., 2004; Varoqueaux et al., 2004; Chih et al., 2005; Levinson et al., 2005; Chubykin et al., 2007). Despite their specific localization, in vitro studies indicate that NLs can induce both excitatory and inhibitory presynaptic specializations (Scheiffele et al., 2000; Graf et al., 2004; Prange et al., 2004; Chih et al., 2005; Levinson et al., 2005; Gerrow et al., 2006). The ability of NLs to regulate excitatory and inhibitory synapses led to the proposal that NLs may comprise the molecular machinery

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that maintains the E/I ratio (Cline, 2005; Levinson and El-Husseini, 2005). Work on cultured neurons shows that abnormal targeting of NLs alters the E/I ratio (Levinson and El-Husseini, 2005; Levinson et al., 2005). Analysis of acute slices from NL knock-out mice demonstrated that specific NLs differentially affect excitatory and inhibitory synapse function (Chubykin et al., 2007). These findings led to the question of whether altered expression of NLs could induce synaptic imbalance in vivo, leading to dysfunction of the CNS.

An important link to CNS disorders was recently revealed when mutations in synaptic proteins were found to be associated with autism, a neurodevelopmental disorder characterized by repetitive/stereotyped behaviour, varying degrees of abnormality in communication ability, and social interactions, along with high incidence of seizure (Konstantareas and Homatidis, 1999; Auranen et al., 2002; Tuchman and Rapin, 2002; Jamain et al., 2003; Rubenstein and Merzenich, 2003; Zoghbi, 2003; Laumonnier et al., 2004; Lise´ and El-Husseini, 2006; Christ et al., 2007; Dover and Le Couteur, 2007; Garber, 2007; Rutherford et al., 2007). Rearrangements of chromosomal regions harboring NL1,2, and mutations in NL3,4 have been detected in families with autism. One particular NL3 mutation associated with autism, Arg451Cys, when introduced into mice, leads to enhanced inhibition and impaired social interactions (Tabuchi et al., 2007). Single-copy chromosomal deletion of a region containing α/β-Nrxs in families with autism demonstrates the influence of changes in gene dose in autism (Szatmari et al., 2007). In addition to single gene alterations, recent work has shown that many autistic patients have novel deletions and duplications in their genomes (Sebat et al., 2007). Thus, both reduced and enhanced expression of affected genes can contribute to the manifestation of autism.
Both loss- and gain-of-function studies indicate that altered amounts of NLs result in aberrant synapse maturation and altered neuronal excitability. It remains unclear, however, whether changes in expression of single NLs in vivo will disturb the E/I balance and result in behavioural deficits. In the present study, we use transgenic (Tg) mice to examine whether expression of specific NLs results in synaptic imbalance and altered neuronal excitability in vivo. Our results reveal that enhanced expression of NL2 induces aberrant synapse maturation and altered neuronal excitability, leading to behavioural deficits.

2.2. Materials and Methods

2.2.1. Generation and Genotyping of Transgenic Mice

NL1 and NL2 transgenes were expressed under control of the Thy1 promoter for neuron specific expression. The NL1 specific sequence (1AB splice variant) was generated by insertion of a Sal I site into the 3’ Spe I site of mouse NL1 cDNA tagged with HA following the signal sequence. This sequence was then amplified using Sal I and Xho I anchored primers. NL2 specific sequence was similarly amplified via Sal I (inserted into Spe I site at 3’ end) and Xho I anchored primers from a plasmid containing rat NL2 cDNA tagged with HA following the inserted NL1 signal sequence. Following restriction digestion, these fragments were then cloned into the Xho I site of the Thy1.2 plasmid (Figure 2.1A). The plasmid was cut using Pme I and Pvu I to remove bacterial backbone, and the resulting Thy1.2 NL1 and NL2 HA fragments were used to generate NL1 and NL2 transgenic mice (TgNL1 and TgNL2). Multiple strains were generated transgene from founders via backcrossing to C57BL/6. TgNL2 female founders were unsuccessful at raising pups despite being fertile and able to copulate. Founders with 2-fold or
Figure 2.1. Mice expressing neuroligin 2 display a neurological phenotype related to levels of expression. A. Schematic diagram of HA tagged NL1 and NL2 constructs in the Thy1.2 vector. B. Assessment of transgene expression level in TgNL1.6, TgNL1.7 and TgNL2.6 strains using immunoblotting for HA. Numbers in boxes show quantification of total protein load from coomassie stained gels. C. Confirmation of generated NL2 rabbit antibody specificity (ABR) on COS-7 cell lysates transfected with HA-NL1, HA-NL2 or HA-NL3 (left 3 lanes), and on whole brain lysates from wildtype and TgNL2.6 mice (right 2 lanes). D. Western blotting analysis of whole brain lysates of endogenous and HA-NL2 expression using NL2 and HA antibodies. E. Limb clasping in TgNL2 mice (right panel) not seen in wildtype littermates or TgNL1 mice. F. Table comparing the level of HA-NL2 across TgNL2 strains relative to endogenous NL2. The severity and frequency of limb clasping parallels the expression level of TgNL2 but not TgNL1 mice. Data shown are means ± SEMs.
greater expression of NL2 (TgNL2.1, 2.4, 2.5) died by 24 weeks postnatal, and these strains failed to produce viable offspring. Two primary strains from low expressing male founders (TgNL2.6 and TgNL2.7) were successfully mated, however TgNL2.7 female offspring were unsuccessful at rearing pups. Attempts were made to maintain the TgNL2.7 strain via ovary transplantation into female C57Bl/6-CBA F1 hybrid female recipients. Similar limitations in producing viable offspring were not seen in TgNL1 mice. The TgNL2.6 strain has moderate expression, marked phenotype, and good viability and consequently was used in all experiments. Whenever possible, experiments were conducted in a higher expressing strain (TgNL2.7) to confirm the data seen in the TgNL2.6 strain. Non-transgenic littermates were used as controls in all experiments (wildtype). Animals were maintained at the Centre for Molecular Medicine and Therapeutics Transgenic Core Facility according to protocols approved by the University of British Columbia Animal Care Committee. Mice were group housed with a 12 hour light-dark cycle with constant temperature. Genotype-blinded behavioural assessments were conducted on transgenic and wildtype mice during the light phase.

Germ line transmission of the transgenes was detected using PCR (Figure 2.2A) with primers spanning from Thy1 into the NL1 signal sequence to recognize both transgenes, and specific primers were also designed to differentiate TgNL1 positives from TgNL2 positives. The primer sequences are as follows: Both NL1 and NL2 transgenes – Thy1 5’-TAGGCTCCCACCTCCTGTCGCC-3’, NLss 5’-GCTATGATGGCATGTGTGGTCGCTG-3’; Transgenic NL1 - NL1; both endogenous and transgenic NL2 - NL2 intron/exon for 5’-CCTGTGTCAACCTTGCTCC-3’, NL2 intron/exon rev 5’-CCTGTGATCCGAGATCC-3’.
Figure 2.2. Genotyping, antibody generation and basic characterization. A. Genotyping of mice using two sets of primers designed to recognize the NL1 transgene (NL1trans – 650 bp) and intron-exon spanning primers designed to recognize both endogenous (420 bp) and transgenic (250 bp) NL2 (NL2endo&trans). B. NL2 rabbit antibody (green) gives punctate staining of clusters which colocalize primarily with VGAT (blue) and rarely with VGlut (red) in rat cortical and hippocampal cultures. C. Staining for NL2 (red) and HA (green) in TgNL2.6 mouse cortical tissue shows high degree of colocalization. Scale bar = 5μm. D. Staining for HA (green) DAPI (blue) and MAP-2 (red) in cortical cultures generated from TgNL2.6 mouse pups at postnatal day 1 (P1). HA staining is seen exclusively in neurons (DAPI positive nuclei that are also positive for HA and MAP-2) and reveals transgene expression in a large number of cells.
2.2.2. Behavioural Assessments

Preliminary Screen: The preliminary screen were based on the modified SHIRPA protocol employed by European Mouse Phenotyping Resource of Standardised Screens (EMPReSS) designed to evaluate the basic phenotype of transgenic mouse strains (Brown et al., 2005; Brown et al., 2006). Please see Appendix for full rating scales used in the behaviour screen.

Open Field: Open field behaviour of mice was assessed using the Noldus Ethovision Video Tracking system (Noldus, Wageningen, the Netherlands). Results from tracking analysis were analyzed using ANOVA to compare means. Stereotyped jumping, and digging and grooming patterns of behaviour were manually scored from video recordings of open field behaviour (Wt n=7; TgNL2.6 n=8). The incidence and duration of each behaviour was recorded, with the termination of each incidence being determined by interruption with another behaviour (example – termination of a grooming bout by initiation of forward locomotion). Since high levels of stereotyped jumping behaviour have the potential to interfere with the assessment of other complex behaviours, all mice were prescreened prior to subsequent behavioural assessment of anxiety or social behaviour. Animals found to have more than one episode of stereotyped jumping during a five minute prescreening were excluded from subsequent tasks. For prescreening and to assess jumping stereotypies in larger numbers of mice (n=50), a genotype blinded observer tallied the incidence of jumping stereotypies during five minute open field sessions, with the observer sitting behind a curtain watching live video feed.

Anxiety Behaviour: For the light-dark exploration test, mice were placed into the light chamber of a standard 2-chamber arena (Holmes et al 2002; Holmes et al., 2003). The arena is composed of a larger chamber that is transparent and brightly illuminated from above, and a
smaller chamber that is black walled and not illuminated. The two chambers were separated by a partition with a small doorway to allow the animal to freely pass from chamber to chamber while exploring for 10 minutes. Videos (30 fps) were digitized and scored manually for number of light↔dark chamber transitions, and time spent in each chamber.

The elevated plus maze was constructed based on that previously described (Holmes et al., 2003; Holmes et al., 2002) and was composed of two open and two closed arms extending from a central platform, elevated to a height of 40 cm above the floor. Test sessions were initiated by placing individual animals in the center square facing an open arm, and were terminated after 5 minutes of free exploration. Digitized video recordings (30 fps) were scored manually for the number of entries (all four paws into the arm) into open and closed arms, and time spent in open and closed arms.

Social Behaviour: For the reciprocal social interaction tests, test (wildtype and TgNL2.6) and target (FVB) juvenile (six week old) male mice were simultaneously placed into a clean, neutral homecage. The FVB strain was used to prevent possible effects of recognition via immune and olfactory cues (Boehm and Zufall, 2006) and to facilitate scoring due to coat color differences. Mice were tested in a behaviour suite that was isolated from both colony rooms where the separate mouse strains were housed. Mice were allowed to interact for 5 minutes while being video recorded from above. Videos (30 fps) were digitized and scored manually for incidence and duration of interactions, as well as which mouse was the primary initiator of each interaction. Due to multiple (4) t-tests being performed on the same data set, a Bonferroni correction factor for the t-tests run on the reciprocal social interaction test was used. This was
calculated by dividing the standard significance level (.05, .01, and .001) by the number of t-tests used (4), resulting in a Bonferroni corrected level of 0.013; 0.003; <0.001 respectively.

For the social approach task, a three chambered maze was used. The dimensions of each of the three chambers of the apparatus were 20cm (L) x 40.5cm (W) x 22cm (H), modeled after that constructed and utilised by Dr. JN Crawley’s group in Nadler et al., 2004, Moy et al., 2004. In the 3-chambered social behaviour paradigm, test-mice are placed in the center (empty) chamber and can access chambers to the left and right, which contain small cylindrical mesh cages. After habituation to the empty apparatus (10 min), the test mouse is blocked in the center chamber while a novel mouse (FVB – 6 week old juvenile) and a novel object (small rubber ball) are introduced within the mesh cages in the left and right chambers (randomized respectively). The test-mouse is then allowed to explore the apparatus again (20 min) and is monitored for preference of the novel mouse or novel object chambers.

Parameters in different zones of the three chambered apparatus were assessed using Noldus ethovision software (Wageningen, The Netherlands) from digitized video recordings (30 fps), by defining each of the three chambers as separate zones, and then assessing the time spent, speed travelled and number of rears in each zone. For both social paradigms used, the experimenter left the arena enclosed by a curtain after initiating a trial, to avoid possible influence of the experimenter on social behaviours of mice. 3’.

2.2.3. In Situ Hybridization

In situ hybridization experiments were performed on PND 28 mice as previously described (Wisden and Morris, 2002). The antisense oligonucleotides 5’ – GCCTCAAAGTTGCTGGTGTA
ACCACACAGGCAGCATGATGGCCGGCA -3 and 5'- GCCAGAAGGCCACCTTGTTGGCA CGGTAGTTGTCACGCACGCGTGG -3' corresponding to nt 341-386 and 1759-1804 respectively, of the rat neuroligin 2 ORF were used simultaneously to detect neuroligin 2 RNA, and the antisense oligonucleotide 5'- CGAGATCTAGCGTAATCTGGAACATCGTATGGGT AGACCGGTAG-3' containing the HA epitope and adjacent neuroligin 2 sequences was used to detect transgene expression. Briefly, the oligonucleotides were 3' end labeled using terminal deoxynucleotidyl transferase and (α)-35S dATP. Sections were hybridized overnight, washed in 1X SSC at 60o C for 20 minutes and exposed to film. The specificity of the three oligonucleotides was confirmed by the absence of a signal on the autoradiograph when incubated in the presence of excess unlabelled oligonucleotide and by the failure of the HA-containing antisense oligonucleotide to detect a signal in wildtype brain sections (not shown).

2.2.4. Western Blotting

Brain tissue was rapidly harvested and homogenized in TEEN buffer (50mM Tris-HCl, 1mM EGTA, 150mM NaCl) supplemented with a protease inhibitor mixture and tablet (1 for 10 mL; Roche Applied Science). Following homogenization protein concentration was assessed using a BCA assay (Pierce) and boiled in SDS page sample buffer with 10% β-mercaptoethanol for 3 min. After SDS page, Western blot signals were detected using ECL (Amersham Biosciences) or an Odyssey machine (Li-Cor). For quantification, specific protein levels were normalized to total protein load, as assessed by coomassie staining. Results were quantified using Image J and were analyzed using t-test to compare means. For antibodies used see below. WB:
Wildtype n=7; TgNL2.6 n=9; TgNL2.7 n=2; TgNL2.1, 2.4, 2.5 founders n=1; assessed in 4 independent experiments.

### 2.2.5. Immunohistochemistry

For histology and immunohistochemistry animals were either transcardially perfused with a 4% solution of paraformaldehyde in PBS (non-fluorescent IHC), or brains were harvested fresh and flash frozen in OCT embedding medium in liquid nitrogen cooled isopentane (fluorescent synaptic protein IHC). Sections were cut on a cryostat at thicknesses of 8um (synaptic protein IHC) or 30um (Nissl stain, HA localization) and were incubated in blocking solution (2.5% BSA, 0.1% triton-x, 0.02% sodium azide in PBS) for 45 minutes, followed by primary antibody incubations diluted in blocking solution overnight at 4°C. Primary antibodies used were anti-HA rat (Boehringer Mannheim); anti-NL2 rabbit (generated using a synthetic peptide; ABR; Figure 2.1C,D, Figure 2.2B,C); anti-synaptophysin rabbit (Zymed); anti-syntaxin mouse (Chemicon); anti-VGlut 1 and 2 guinea pig (Chemicon); anti-VGAT mouse (Synaptic Systems); anti-PSD-95 mouse (ABR); anti-Gephyrin rabbit (Alexis). Following washing, sections were incubated with either biotinylated secondary antibodies (non-fluorescent IHC; Vector) or Alexa conjugated secondary antibodies (fluorescent IHC; Invitrogen Molecular Probes). Non-fluorescent immunostaining was visualized using avidin-biotin conjugation (Vectastain elite standard kit; Vector) to the secondary antibody, followed by diaminobenzene (Vector). Fluorescent immunostaining (VGlut and VGAT immuno: Wildtype n=9, TgNL2.6 n=9) and colocalization analysis (HA with synaptic markers: Wildtype n=9, TgNL2.6 n=9). Immunostaining and co-
localization was quantified using Northern eclipse, and results were analyzed using T-tests to compare means.

2.2.6. Electron Microscopy

Tissue Preparation: Tissue for electron microscopy was harvested fresh and cut using a vibratome in room temperature ACSF, followed by rapid fixation in 6% glutaraldehyde, 1% paraformaldehyde, 2mM CaCl2, 4mM MgCl2 in 0.1M Cacodylate buffer. Sections were examined under a dissecting microscope and regions of the medial frontal cortex were isolated (bregma: 2.71-2.22mm). These tissue blocks were then washed in 0.1M cacodylate, postfixed in 2% osmium tetroxide, 1.5% potassium ferrocyanide in 0.1M cacodylate buffer for 2 h, and en bloc stained with 2% uranyl acetate for 45 min. Samples were then dehydrated through a series of alcohols before being transferred into propylene oxide and gradually embedded in Eponate resin. 1μm sections were taken from prospective tissue blocks and examined under a light microscope to ensure a consistent location was selected. These sections were also used for neuron counting. All tissue samples were coded with respect to genotype before electron microscopic analysis of number and morphology.

Analyses: Assessments of the size of synaptic elements were measured using Image J on 3 digital micrographs across six serial sections (measurements taken every other section). These numbers were then averaged to ensure that measurements were not influenced by the 2-dimensional position within the synapse. Synaptic vesicles were manually counted in high magnification images, and were classified according to their location within the presynaptic bouton (docked; reserve pool). Synapses were counted in fields of 100μm2. Within these
fields, synapses were classified as either Type I (asymmetric excitatory) or Type II (symmetric inhibitory) on the basis of synaptic vesicle and postsynaptic density morphology. Type I synapses were identified by the presence of a thickened post-synaptic density and round synaptic vesicles within the presynaptic terminal, whereas Type II synapses were identified by the presence of at least three oval or flattened synaptic vesicles and no thickening of the postsynaptic membrane. Wildtype n=4 mice, TgNL2.6 n=4 mice, TgNL2.7 n=3 mice, with 16 fields assessed per group. Results of synapse assessments were compared using ANOVA.

2.2.7. Whole-Cell Patch Clamp Recordings

Slice Preparation: Slices of the frontal cortex (300 μm) were prepared using routine methods used previously (Wu et al., 2007). Adult male mice (8-12 weeks) were anesthetized with 1-2 % halothane and decapitated for slice preparation. Slices were transferred to a room temperature submerged recovery chamber with oxygenated (95% O2 and 5% CO2) artificial cerebrospinal fluid (ACSF) containing (in mM: 124 NaCl, 2.5 KCl, 2 CaCl2, 1 MgSO4, 25 NaHCO3, 1 NaH2PO4, 10 glucose) at room temperature for at least 1 h.

Recording: Slices were placed in a recording chamber on the stage of an Axioskop 2FS microscope (Zeiss, Thornwood, NY) equipped with infrared DIC optics for patch clamp recordings. Postsynaptic currents were recorded with an Axon 200B amplifier (Molecular Devices, Union City, CA). Stimulations were delivered using a bipolar tungsten stimulating electrode locally placed in the prefrontal cortex. Electric square-wave voltage pulse (200 μs, 5-10 V) was generated using a Grass S88 stimulator (Grass instrument Co., Quincy, MA) attached to a Grass SIUSD isolator unit. Recording electrodes (3-5 MΩ) contained an internal solution
composed of (in mM): Kgluconate, 120; NaCl, 5; MgCl2 1; EGTA, 0.5; Phosphocreatine, 10, Mg-ATP, 2; Na3GTP, 0.1; HEPES, 10; pH 7.2; 280-300 mOsmol. The membrane potential was held at -70 mV throughout all experiments. When recording GABAA receptor-mediated currents, a holding potential of +10 mV was used. In addition, K-gluconate was replaced by equimolar CsMeSO3 and QX-314 chloride (5 mM) was added in internal solution. mIPSC Wildtype n=22, TgNL2.6 n=23; mEPSC Wildtype n=16, TgNL2.6=17. Results were compared using t-tests to compare means.

2.2.8. In vivo EEG

Surgical Preparation: Surgery was performed on 10 TgNL2 and 5 wildtype mice (12-21wks) for EEG studies. Surgical procedures conformed to the recommendations of the Canadian Council on Animal Care and the University of Toronto Animal Care Committee approved the protocols. Mice were anesthetized with IP injection of a mixture of xylazine (10 mg/kg) and ketamine (130 mg/kg). Oxygen (30%) was delivered to the mice via nose cone throughout the surgery and halothane was used for supplemental anesthesia. All electrodes were pre-attached to a miniature connector. The abdomen and scalp of mice was shaved and then cleaned with iodine (Triadine) and 70% alcohol. The head of the mouse was fixed into a stereotaxic adapter (GENEQ Inc. Model 463013, Montreal, Quebec City, CA) and mounted on a stereotaxic frame (Kopf Model 962, Tujunga, CA, USA). The scalp was incised and the skull exposed. Three small holes (1.19 mm diameter) were drilled into the skull for differential frontal-parietal recordings. Electrodes, consisting of a wire attached to a jewelers screw (with the contact end ground flat), were fixed into the holes to record EEG at the following coordinates relative to bregma: frontal
cortex (AP 2.2, ML 1), parietal cortex (AP -2.2, ML-2.5) and ground (AP -3.0, ML 3.0). Dorsal neck muscles were also exposed and Teflon coated stainless steel electrodes (Cooner Wire) were sutured (4.0 silk) to each muscle to record neck EMG. The wires and connector were secured to the skull with dental cement and cyanoacrylate glue (Krazy glue). Mice were injected, intraoperatively (SC) with buprenorphine (0.1 mg/kg) as an analgesic, and 1.0 ml sterile saline (IP) for hydration. Mice recovered on a warm heating pad until they showed signs of ambulation, and mice were permitted to recover ~7 days prior to recording. The animals recovered well from surgery as indicated by normal body weight, shiny coat, activity and normal eating habits.

Recording: Recordings were commenced at 0900 at a time when the animals normally sleep. On the day of experiment, a lightweight cable was connected to the assembly on the head of the animal. The signals were routed through a commutator (Crist Instruments, Hagerstown, MD, USA). The mice were placed in a clear plastic container situated in a cubicle (ENV-017M, Med Associates, St. Albans, VT, USA). Electrophysiological signals were amplified and filtered (Super-Z headstage amplifiers and BMA-400 amplifiers and filters, CWE Inc., Ardmore, PA, USA) as follows; EEG 1000X at 1-100Hz, EKG 1000-5000X at 50-100Hz, and EMG 2000X at 100-1000Hz. Neck EMG signals were digitized, smoothed (25 msec time constant) and rectified (Spike2 software, 1401 interface, CED Ltd., Cambridge, UK) and recorded on a computer.

EEG Analyses: Periods of wakefulness, NREM sleep, and REM sleep were identified visually off-line, using standard EEG and EMG criteria. A minimum of 2 periods was obtained from each state, with nearly all states occurring within 3 hours of recording. To insure that the analyses would not be biased, periods of NREM sleep were analyzed just before the transition to REM
sleep. All analyzed data was copied to a spreadsheet and sorted according to the sleep or wakefulness states. Seizures were scored as repetitive high voltage (3-4 times baseline) spike-wave discharges occurring for at least 1 sec. The duration of seizures we quantified for each behavioural state and the percentage time spent with seizures in a particular state was also quantified.

2.3. Results

2.3.1. Limb Clasping Reveals a Neurological Phenotype in Mice Expressing Neuroligin 2 that is not Observed in Mice Expressing Neuroligin 1

We used a gain-of-function approach to examine whether manipulations of the levels of NLs in vivo influence synapse function leading to altered behaviour. For these experiments, transgenic animals overexpressing NL1 (TgNL1) and NL2 (TgNL2) were generated (Figure 2.1A,B). The transgenes were under the control of Thy1 promoter, which allows for expression in various brain regions at early stages of development. To facilitate detection of the transgene, influenza hemagglutinin (HA) tags were inserted (between amino acids 45 and 46) immediately after the NL1 signal sequence. To compare transgene expression and localization with wild-type NL2, a specific antibody was generated. This antibody was tested for specificity on lysates from Cos cells expressing HA-NL1, HA-NL2, or HA-NL3 (Figure 2.1C). In addition, the antibody was tested in cultured hippocampal neurons for predominant colocalization with vesicular GABA transporter (VGAT) (Figure 2.2B) and in tissue from TgNL2 mice for correspondence with HA-tagged transgene (Figure 2.2C). The generated TgNL1 and TgNL2 strains were viable; however, TgNL2 mice exhibited a striking and distinct phenotype. TgNL2
mice with moderate to high levels of expression (Figure 2.1D,F) showed reduced lifespan and capacity to produce viable offspring. One additional feature of the TgNL2 phenotype is limb clasping (Figure 2.1E), a defect associated with mouse models of neurological disorders such as Rett syndrome (Gemelli et al., 2006). This feature was not observed at comparable levels in TgNL1 strains with similar transgene expression (Figure 2.1B) (1.25- to 1.71-fold TgNL2.6 HA expression).

2.3.2. Behavioural Test Battery Demonstrates a Consistent, Dose Dependent Phenotype in Mice Expressing Neuroligin 2 not Observed in Mice Expressing Neuroligin 1

The preliminary behavioural screen was conducted on all founders and strains generated based on the modified SHIRPA screen from the EMPReSS (Brown et al., 2005, 2006). The preliminary screen revealed striking and consistent abnormalities among all TgNL2 strains generated that increased with level of expression (Table 2.1, - indicates no difference from wild type). Animals with twofold or greater expression of NL2 (determined by Western blot; TgNL2.1, TgNL2.4, and TgNL2.5) showed the most striking phenotype with frequent limb clasping and death by 24 postnatal weeks, and these strains also failed to produce viable offspring. The primary strain has moderate expression level and phenotype (TgNL2.6; 1.6-fold wild-type NL2 expression) and has reproduced successfully over nine generations; thus, most of the analysis performed was on this strain. Whenever possible, results obtained from a higher expressing strain (TgNL2.7; 1.9-fold wildtype NL2) are included for comparison.

**Table 2.1.** Summary of the preliminary screen conducted on all strains. Table shows scores for Wt, TgNL1, and TgNL2 strains, transgenic strains are arranged in order of increasing expression of transgene. Dash mark (-) = normal or no impairment; in the case of impairment, increasing values indicate increased impairment, and a brief description is provided. Cells shaded in light
grey represent assessments where transgenic mice showed difference from wildtype littermates.

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In general, TgNL2 mice are characterized by a reduction in body weight (Figure 2.3B–D), limb clasp ing (Figure 2.1E,F), Straub tail, transient episodes of kyphosis (Figure 2.3A), as well as enhanced basal activity and enhanced startle response to a 90 dB click (Table 2.1). However, no overt changes were observed in autonomic indicators such as palpebral closure and piloerection (Table 2.1, Autonomic). In addition, somatosensory, visual, auditory, and olfactory systems were also found to be intact in TgNL2 mice using a battery of multiple tests (Table 2.1, Sensory). Basic muscle and motor assessments in TgNL2 mice revealed normal body and limb tone, and intact righting reflex (Table 2.1, Muscle and motor). The drastic phenotypic changes observed on relatively small alterations in transgene expression (1.6- to 2-fold endogenous NL2), combined with the dose-dependent change in the severity of phenotype, indicates that the effects seen are not because of excessive protein overexpression in vivo. Moreover, the lack of these abnormalities in TgNL1 further suggests that the observed changes in TgNL2 mice have resulted from alterations attributable to specific manipulation of NL2 levels in vivo.
**Figure 2.3. Observation of transient kyphosis and reduction in body weight in mice expressing neurolin 2.** A. Intermittent kyphosis was observed to varying degrees in all TgNL2 strains but not in wildtype or TgNL1 strains. B. Representative image of age and sex matched mice comparing the body size of wildtype, TgNL2.6 and TgNL2.7 mice. C. Time course comparison of body weight (Repeated Measures ANOVA genotype by week interaction $F(5,85)=20.24$, $p<.001$) in wildtype and TgNL2.6 mice through 6 weeks postnatal. D. Comparison of wildtype, TgNL1.7, TgNL2.6 and TgNL2.7 body weights ($Wt=20.29±0.42$; TgNL1.7=16.89±1.16; TgNL2.6=11.54±8.55; TgNL2.7 5.93±0.48; ANOVA $F(3,18)=71.89$, $p<.001$, post hoc tests, $p=.004$ $Wt$ vs TgNL1.7, $p<.001$ $Wt$ vs TgNL2.6, $p<.001$ $Wt$ vs TgNL2.7) at 4 weeks postnatal. Data shown are means ± SEMs. Weight time course, wildtype $n=5$, TgNL2.6 $n=5$; strain weight comparison, wildtype $n=6$, TgNL1.7 $n=5$, TgNL2.6 $n=6$, TgNL2.7 $n=4$. 
2.3.3. Expressed Neuroligin 2 is Distributed Throughout the Neuroaxis in Neuronal Cells and is Predominantly Localized to Inhibitory Synaptic Contacts

To assess exogenous NL2 expression in detail, multiple approaches were used. Both immunohistochemistry and in situ hybridization revealed broad distribution of the HA-NL2 transcript and protein in TgNL2.6 (Figure 2.4A–C). Despite varied levels of expression between transgenic strains as revealed by Western blotting, the pattern of HA-NL2 staining was consistent. HANL2 was expressed throughout the neuroaxis, with high levels in the cortex and limbic structures, such as amygdala and hippocampus (Figure 2.4B,C). Staining of cortical cultures from TgNL2.6 confirmed that HA-positive (HA+) labeling was exclusive to neuronal cells (4,6-diamidino-2-phenylindole-positive, MAP-2+) and could be detected in large numbers of neurons (Figure 2.2D). Similar to wildtype NL2, HA-NL2 predominantly colocalized with inhibitory ( gephyrin) and to a lesser extent with excitatory (PSD-95) postsynaptic markers, demonstrating that exogenous HA-NL2 is not mislocalized (Figure 2.4D,E).

2.3.4. Enhancement of Markers of Presynaptic Terminals in Mice Expressing Neuroligin 2

Given the implication of NLs in modulation of the E/I ratio, we analyzed changes in the content of excitatory and inhibitory synapses in TgNL2 mice. Quantitative Western blotting analysis of forebrain lysates revealed significant changes in the amounts of VGAT, vesicular glutamate transporter (VGluT) and syntaxin but not in the expression of PSD-95 and gephyrin (Figure 2.5A,B), suggesting a change in the content of presynaptic but not postsynaptic elements of both excitatory and inhibitory synapses. Assessments of other NL family members revealed no change in NL1 but a significant decrease in NL3 expression levels in TgNL2 brain lysates (Figure
Figure 2.4. Neuroligin 2 transgene distribution and localization. A. In situ hybridization analysis of HA-containing transcript in TgNL2.6 (left) and of NL2-containing RNA in TgNL2.6 (center) and wildtype (right). Scale bar = 3mm. B. DAB immunohistochemistry for HA in sagittal sections from wildtype and TgNL2.6. Scale bar = 3mm. C. HA DAB immunostaining in coronal sections through the frontal cortex (bregma: 2.71 – 2.22mm) and hippocampus/amygdala (bregma: -1.28 – -1.64mm) of TgNL2.6 brain. Scale bar = 1mm. D. Confocal analysis of co-localization of HA-NL2 (green) and excitatory (PSD-95, red, top panel) and inhibitory (gephyrin, red, bottom panel) synaptic markers in the frontal cortex. Scale bar = 2μm. E. Quantification of excitatory (PSD-95; Wt=24.21%±5.50; TgNL2.6=35.20%±3.51; t-test p=.181) and inhibitory (gephyrin; Wt=73.60%±5.86; TgNL2.6=61.91±5.38; t-test p=.176) marker co-localization with endogenous NL2 in wildtype frontal cortex compared with HA-NL2 in TgNL2.6 frontal cortex. Data shown are means ± SEMs; Wt, TgNL2.6 n=9; ns, not significant.
Figure 2.5. Western blotting and immunohistochemical assessment of synaptic proteins in mice expressing neuroligin 2. A. Representative blots for synaptic proteins assayed in wildtype and TgNL2.6 mice. B. Quantification of western blot normalized to total protein load from coomassie stained gels, and compared to wildtype expression level. C. Confocal microscopy of VGluT (centre, green) and VGAT (right, red) staining in wildtype (top) and TgNL2.6 (bottom) medial prefrontal cortex (MPFC). Scale bar = 5 μm. D. Quantification of the average integrated intensity of VGluT (Wt=37.37±1.00; TgNL2.6=48.29±3.29; t-test p=.033) and VGAT (Wt=30.26±2.02; TgNL2.6=49.76±1.53; t-test p<.001) puncta in MPFC. E. The E/I ratio as expressed by the ratio of VGluT intensity:VGAT intensity (Wt=1.28±0.09; TgNL2.6=1.00±0.07; t-test p=.028). Data shown are means ± SE±Ms; Wt, TgNL2.6 n=9.
Interestingly, TgNL1 mice show opposing alterations in related protein expression levels, with a significant increase in both NL3 and PSD-95 (data not shown).

Immunostaining of frontal cortex sections from P28 TgNL2 mice showed altered VGAT and VGLuT staining intensity (Figure 2.5C,D). Consistent with the enrichment of NL2 at the majority of GABAergic synapses, more robust increases were seen in the average integrated intensity (VGAT, 1.7-fold; VGLuT, 1.3-fold) of inhibitory contacts in the frontal cortex. The ratio of VGLuT to VGAT staining intensity in the frontal cortex was significantly decreased in TgNL2 when compared with wild-type littermates (Figure 2.5E) revealing a bias toward increased inhibition during in vivo expression of NL2.

2.3.5. EM Analysis Reveals Changes in Synapse Morphology and an Increase in Inhibitory Contacts in Frontal Cortex of Mice Expressing Neuroligin 2

Ultrastructural EM analysis of medial prefrontal cortex (MPFC) of TgNL2.6 and TgNL2.7 brain demonstrated changes in the morphology of synaptic components (Figure 2.6C) and synapse density. Marked increases were observed in the number of vesicles in the reserve pool (Figure 2.6A,D), as well as the area of symmetric (type II, typically inhibitory) presynaptic compartments (Figure 2.6A,E) and contact length of symmetric synapses (Figure 2.6A,F) in TgNL2 mice. In addition, a small but significant change was observed in asymmetric (type I, typically excitatory) presynaptic compartment area (Figure 2.6B,E). No change was observed in the average length of postsynaptic densities in TgNL2 mice (Figure 2.6B,D). Using unbiased stereological analysis, a modest increase in the total density of synapses, as well as the density of symmetrical synapses was observed in both TgNL2.6 and TgNL2.7 (Figure 2.6G,H). No
Figure 2.6. Synaptic abnormalities in mice expressing neuroligin 2. A. and B. Representative electron micrographs of symmetric (A) and asymmetric (B) synapses in wildtype and TgNL2.6 MPFC. Scale bar = 500 nm. C. Synaptic elements quantified are highlighted. D. and E. Quantification of the length of the PSD (Wt=146.05±7.78; TgNL2.6=180.00±15.14; TgNL2.7=139.96±10.35), and number of vesicles in the reserve pool (Wt=30.93±3.24; TgNL2.6=51.87±6.37; TgNL2.7=69.27±8.87; ANOVA, F(2,42)=8.52, p=0.001, post hoc tests, p=.030 Wt vs TgNL2.6, p<.001 Wt vs TgNL2.7), and presynaptic compartment area (symmetric: Wt=501.54±71.79; TgNL2.6=1020.11±131.01; TgNL2.7=1436.04±157.72; ANOVA, F(2,42)=8.57, p<.001 Wt vs TgNL2.6, p<.001 Wt vs TgNL2.7; asymmetric: Wt=572.95±44.52; TgNL2.6=747.99±69.14; TgNL2.7=934.83±145.27; ANOVA, F(2,138)=4.75, p=.010 Wt vs TgNL2.6, p=.048 Wt vs TgNL2.7) in wildtype, TgNL2.6 and TgNL2.7 MPFC. F. Analysis of the length of contact between symmetric pre- and postsynaptic compartments (Wt=254.34±32.46; TgNL2.6=493.87±57.00; TgNL2.7=465.59 ±66.36; ANOVA, F(2,31)=4.03, p=.028; post hoc tests, p=.009 Wt vs TgNL2.6, p=.039 Wt vs TgNL2.7) in wildtype, TgNL2.6 and TgNL2.7 symmetric synapses. G. and H. Number of total (Wt=8.69±0.34; TgNL2.6=10.42±0.34; TgNL2.7=11.81±0.88; ANOVA, F(2,45)=7.28, p=.002; post hoc tests, p=.040 Wt vs TgNL2.6, p<.001 Wt vs TgNL2.7), symmetric (Wt=7.30±0.35; TgNL2.6=7.99±0.44; TgNL2.7=8.69±0.78; ANOVA, F(2,45)=1.58, p=.219), and asymmetric (Wt=1.16±0.28; TgNL2.6=2.32±0.28; TgNL2.7=3.13±0.49; ANOVA, F(2,45)=7.55, p=.002; post hoc tests, p=.028 Wt vs TgNL2.6, p<.001 Wt vs TgNL2.7) synapses per 100 μm² field of MPFC neuropil. I. The E/I ratio as expressed by the ratio of asymmetric: symmetric synapses (Wt=5.89±0.60; TgNL2.6=4.14±0.53; TgNL2.7=3.67±0.60; ANOVA, F(2,45)=4.09, p=.024; post hoc tests, p=.039 Wt vs TgNL2.6, p=.100 Wt vs TgNL2.7). Data shown are means ± SEMs; Synapse density: Wildtype, TgNL2.6 N=4 mice, n=16 fields; TgNL2.7 N=3 mice, n=16 fields. Straight line indicates overall significance, whereas brackets indicate significance from post-hoc tests.
Changes were seen in the total number of neurons in the frontal cortex (Figure 2.7A,B). By dividing the density of asymmetric synapses by the density of symmetric synapses, we can estimate a shift in the E/I ratio toward inhibition in TgNL2 frontal cortex compared with littermate controls (Figure 2.6i), supporting the finding of a decreased ratio of VGlut to VGAT immunostaining in TgNL2.6 frontal cortex.

Together, these results suggest an effect of NL2 in vivo on the modulation of synapse morphology with primary effects on presynaptic terminals and, in particular, symmetric synapses. The small effects of NL2 on asymmetric presynaptic terminals are not surprising because endogenous NL2 can be found at 20–30% of excitatory synapses (Figure 2.4E). Given the effects on symmetrical synapse density, it is also possible that changes observed in the morphology of asymmetrical terminals are a means of compensation. The greater degree of change observed in inhibitory versus excitatory synapses suggest that enhanced expression of NL2 results in an overall reduction in the E/I ratio, revealing that a small increase in NL2 expression results in alterations in synaptic balance in frontal cortex.

2.3.6. Altered Synaptic Transmission in Prefrontal Cortex of Mice Expressing Neuroligin 2

Whole-cell patch-clamp recordings were performed in pyramidal neurons in layer II/III of wild-type and TgNL2.6 prefrontal cortex. Recording of spontaneous activity revealed that the frequency of miniature IPSCs (mIPSCs) is increased in TgNL2 mice compared with those in wild-type mice (Figure 2.8A,B). However, the mIPSC amplitude was not altered in the prefrontal cortex of TgNL2 mice (Figure 2.8A,B). In contrast to inhibitory currents, neither frequency (Figure 2.8C,D) nor amplitude (Figure 2.8C,D) of mEPSCs in TgNL2 mice were found to be
Figure 2.7. Light and electron microscopic analysis of neuron and synapse number. A. Comparison of fields from wildtype (left panel) and TgNL2.6 (right panel) medial prefrontal cortex (1μm thick section) stained with toluidine blue. B. Graph comparing the number of neurons per 20μm² in medial prefrontal cortex (Wt=120.63±10.19; TgNL2.6=117.35±6.61; t-test p=.639) of wildtype and TgNL2.6 mice.
Figure 2.8. Increased inhibitory synaptic transmission in pyramidal neurons of prefrontal cortex in mice expressing neuroligin 2. A. Representative traces showing the mIPSCs in wildtype and TgNL2.6 mice. TTX (1 μM), CNQX (20 μM) and AP5 (50 μM) was added in the ACSF during mIPSC recordings. B. Statistical results showing the significant increase of mIPSC frequency (left; Wt=2.3±0.3, n=22; TgNL2.6=3.6±0.4, n=23; t-test p<.050) but not amplitude (right; Wt=13.1±0.5, n=22; TgNL2.6=13.5±0.3, n=23; t-test p=0.55) in TgNL2.6 mice as compared to wildtype mice. C. Typical traces showing the mEPSCs recorded in wildtype (left) and TgNL2.6 mice (right). TTX (1 μM), picrotoxin (100 μM) and AP5 (50 μM) was added in the ACSF during mEPSC recordings. D. Statistical results showing normal mEPSC frequency (left; Wt=1.6±0.3, n=16; TgNL2.6=2.0±0.3, n = 17; t-test p=0.24) or amplitude (right; Wt=9.2±0.3, n=16; TgNL2.6=8.7±0.2, n=17; t-test p=0.21) in TgNL2.6 mice as compared to wildtype mice. Data shown are means ± SEMs.
A

**mIPSCs**

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**mEPSCs**

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**mEPSC frequency (Hz)**

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**mEPSC amplitude (pA)**

<table>
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significantly different from those in the prefrontal cortex of wild-type mice (Figure 2.8A–C). These results are consistent with the EM data suggesting a primary effect of NL2 expression on modulating inhibitory synapse function.

2.3.7. Altered Neuroligin 2 Expression Leads to Spontaneous Stereotypies and Anxiety Behaviour

To assess the behaviour of TgNL2 mice in more detail, we next used the open field paradigm. When in the open field, TgNL2.6 mice display spontaneous jumping stereotypies in the corners of the arena (Figure 2.9A). Sterotyped patterns of behaviour can be induced in animals by treatment with amphetamines and cocaine or by deprivation (Powell et al., 1999; Würbel, 2001) and have also been observed in mouse models of mental retardation such as the Down syndrome model Ts65Dn (Turner et al., 2001). Sterotyped jumping behaviour is typically preceded by rearing against the arena wall and is characterized by repeated jumping vertically on hindlegs and balancing on a rigid tail (Würbel and Stauffacher, 1996; Garner and Mason, 2002). The jumping stereotypy was also observed when TgNL2.6 mice were in the home cage (in the presence of social and environmental enrichment); however, repetitive jumping was never observed in wild-type littermates (n=50). Not all TgNL2.6 mice showed stereotypies during open field observation, and it was found that 44% of TgNL2.6 males (n=50) show more than one episode of stereotypy during a 5 min open field exploration (Figure 2.9B). TgNL2.6 mice showing stereotypies typically display high levels of this behaviour, with an average of 10 episodes over the 5 min period (Figure 2.9B). TgNL2.6 mice displaying more than one episode of jumping stereotypy during a 5 min prescreening session in the open field were excluded from behavioural assessments of anxiety and social behaviour to avoid possible
Figure 2.9. Neuroligin 2 expressing mice display spontaneous jumping stereotypies and anxiety behaviour. A. Graph plotting the incidence of stereotyped jumping (Wt=0; TgNL2.6=5.58±2.53), digging (Wt=8.50±1.31; TgNL2.6=10.75±2.63; t-test p=.461) and grooming (Wt=3.63±0.78; TgNL2.6=4.83±0.81; t-test p=.318) behaviours in the open field (Wt n=7; TgNL2.6 n=8). Signifies that wildtype animals did not demonstrate this behaviour, under any of the conditions examined. B. Table showing the percentage of wildtype (0%) and TgNL2.6 (44%) mice showing stereotypies in a larger population (n=50), and of these mice, the average number of stereotypies shown in a five minute open field session. C. Representative paths of wildtype (left panel) and TgNL2 (right panel) mice in the open field arena. D. Assessment of the cumulative distance from the arena border (Wt=17965.74±379.79; TgNL2.6=15088.26±709.59; TgNL2.7=9784.31 ±570.32; ANOVA, F(2,17)=30.51, p<0.001, post hoc tests, p=.001 Wt vs TgNL2.6, p<0.001 Wt vs TgNL2.7) in the open field task. E. Percentage of time spent in the dark chamber (Wt=64.90±4.21; TgNL2.6=81.58±3.55; t-test p=.022) by wildtype and TgNL2.6 mice during the light-dark exploration test. F. Assessment of the percentage of open arm time (Wt=34.17±2.92; TgNL2.6=18.94±1.45; t-test p<.001) and open arm entries (Wt=38.86±1.39; TgNL2.6=27.46±2.15; t-test <.001) by wildtype and TgNL2.6 mice in the elevated plus maze. Data shown are means ± SEMs. Open Field wildtype n=7, TgNL2.6 n=8, TgNL2.7 n=2; light dark exploration test wildtype, TgNL2.6 n=8; elevated plus maze wildtype, TgNL2.6 n=8. Straight line indicates overall significance, whereas brackets indicate significance from post-hoc tests.
confounds of this behaviour (Garner, 2005). Other types of repeated behaviour patterns were also monitored in the open field, such as digging and grooming, but the incidence of these types of behaviours was not significantly different from wild-type littermates (Figure 2.9A). It has been shown that stereotyped patterns of behaviour that result from drug sensitization can be blocked by antagonists or induced by agonists of the GABAergic system (Karler et al., 1995); thus, it is possible that this behaviour arises as a result of the alterations in GABAergic transmission observed in the frontal cortex (Karler et al., 1997) of TgNL2 mice.

During the open field test, we also observed indications of anxiety behaviour in TgNL2 mice. Both TgNL2.6 and TgNL2.7 mice show thigmotaxis (Figure 2.9C), as demonstrated by a decrease in both average (Figure 2.10A) and cumulative (Figure 2.9D) distance from the arena border compared with wild-type mice. TgNL2 mice also show increased rearing frequency, which is further indicative of anxiety (Figure 2.10B). However, no changes were observed in average speed traveled while exploring the open field (data not shown; wild type, 4.75 cm/s; TgNL2.6, 6.07 cm/s; TgNL2.7, 6.18 cm/s; p = 0.169) or total distance traveled in the open field (Figure 2.10C), demonstrating that TgNL2 animals do not have motor impairments or lack exploratory motivation.

To confirm that the thigmotaxis observed in the open field was indicative of anxiety, we also compared the moderate expressing TgNL2.6 strain with wild-type littermates in the light-dark exploration test. Observation of TgNL2.6 mice in the light/dark test revealed an increase in the percentage of time spent in the dark compartment compared with wild-type littermates (Figure 2.9E). However, no change was observed in the number of transitions between the light and dark compartments (Figure 2.10D), which is an additional measure of anxiety-like
Figure 2.10. Anxiety-like behaviour in mice expressing neuroligin 2. A. Graph showing the average distance from the arena border during open field exploration (Wt=5.18±0.08; TgNL2.6=4.33±0.10; TgNL2.7=3.26±0.19; ANOVA F(2,17)=63.87, p<.001, post hoc tests, p<.001 Wt vs TgNL2.6, p<.001 Wt vs TgNL2.7) in wildtype, TgNL2.6 and TgNL2.7 mice. B. Total number of rears during open field exploration (Wt=46.50±6.80; TgNL2.6=110.50±18.84; TgNL2.7=89.00±15.00; ANOVA F(2,17)=8.50, p=.003, post hoc tests, p=.001 Wt vs TgNL2.6, p=.048 Wt vs TgNL2.7) by wildtype, TgNL2.6 and TgNL2.7 mice. C. Total distance travelled (Wt=2892.12±161.65; TgNL2.6=3505.19±377.95; TgNL2.7=3705.59±1919.37) by wildtype, TgNL2.6 and TgNL2.7 mice during exploration of the open field. D. Number of transitions (Wt=21.50±2.50; TgNL2.6=24.25±1.58; t-test p=.386) by wildtype and TgNL2.6 mice between light and dark chambers during the light-dark exploration test. E. Total number of arm entries (Wt=16.86±2.85; TgNL2.6=24.78±2.26; t-test p=.078) by wildtype and TgNL2.6 mice during the elevated plus maze assessment. Straight line indicates overall significance, whereas brackets indicate significance from post-hoc tests.
A
Avg Distance Border (cm)

B
Total Number of Rears

C
Total Distance Travelled (cm)

D
Light-Dark Transitions

E
Total Arm Entries
behaviour in the light/dark test. It has been reported that the most consistent measure of anxiety using the light/dark test is the percentage of time spent in each compartment, and transitions have been suggested to be influenced by activity or exploration (Belzung et al., 1987; Hascoët and Bourin, 1998; Bourin and Hascoët, 2003). Although it is unclear why TgNL2 mice display an anxiety-like phenotype on only one of the two measures for the light/dark task, it is possible that the high level of basal activity observed in TgNL2 may obscure detection of differences in the number of transitions.

To further confirm an anxiety-like phenotype, mice were also tested in the elevated plus maze. TgNL2.6 mice demonstrated a reduction in the percentage of time spent in the open arms (Figure 2.9F), as well as in the percentage of entries made into open arms (Figure 2.9F) of the elevated plus maze compared with wild-type littermate controls. No difference was observed in the total number of entries (Figure 2.10E), supporting the idea that TgNL2.6 performance on this task is not limited by motor impairments or lack of exploratory motivation. Overall, these three assessments indicate an increase in anxiety-like behaviour in mice expressing NL2 compared with their littermate controls.

2.3.8. Mice Expressing Neuroligin 2 Display Abnormalities in Social Behaviour

To assess reciprocal social interactions of TgNL2 mice, male mice were placed into a neutral home cage with a novel juvenile target male of a different strain (FVB). The two animals were allowed to freely interact over the course of 5 min, and the number and duration of interactions were manually assessed from video recordings. A striking reduction in the total time of interaction with the novel target mouse was observed in TgNL2 mice when compared
with wild-type littermates (Figure 2.11A). Although TgNL2.6 mice did not show a difference in the total number of interactions with novel juveniles (Figure 2.11C), the average duration of individual interactions was significantly reduced (Figure 2.11B). The lack of change in the total number of interactions is likely to result from increased initiation of interactions by the freely moving target mouse (Figure 2.11D).

To further assess social behaviour and rule out a general defect in their interaction with novel stimuli, we assessed TgNL2.6 mice in a counterbalanced social approach apparatus based on the task developed by the laboratory of Dr. J. N. Crawley (Figure 2.11E) (Moy et al., 2004; Nadler et al., 2004). In this three-chambered apparatus, wild-type mice show a strong preference for the chamber containing the novel mouse over either the center or counterbalanced novel object chamber. This effect can be seen in the total amount of time spent in the chamber with the novel mouse (Figure 2.11F). In contrast, TgNL2.6 mice do not show this preference, with no significant difference between the time spent in the novel mouse chamber compared with the novel object chamber. In addition, wild-type mice also display preference for the novel mouse chamber by the number of investigative rears made in this chamber (Figure 2.11G), indicating a high level of exploration of the novel mouse. In contrast, TgNL2.6 mice do not demonstrate preference for the novel mouse chamber over the novel object chamber in terms of high levels of rearing. This result of reduced social approach in the three-chambered apparatus, in conjunction with reduced reciprocal social interactions, demonstrates that TgNL2 animals are impaired in the natural preference for social interaction seen in wild-type mice.
Figure 2.11. Deficits in social interactions in mice expressing neuroligin 2. A. Assessment of the total time wildtype or TgNL2.6 mice spent interacting with a novel mouse (Wt=124.01±9.04; TgNL2.6=54.29±6.69; t-test p=.001) in a neutral arena over a 5 min period. B. Graph showing the average length of individual interactions between wildtype or TgNL2.6 mice and a novel mouse (Wt=6.68±0.84; TgNL2.6=2.84±0.26; t-test p=.004). C. No difference was observed in the total number of interactions (Wt=20.40±1.40; TgNL2.6=19.20±1.41; t-test p=.527) between wildtype or TgNL2.6 mice and novel mice. D. Proportion of interactions initiated by either wildtype or TgNL2.6 mice with a novel mouse (Wt=52.80±1.67; TgNL2.6=23.35±3.59; t-test p<.001). A.-D. Bonferroni corrected significance levels for multiple t-tests used: 0.013; 0.003; <0.001. E. Schematic of the 3-chambered social behaviour apparatus. F. Analysis of time spent (Wt novel mouse=654.78±81.10; Wt novel object=350.60±51.71; TgNL2.6 novel mouse=406.32±31.78; TgNL2.6 novel object=539.75±45.10; ANOVA F(3,28)=6.077 p=.003, post hoc tests, p=.001 Wt novel mouse vs Wt novel object, p=.100 TgNL2.6 novel mouse vs TgNL2.6 novel object) in the novel mouse and novel object chambers of the social apparatus. G. Frequency of rearing (active exploration) in the chambers of the social apparatus (Wt novel mouse=115.50±23.61; Wt novel object=59.50±7.84; TgNL2.6 novel mouse=94.00±12.20; TgNL2.6 novel object=89.50±8.44; ANOVA F(3,28)=2.54 p=.077, post hoc tests, p=.011 Wt novel mouse vs Wt novel object, p=.828 TgNL2.6 novel mouse vs TgNL2.6 novel object). Data shown are means ± SEMs. Reciprocal social interaction wildtype, TgNL2.6 n=10; 3-chamber social approach behaviour wildtype, TgNL2.6 n=8.
2.3.9. Chronic EEG Recording in Freely Moving Neuroligin 2 Transgenic Mice Reveals Bilateral Spike-Wave Discharges

Synapse anomalies and behavioural observations prompted assessment of spike wave patterns via frontoparietal EEG recordings in TgNL2 mice. Neck electromyogram (EMG) was recorded to establish whether spiking occurred during different sleep and wake states. Eight of the 10 TgNL2 animals studied demonstrated brief bilateral bursting activity characterized by spike-wave discharge of ~6–8Hz (Figure 2.12A,B). The spike-wave episodes were brief, did not always occur with specific behaviours, and could be identified in both awake and sleep states (Figure 2.12C). The results indicate that even a mild increase in the expression of NL2 results in spiking activity. Despite the detection of spiking activity by EEG, TgNL2.6 mice did not exhibit any overt visible signs of seizure. The spiking activity observed by frontoparietal EEG may be attributable to desynchronized cortical activity patterns or as a result of spreading excitation from other brain regions.

2.4. Discussion

Our analysis reveals that manipulation of NL2 expression results in altered synapse morphology and function. Previous studies indicate that altered levels of individual NLs at particular synaptic sites modulate synapse maturation and neuronal excitability (Levinson and El-Husseini, 2005). Consistent with these findings, EM and electrophysiological analyses of frontal cortical areas in transgenic NL2 mice reveal an increase in the size and number of inhibitory synaptic contacts and enhanced frequency of presynaptic currents. Thus, the net result is potentiation of inhibitory responses in the frontal cortex, revealing a shift in the balance toward inhibition.
Figure 2.12. Seizure spiking activity as observed via freely moving EEG recording in mice expressing neuroligin 2. A. and B. Representative traces of differential recordings from neck EMG and left and right frontal-parietal (FP) EEG from wildtype (A) and TgNL2 (B) mice. TgNL2 mice exhibit bilaterally synchronous bursting activity characterized by spike-wave discharge around 7Hz. C. Spiking activity was observed in 8 of 10 TgNL2 mice tested and was detected in all stages of sleep, but most consistently in wakefulness and REM sleep. Abbreviations: REM; Rapid Eye Movement, NREM; Non-Rapid Eye Movement.
A

Wildtype

Neck EMG 400μV

Left FP EEG 200μV

Right FP EEG 200μV

B

TgNL2.6

Neck EMG 400μV

Left FP EEG 200μV

Right FP EEG 200μV

C

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<td>26.0</td>
<td>6.95</td>
<td>wake, REM</td>
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Mean  1.7          11.3          7.43
SEM ± 0.2          2.9           1.06
The observed phenotypic changes were attributable to small alteration in NL2 expression (1.6-to 2-fold above endogenous levels of NL2). These findings indicate that the effects seen are not attributable to excessive protein overexpression in vivo. Interestingly, the severity of behavioural changes correlates with the level of NL2 expression, suggesting dose-dependent changes in synapse function and behaviour. The lack of related abnormalities in TgNL1 further suggest that the observed behavioural changes in TgNL2 mice resulted from alterations in synaptic maturation and/or function attributable to specific manipulation of NL2 levels in vivo. Importantly, neither TgNL1 nor TgNL2 strains display deficits in basic sensory, reflexive, or motor function using the modified SHIRPA screen, making them feasible candidates for additional behavioural characterization. Intact motor function and exploratory motivation was further confirmed in TgNL2 mice in open field, elevated plus, and social approach tasks in which no deficits were observed in control measures of speed, distance traveled, or total entries.

The differential enrichment of specific NLs to particular synaptic sites in vivo suggests that NL3 and NL4 mainly modulate excitatory synaptic function, whereas NL2 is associated with modulation of inhibitory synaptic transmission (Song et al., 1999; Dean et al., 2003; Prange et al., 2004; Varoqueaux et al., 2004, 2006; Chubykin et al., 2005, 2007; Levinson and El-Husseini, 2005; Levinson et al., 2005; Sara et al., 2005; Dean and Dresbach, 2006). In particular, loss of NL2 has been shown to specifically alter inhibitory synapse function in vivo. Consistent with these findings, our results show that enhanced expression of NL2 results in a significant increase in inhibitory synapse maturation and transmission in cortical areas. However, it is important to note that a small but significant change in excitatory synapse morphology was also observed in TgNL2 mice, indicating that NL2 expression can influence both excitatory and
inhibitory contact maturation, although with pronounced effects on inhibitory contacts. These data suggest that NL2 function \textit{in vivo} is not fully restricted to inhibitory synapse maturation. These findings also hint to some overlapping and redundant functions of members of the NL family \textit{in vivo}. In support of some functional redundancy between NLs, knock-out of NL1–NL3 is lethal, whereas all of the single and double knock-out combinations are viable (Varoqueaux et al., 2006). The finding that endogenous NL2 is found at 20–30% of excitatory synapses and the recent work that demonstrates that NLs can form heteroligomers (Budreck and Scheiffele, 2007) lend additional support to this notion.

The mechanism underlying the lack of change in inhibitory postsynaptic responses remains unclear. It is possible that recruitment of GABA receptors at inhibitory synapses is more constrained and consequently the presynaptic changes seen did not lead to an increase in recruitment of postsynaptic GABA receptors. This could be caused by enhanced levels of NL2, which can act to disperse GABA receptors and reduce inhibitory currents (Graf et al., 2004).

The changes observed including limb clasping, repetitive behaviours, anxiety, and social dysfunction are similar to those observed in animal models of Rett syndrome (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002; Pelka et al., 2006). Some of these phenotypic characteristics also resemble aspects of human disorders, including Rett syndrome and autism (Hagberg, 2002; Rubenstein and Merzenich, 2003; Zoghbi, 2003; Dover and Le Couteur, 2007; Lewis et al., 2007). Autism, a genetically linked disorder, is characterized by varying degrees of abnormality in communication ability, social interactions, repetitive and stereotyped behaviour, as well as high incidence of seizure (Tuchman and Rapin, 2002; Rubenstein and Merzenich, 2003; Zoghbi, 2003; Christ et al., 2007; Dover and Le Couteur, 2007; Rutherford et al., 2007).
Of particular interest, recent studies have shown rearrangement of chromosomal regions harboring NL1 and NL2, as well as mutations in NL3 and NL4 in families with autism (Konstantareas and Homatidis, 1999; Auranen et al., 2002; Jamain et al., 2003; Laumonnier et al., 2004; Lise´ and El-Husseini, 2006). A single-copy chromosomal deletion of a region containing α/β-Nrxs indicates that altered amounts of these proteins is sufficient to confer the behavioural changes associated with autism (Szatmari et al., 2007). In addition to single gene alterations, recent studies have shown that many autistic patients have novel deletions and duplications in their genomes (Sebat et al., 2007). Neuroimaging and postmortem studies of patients with autism suggest that an alteration in the ratio of E/I in neural circuits underlies the dysfunctions characteristic of autism (Rubenstein and Merzenich, 2003). The theory proposed by Rubenstein and Merzenich suggests that the dysfunction in autism could result from a shift toward excitation, but, in mouse models of related disorders such as Rett syndrome (Dani et al., 2005), the trend appears to favour inhibition. In addition, increased inhibition was observed in mice expressing an autism-related mutation of NL3, and this was shown to result in altered social interaction (Tabuchi et al., 2007). In this regard, a gain of NL2 function may result in a shift toward increased inhibition, mimicking the reduced function of the mutant forms of NL3 or NL4 most commonly associated with autism. Thus, as also observed for models of Rett syndrome (Collins et al., 2004; Gemelli et al., 2006), both reduced and enhanced expression of affected genes may model the underlying cause of neurodevelopmental disorders such as autism.

Several lines of evidence indicate multiple deficits in the structure and function of the brain in individuals with autism. In particular, there is strong evidence supporting that abnormalities in the frontal cortex and the amygdala contribute highly to deficits in social behaviour and
anxiety. In particular, previous studies have shown a link between increased excitation in the amygdala and anxiety (Davis et al., 1994). Amygdala excitability can also be increased via disinhibition (decreasing inhibitory transmission from other brain regions). For instance, increased inhibition within the prefrontal cortex, which normally acts to inhibit the amygdala (Rosenkranz et al., 2003; Quirk and Gehlert, 2003; Quirk et al., 2003), can lead to increased excitation (disinhibition) in the amygdala and, hence, anxiety (Davis et al., 1994; Berkowitz et al., 2007; Bishop, 2007). Changes in the morphology and density of inhibitory synapses and increases in mIPSC frequency in the frontal cortex observed in the present work may therefore disinhibit the amygdala and contribute, at least in part, to the enhanced anxiety in TgNL2 mice. In relation to altered frontal cortex–amygdala projections, our data may also shed light on a hypothesis that suggests that the dysfunction in autism may result from excessive and unselective connectivity in local frontal cortex circuitry, paired with impoverished long-range connectivity to other systems such as the amygdala (Courchesne and Pierce, 2005).

The incidence of frontoparietal seizure spiking, in light of increased prefrontal cortex inhibition, is particularly interesting because prevailing theories suggest that seizure activity results from hypersynchronous neuronal activity and is commonly associated with increased E/I ratios (Kofke et al., 1997; Stief et al., 2007). However, recent reports have also shown that focal seizure activity can result from desynchronization of firing caused by increased inhibitory feedback (Netoff and Schiff, 2002; Mormann et al., 2003; Klaassen et al., 2006). Thus, increased inhibition observed in frontal cortex of TgNL2 mice could underlie the cortical seizure spiking activity detected by freely moving EEG recording. Clinically apparent seizures occur in 30% of autistic individuals (Gillberg and Billstedt, 2000), whereas 50–70% of autistic individuals display ongoing “sharp-spike” activity during sleep (Lewine et al., 1999; Wheless et
The incidence of seizure spiking in TgNL2 mice may relate to cortical–limbic dysfunction discussed above in relation to autism, which is also implicated in temporal lobe epilepsy (Aroniadou-Anderjaska et al., 2007). In keeping with the idea of frontal cortex inhibition leading to amygdala disinhibition in TgNL2 mice, altered neuronal excitability in the amygdala could generate spontaneous epileptiform activity, which will subsequently spread to the other brain areas (Aroniadou-Anderjaska et al., 2007). Overall, the spiking activity observed may be directly attributable to alterations in cortical synchrony cause by increased inhibition or indirectly as a result of overexcitation in the amygdala spreading to the cortex. Future studies are needed to determine whether enhanced inhibition in the frontal cortex can be related to alterations in the excitability of the amygdala.

Our new findings combined with the link between mutations in NLs/Nrxs and autism may provide the neuronal basis for alterations in neuronal excitability, seizure spiking activity, stereotypies, anxiety, and impaired social interactions associated with autism (Konstantareas and Homatidis, 1999; Auranen et al., 2002; Jamain et al., 2003; Laumonnier et al., 2004; Lise´ and El-Husseini, 2006; Szatmari et al., 2007). In conclusion, we have discovered synapse and behavioural abnormalities in mice with altered expression of particular members of the NL family that recapitulate multiple aspects of behavioural changes associated with neurodevelopmental disorders such as Rett syndrome and autism. These include impaired social interactions, stereotyped patterns of behaviour, and enhanced incidence of seizure spiking, as depicted by EEG analysis in freely moving animals. We also show that a small change in NL2 expression results in synaptic abnormalities in cortical networks, which are thought to underlie the complex behavioural alterations seen in autism. These findings may provide the neural basis for synaptic imbalance and altered behaviour associated with autism.
2.5. Bibliography


3. LEARNING DEFICITS, IMPAIRED LTP AND ALTERED SYNAPTIC EXCITATION-INHIBITION RATIO IN MICE OVER-EXPRESSING NEUROLIGIN 1

3.1. Introduction

The development of synaptic networks depends on the synchronized actions of several guidance and cell-adhesion proteins during synaptogenesis (Piechotta et al., 2006) as well as synaptic activity later in development (Waites et al., 2005). Although somewhat distinct, some proteins play critical roles in both synaptogenesis and synapse maturation (Contractor and Heinemann, 2002; Luthl et al., 1994; Tang et al., 1998). For example, the postsynaptic density protein PSD-95 can drive glutamate synapse maturation (El-Husseini et al., 2000a), while also playing a critical role in experience dependent synapse stabilization (Ehrlich et al., 2007) and long-term potentiation (LTP) of synaptic efficacy (Ehrlich and Malinow, 2004; Migaud et al., 1998). The NL family of postsynaptic transmembrane proteins (NL1-4) is active early in development (Craig and Kang, 2007; Lise and El-Husseini, 2006). NLS were first identified as binding partners of Nrx, presynaptic cell adhesion molecules (Rowen et al., 2002; Tabuchi and Sudhof, 2002). The extracellular domain of NLS binds to Nrxs in a Ca²⁺ dependent manner, thereby bridging the synaptic cleft and linking NLS to the exocytotic machinery (Dean et al., 2003; Rao et al., 2000). The NL1 intracellular PDZ binding domain interacts with PSD-95, which clusters N-methyl-Daspartate receptors (NMDARs) and controls AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor numbers (Beique and Andrade, 2003; Ehrlich et al., 2007; Ehrlich and Malinow, 2004; El-Husseini et al., 2000b; Prange et al., 2004; Schnell et al.,

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2002; Stein et al., 2003). In addition, the NL1/PSD-95 complex is able to modulate neurotransmitter release probability via trans-synaptic protein-protein interactions (Futai et al., 2007). NLs have also been implicated in regulating the ratio of excitatory to inhibitory synapses (Chih et al., 2006; Graf et al., 2006; Kang et al., 2008; Levinson et al., 2005; Nam and Chen, 2005; Prange et al., 2004). *In vitro* studies have shown that the expression of individual NLs is sufficient to alter the density of excitatory and inhibitory synapses (Scheiffele et al., 2000; Graf et al., 2004; Prange et al., 2004; Chih et al., 2005; Levinson et al., 2005; Nam and Chen, 2005; Gerrow et al., 2006), biasing the E/I ratio. Specifically, NL1 has been shown to affect excitatory synaptic responses, whereas NL2 has been demonstrated to influence inhibitory synaptic responses (Chubykin et al., 2007). Since inhibition of NMDARs or CaM-Kinase II blocks NL1 activity, while inhibition of general synaptic activity suppresses the effects of NL2, NLs function in an activity-dependent manner (Chubykin et al., 2007).

Despite the body of research implicating NLs as key molecules regulating synapse maturation and specificity, a role for NLs in synaptic plasticity, outside of the developmental setting, has not been established. The present experiments examine the influence of NL1 expression on synapse maturation, synaptic plasticity, synapse specificity and learning using transgenic mice over-expressing NL1.

### 3.2. Materials and Methods

#### 3.2.1. Generation and Maintenance of Transgenic Mice

The NL1 transgene was expressed under control of the Thy1 promoter to drive neuron specific expression (Caroni, 1997). The sequence for NL1 was generated by insertion of a Sal I site into
the 3’ Spe I site of mouse NL1 cDNA (Hines, in press). Briefly, the HA tag was inserted following the signal sequence. NL1 was then amplified using Sal I and Xho I anchored primers. Following restriction digestion, these fragments were then cloned into the Xho I site of the Thy1.2 plasmid. The NL1-Thy1.2 plasmid was cut using Pme I and Pvu I enzymes to remove the bacterial backbone, and the resulting Thy1.2 NL1 fragments were used to generate NL1 transgenic mice (TgNL1). Distinct strains were generated from multiple founders via backcrossing to the C57BL/6 strain. Two primary strains with similar NL1 expression levels (TgNL1.6 and TgNL1.7) have been maintained on the C57/BL/6 background for 8 generations.

Mice were maintained at the University of British Columbia Animal Resource Unit, the Centre for Molecular Medicine and Therapeutics, or the Division of Medical Sciences at the University of Victoria according to protocols approved by the University of British Columbia/University of Victoria Animal Care Committees. Mice were group housed with a 12 hour light-dark cycle with constant temperature. Behavioural assessments were conducted during the light phase. All experiments were conducted genotype-blinded with wild-type (Wt) littermates used as control animals.

3.2.2. Genotyping

Germ line transmission of the TgNL1 transgene was detected using PCR, with primers spanning from Thy1 into the NL1 signal sequence. The primer sequences are as follows:

Thy1 5’-TAGGCTCCCACCTCCTTGGC-3’, NLs 5’-GCTATGATGGCATGTGTTGC-3’
3.2.3. Behaviour

Preliminary Screen: Assessments made in the preliminary screen were based on the modified SHIRPA protocol from the EMPReSS screen, designed to evaluate the basic phenotype of transgenic mouse strains (Rogers et al., 2001). The tasks of this screen were designed to rule out obvious alterations in sensory, motor and reflexive behaviours while evaluating the possibility of neurological dysfunction through several assessments.

Open Field: Open field exploratory behaviour of mice was assessed using a Logitech™ QuickCam webcam and Any-Maze™ software for video tracking. Mice were allowed to freely explore the novel environment for a period of 30 minutes. Parameters measured include total distance traveled, average speed, number of line crossings between edge and field zones, as well as mean speed on the open field or at the edge, respectively. Further parameters analyzed for the different zones include duration of mean visit, traveled distance, time spent, and the number of defecation boli. Results from tracking analysis were compared using one-way ANOVA (Wt n=12; TgNL1 n=14).

Elevated plus maze: Exploratory behaviour of Wt (n=12) and TgNL1 (n=10) mice was recorded in the elevated plus maze for 10 minutes using a Logitech™ QuickCam and Any-Maze™ software. Measurements included: average distance, speed, line crossings, time spend in the open, risk and safe zones as well as latency to first entry of both open and risk zones. Statistical significance was tested using two-tailed Student's t-tests.

Water Maze Visual testing: Wt (n=10) and TgNL1 (n=10) mice were initially tested in a plus shaped water maze for their ability to locate a visible platform in two sessions consisting of 3 consecutive trials. Mean latency, distance, speed, time immobile, and the number of line
crossings were calculated for each animal and statistic significance was tested by two-tailed Student's t-test.

Water Maze Learning and Memory experiments: In order to prevent excessive floating behaviour, Wt (n=10) and TgNL1 (n=10) mice were initially trained in the smaller plus-shaped water maze, followed by the standard Morris water maze. Training procedures for both water mazes have been described in detail elsewhere (Brandeis et al., 1989; van Praag et al., 1999; Van Dam et al., 2000; Vloeberghs et al., 2006). Briefly, animals were trained daily in two sessions consisting of 3 consecutive trials for 6 to 9 days followed by the probe test and reverse as well as working memory training in the same manner. The maximum time given for each trial was 60s in the plus-shaped and 90s in the Morris water maze, thereafter animals were guided to the platform. Each animal was allowed to remain on the platform for 15s, before it was taken back to the home cage. Tracking was done by a Logitech™ QuickCam webcam and data was recorded and analyzed using Any-Maze™ and Graph Pad Prism 5 software. Acquisition curves were compared with two-way analysis of variance for repeated measurements (RM ANOVA), while the probe trials were assessed by one-way ANOVA. Pair-wise comparisons were performed by Bonferroni post-hoc testing.

3.2.4. Lysate Preparation and Western Blotting

Lysate Preparation: Rapidly extracted tissues were homogenized in 3ml/g HEPES-buffer (10 mM HEPES, pH 7.5; 1 mM EGTA; 0.1 mM MgCl2; 0.15 M NaCl; 1x protease inhibitor cocktail (Roche Applied Science)) and cell debris was removed by centrifugation at 16000g at 4°C for 1hr.
Western Blotting (WB): Obtained supernatants were subjected to SDS-PAGE and analyzed by immunoblot. Staining for Comassie and actin was performed to determine the protein content of each sample. Protein levels were quantified using Image J software, and statistical significance of normalized measurements was tested by two-tailed Student's t-tests.

3.2.5. Golgi Cox Impregnation and Spine Morphometry Measurements

Two and a half month old mice were anesthetized with an overdose of sodium pentobarbital and perfused transcardially with 0.9% saline. Brains were then quickly removed from the skull, submersed in Golgi Cox solution (208mM K2Cr2O7, 260mM HgCl2 and 43mM K2CrO4 in dH2O) and stored for 7 days in the dark. After replacement of Golgi Cox solution with 30% sucrose buffered in phosphate (PBS), brains were stored in the dark for 3 more days. Thereafter, 150µm thick vibratome sections were collected, mounted onto 2% gelatinized slides and developed by a modified Golgi Cox technique (Glaser and Van der Loos, 1981). Following rapid dehydration, sections were embedded in Permount™ and coverslipped.

The density (spines per µm), as well as the head and neck size of pyramidal cells from cortical layer 5 and the CA1 region were analyzed using Image J software. Results are shown as percent change normalized to Wt measurements. Statistical significance was analyzed by two-way ANOVA and subsequent Bonferroni post-hoc.
3.2.6. Immunostaining

For immunohistochemistry (IHC), animals were perfused transcardially with a 4% solution of paraformaldehyde in PBS (for non-fluorescent IHC) or brains were harvested fresh and flash frozen with OCT/isopentane embedding medium in liquid nitrogen (for fluorescent synaptic protein IHC). Sections were cut on a cryostat at thicknesses of 10 µm (synaptic protein IHC) or 30µm (HA localization).

Sections were incubated in blocking solution (2.5% BSA, 10% normal goat serum, 0.1% triton-x 100, 0.02% sodium azide in PBS) for 60 minutes, followed by primary antibody incubations (diluted in blocking solution) overnight at 4°C. Following washing with 1xPBS-triton (1%), sections were incubated with secondary antibodies. Non-fluorescent immunostaining was visualized using avidin-biotin conjugation (Vectastain elite standard kit; Vector) to the secondary antibody, followed by diaminobenzene (Vector). Statistical significance was analyzed using two-tailed Student's t-tests.

3.2.7. Antibodies

Primary antibodies used were rat anti-HA (Boehringer Mannheim); rabbit anti-PSD-95 (generated by ABR); mouse anti-PSD-95 (ABR); rabbit anti-NL1 (Synaptic Systems); rabbit anti-NL2 (generated using a synthetic peptide by ABR); rabbit anti-NL3 (generated by ABR); rabbit anti-synaptophysin (Zymed); mouse anti-syntaxin 1 (Chemicon); rabbit anti-Synaptotagmin 1 (Synaptic Systems); guinea pig anti-VGluT 1 & 2 (Chemicon); rabbit anti-GluR2/3 (Chemicon); mouse anti-VGAT (Synaptic Systems); rabbit anti-Gephyrin (Alexis). Secondary antibodies used include peroxidase coupled donkey anti-rabbit and sheep anti-mouse IgG (WB; GE Healthcare),
biotinylated secondary antibodies (non-fluorescent IHC; Vector) or Alexa conjugated secondary antibodies (fluorescent IHC; Invitrogen Molecular Probes).

3.2.8. Electron Microscopy

Tissue Preparation: Tissue for electron microscopy (EM) was harvested fresh and cut using a vibratome in room temperature ACSF, followed by rapid fixation in 6% glutaraldehyde, 1% paraformaldehyde, 2mM CaCl2, 4mM MgCl2 in 0.1M cacodylate buffer. Sections were examined under a dissecting microscope and CA1 stratum radiatum regions of the hippocampus were isolated (bregma: -1.64 mm). Tissue blocks were washed in 0.1M cacodylate, postfixied in 2% osmium tetroxide, 1.5% potassium ferrocyanide in 0.1M cacodylate buffer for 2 h, followed by en bloc staining with 2% uranyl acetate for 45 min. Samples were then dehydrated by immersion in increasing concentrations of alcohol before being transferred into propylene oxide and gradually embedded in Eponate resin. Sections (1μm) were taken from prospective tissue blocks and examined under a light microscope to ensure a consistent location was selected. All tissue samples were coded with respect to genotype before electron microscopic imaging and analysis of number and morphology.

Analyses: The size of synaptic elements was measured using Image J on digital micrographs taken across six serial sections in alternation (n=4 animals for each group). These numbers were then averaged to ensure that measurements were not influenced by the 2-dimensional position within the synapse. Synaptic vesicles were manually counted in high magnification images, and were classified according to their location within the presynaptic bouton (docked or reserve pool).
For assessment of synapse number, synapses were counted in fields of 100μm². Within these fields, synapses were classified as either Type I (asymmetric excitatory) or Type II (symmetric inhibitory) on the basis of synaptic vesicle and postsynaptic density (PSD) morphology. Type I synapses were identified by the presence of a post-synaptic density and round synaptic vesicles within the presynaptic terminal, whereas Type II synapses were identified by the presence of at least three oval or flattened synaptic vesicles and the absence of a post-synaptic density (Uchizono, 1965). The data was analyzed using ANOVA, with the LSD post hoc test applied to significant ANOVA comparisons.

3.2.9. Electrophysiology

Field Recordings: Adult TgNL1 mice (TgNL1.6 n=6; TgNL1.7 n=9) and their Wt littermates (Wt; n=12) were used for electrophysiology experiments as previously described (Christie et al., 2005). Briefly, brains were rapidly removed while submerged in chilled sucrose artificial cerebrospinal fluid (sACSF; pH 7.2) containing (in mM) 110.00 sucrose, 60.00 NaCl, 3.00 KCl, 1.25 NaH₂PO₄, 28.00 NaHCO₃, 0.50 CaCl₂, 7.00 MgCl₂, 5.00 dextrose and 0.60 ascorbate, and saturated with 95% O₂-5% CO₂. Transverse slices containing the hippocampal formation were sectioned at 400 μm using a vibratome kept at 4°C (Vibratome 1500, Ted Pella Inc). For recovery, each slice was sequentially placed in a beaker containing warm (30°C) normal ACSF (nACSF; pH 7.2) containing (in mM) 125.00 NaCl, 2.50 KCl, 1.25 NaH₂PO₄, 25.00 NaHCO₃, 2.00 CaCl₂, 1.30 MgCl₂ and 10.00 dextrose, continuously bubbled with 95% O₂-5% CO₂ for ≥60 min. Individual slices were transferred to the recording chamber where they were perfused (2 ml/min) with warm (30°C), bubbled (95% O₂-5% CO₂) nACSF for the duration of each recording.
Responses were obtained using a 1–3 MΩ recording electrode (filled with nACSF) and a MultiClamp 700B (Axon Instruments) amplifier. All data was acquired at 100 kHz. Responses were evoked with a concentric bipolar electrode (FHC) using monophasic negative current pulses (120 µs, 10–80 µA) and a digital stimulus isolation unit (Getting Instruments). An Olympus BX50wi (10x objective) was used to visually position both the recording and stimulating electrodes for each experiment 50-100 µm apart in the stratum radiatum of the CA1 region of the hippocampus. Stimulation intensity was adjusted to yield response amplitudes 40% of the estimated maximum. All evoked responses were initially tested with paired-pulse stimuli (50 ms interpulse interval). During experiments, evoked responses were continuously elicited at 15 s intervals, except during the application of the conditioning stimulation. To assess LTP, a stable baseline was obtained for a minimum of 15 minutes and then conditioning stimuli were administered (four bursts of 50 pulses at 100 Hz; 30 s inter-burst interval). Single pulse stimulation was again initiated immediately following the conditioning stimuli and continued for a minimum of 60 minutes. The initial slope of the negative going waveform was used to assess changes in synaptic efficacy. Paired-pulse (PP) responses are presented as the normalized difference between the slopes of the two fEPSP responses and are presented as a percentage change. LTP was quantified as the percentage change in slope for responses collected following the application of conditioning stimuli. Assessment of disinhibition of the population spike (PS) was obtained by bath application of bicuculline methiodide (5 µM). The amplitude of the population spike was used to assess excitability of the population of cells in the immediate vicinity of the recording electrode. After obtaining a stable response for 15 minutes, bath application the GABA_A antagonist bicuculline methiodide (5 µM) was initiated and maintained for a minimum of 30 minutes. All data
acquisition and analysis was performed using ClampFit 10.2 software (Axon Instruments), Excel (Microsoft) and Statistica 7.0 for PC. Data was analyzed using unpaired t-tests and is presented as the mean ± SEM.

Whole cell Recordings: Similar to slices used for field recordings, for whole cell recordings, transverse slices (400 μm) were obtained from adult TgNL1 mice (n=4) and their Wt littermates (n=4) and submerged in nACSF and maintained at 30°C. Individual slices were transferred to the recording chamber where they were perfused (2 ml/min) with warm (30°C), bubbled (95% O₂-5% CO₂) nACSF for the duration of each recording.

Whole cell currents were obtained using a 5-7 MΩ recording electrode filled with (in mM) 140.00 KMeSO₄, 10.00 HEPES, 4.00 NaCl, 0.10 EGTA, 4.00 ATP, 0.30 GTP, 14.00 Phosphocreatine, and an Axopatch 200B (Axon Instruments) amplifier was used. All data were acquired at 100 kHz. Responses were evoked with concentric bipolar electrodes (FHC) using monophasic negative current pulses (120 μs, 10–80 μA) and a digital stimulus isolation unit (Getting Instruments). An Olympus BX50wi (10x and 40x objectives) was used to visually position electrodes. One stimulating electrode was placed in the Stratum radiatum to evoke EPSCs while a second stimulating electrode was placed directly in the pyramidal cell layer to evoke IPSCs. An input/output curve for excitatory currents (EPSCs) was obtained in CA1 pyramidal neurons by obtaining responses with increasing pulse widths while holding the cells at the reversal potential (Vrev) for GABA receptors (-91 mV). IPSCs were isolated by holding the cells at the Vrev for AMPA and NMDA receptors (+2 mV) and administering identical stimuli. The identity of EPSCs and IPSCs were confirmed using CNQX (20 μM), (50 μM) APV and (10 μM) bicuculline methiodide. All data acquisition and analysis was performed using ClampFit
10.2 software (Axon Instruments), Excel (Microsoft) and Statistica 7.0 for PC. Data was analyzed using unpaired t-tests and is presented as the mean ± SEM.

3.3. Results

3.3.1. The Neuroligin 1 Transgene is Expressed Throughout the Brain and is Localized at Excitatory Synapses

Mice expressing Neuroligin 1 (TgNL1) were generated using a construct composed of an HA tagged version of full length NL1 (details of mouse generation are described in (Hines, in press). There were no significant differences in the expression level of HA-NL1 in either of the transgenic mouse strains (TgNL1.6 and TgNL1.7; Figure 3.1A, B). Western blots revealed HA-NL1 was specifically expressed in brain and to a lesser extend in spinal cord, but not in non-neuronal tissues such as liver or heart (Figure 3.1C). In the brain, DAB immunohistochemistry and WB show that HA-NL1 has a broad distribution, with highest levels of expression in the cortex and hippocampus, intermediate in the striatum and lowest in the cerebellum and olfactory bulb (Figure 3.1D, and Figure 3.2). During brain development, an increase in the expression level of HA-NL1 can be observed during early postnatal development, in particular between postnatal day 10 and 14, while thereafter the expression level remains constant throughout adulthood (Figure 3.1E). Interestingly, the observed increase in HA-NL1 expression is similar to the developmental expression pattern of endogenous NLs (Jamain et al., 2008) and correlates with the development of dendritic spines.
Figure 3.1. Expression of the neuroligin 1 transgene. A. Western blots showing representative samples from Wt, TgNL1.6 and TgNL1.7 whole brain lysate probed for HA and actin as a protein loading control. B. Quantification of HA signal, shown as the percentage signal increase over background. C. Expression of HA-NL1 in different tissues of TgNL1.7 mice. D. Representative sagittal sections from Wt and TgNL1.7 animals stained for HA by DAB immunohistochemistry. E. Expression of HA-NL1 during development. (d=days, m=months). F. Confocal analysis of HA-NL1 localization in TgNL1.7 mice. Top panel: Co-localization of HA-NL1 (green) with a marker of excitatory synapses, PSD-95 (red), in the CA1 region of the hippocampus. Bottom panel: Co-localization of HA-NL1 (green) with a marker of inhibitory synapses, Gephyrin (red), in the CA1 region of the hippocampus. Scale bar: 2 µm. G. Quantification of HANL1 co-localization in TgNL1.7 mice.
Figure 3.2. Expression of neuroligin 1 in different brain regions. Western Blot analysis of HA-NL1 expression in different brain regions of TgNL1.7 mice. het: heterozygous animal. hom: homozygous animal. Minor signals have been observed in the olfactory bulb and the cerebellum of heterozygous animals upon longer western blot exposures (data not shown).
To further address the localization of HA-NL1, the co-localization of HA-NL1 with postsynaptic marker proteins for excitatory (PSD-95) and inhibitory (Gephyrin) synapses was studied. The analysis revealed that 73% of HA-NL1 puncta are localized with puncta positive for the excitatory synapse marker PSD-95. Conversely, 2% are found to be localized with puncta positive for the inhibitory synapse marker Gephyrin (Figure 3.1F, G). Moreover, 61% of puncta positive for PSD-95 and 8% of puncta positive for Gephyrin also contain HA-NL1, reflecting that not all cells express the transgene under the Thy1 promoter. Further HA-NL1 puncta observed may also represent dendritic transport clusters and/or potential new contact sides lacking PSD-95.

3.3.2. Mice Expressing Neuroligin 1 do not show Abnormalities in Sensory, Autonomic or Basic Motor Function

A preliminary phenotype screen, based on the modified SHIRPA protocol from the European Mouse Phenotyping Resource of Standardised Screens (EMPreSS; Rogers et al., 2001), was used to assess the overall health and basic autonomic, sensory, and motor functions of TgNL1 mice (Table 3.1). No alterations were observed in indicators of autonomic function, including palpebral closure, piloerection, and tail position. Similarly, sensory systems were found to be intact with no deficits in transfer arousal and touch escape (somatosensory system); corneal reflex, visual placing and visible platform water maze performance (visual system); pinna reflex and acoustic startle (auditory system), and buried food retrieval (olfactory system). In addition, no changes were observed in body position, body/limb tone, righting reflex, or basal activity levels, indicating no gross motor impairments in TgNL1 mice.
**Table 3.1.** Summary of the preliminary screen conducted on primary neuroligin 1 and neuroligin 2 transgenic strains. Dash mark (-) = normal or no impairment; in the case of impairment, increasing values indicate increased impairment, and a brief description is provided. N/A = Not Assessed.

### A. Sensory

<table>
<thead>
<tr>
<th>Sensory</th>
<th>Wt</th>
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<th>TgNL 1.7</th>
<th>TgNL 2.6</th>
<th>TgNL 2.7</th>
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<tr>
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### B. Motor & Anxiety

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<th>TgNL 1.7</th>
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3.3.3. Specific Learning Impairments are Observed in Mice Expressing Neuroligin 1

Since no deficit was detected in the basic autonomic, sensory, or motor performance of TgNL1 mice, we became interested in the possibility of more subtle defects on complex functions such as learning and memory. To test hippocampal dependent spatial learning and memory, the hidden platform version of the plus-shaped and Morris water maze tests were applied to compare TgNL1 mice (n=10) and their Wt littermate controls (n=10). In both tests, animals learn to escape from the water maze by locating a hidden platform using spatial cues, which requires regular hippocampal function (Morris et al., 1982).
Both, Wt and TgNL1 mice significantly improved their performance in the plus-shaped (Figure 3.3A-C; Figure 3.4), and the standard Morris, water mazes during acquisition training, as indicated by a significant within groups effect of training session (Plus shaped water maze training: Distance $F_{(8,464)}=5.08, p<0.001$ and Latency $F_{(8,464)}=9.62, p<0.001$; Reversal training: Distance $F_{(5,290)}=12.88, p<0.001$; Latency $F_{(5,290)}=16.42, p<0.001$; Morris water maze training: Distance $F_{(5,290)}=6.39, p<0.001$; Latency $F_{(5,290)}=6.91, p<0.001$; Reversal training: Distance $F_{(3,174)}=4.60, p<0.001$; Latency $F_{(3,174)}=2.37, p<0.001$). However, during forward training in the plus shaped water maze, TgNL1 mice show a significant increase in the distance travelled (Figure 3.3B; $F_{(1,464)}=63.05, p<0.001$), the escape latency (Figure 3.3C; $F_{(1,464)}=79.66, p<0.001$) and the number of lines crossed (Figure 3.4; $F_{(1,464)}=67.27, p<0.001$), as well as a significant reduction in the number of correct attempts (Figure 3.4; $F_{(1,464)}=26.13; p<0.001$) when compared to Wt littermates. Moreover, additional training sessions (session 5-9) did not improve the performance of TgNL1 mice any further, nor allow them to reach the performance level of Wt littermates. Given that TgNL1 and Wt mice did not differ in control swim speed (data not shown; $F_{(1,464)}=0.7669, p>0.05$), these differences cannot be explained by locomotor deficits in TgNL1 mice. Instead these results are interpreted as disrupted acquisition of memory for the platform location. The difference in performance observed in TgNL1 mice and their Wt littermates during the first training session might relate to visual testing which immediately preceded water maze testing. It is possible that due to a learning deficit in TgNL1 mice, Wt mice benefit more from the experience of the vision test. In support of this explanation, TgNL1 mice and their Wt littermate controls show an equal performance during the first session of all subsequent water maze tasks (reversal training, two Morris water maze paradigms) when the TgNL1 mice had more time to become familiar with the hidden platform.
Figure 3.3. Impaired memory acquisition in the plus-shaped water maze in mice expressing *neuroligin 1*. A-C. Plus shaped water maze training. D-F. Reverse training. A-B, D-E. Each data point represents mean of summed results per training session. Error bars indicate confidence interval (95%). ***, p<0.001. Black symbols represent TgNL1.7 mice, while white symbols indicate Wt animals. A, D. Path length in meters. B, E. Escape latency in seconds. C, F. Representative track plots from the last training session. S, start. T, target (hidden plat form). G-J. Probe trial. G, I. White bars indicate the mean for Wt and black bars for TgNL1 mice. G. Forward training. I. Reverse training. G, I (time) p>0.05. G (number of entries) F=10.16, p=0.005 (**); I (number of entries) F=55.49, p<0.001 (**). H, J. Representative track plots from the probe trial. S, start. T, target (hidden plat form). H. Forward training. J. Reverse training. Note the cumulated paths at the bottom of the target area in H. resulting from ongoing for and back swimming by the TgNL1 animal, whereas the Wt animal displays repetitive attempts for the former platform location.
Figure 3.4. Impairment in memory acquisition of mice expressing neuroligin 1. A,B. Forward training. C,D. Reverse training. A-D. Each data point represents mean of summed results per training session. Error bars indicate confidence interval (95%). ***, p<0.001; *, p<0.05. Black symbols represent TgNL1.7 mice, while white symbols indicate Wt animals. A,C. Number of line crossings. B,D. Number of correct attempts.
condition. Consistent with forward training, the reverse training (Figure 3.3D-F, Figure 3.4) revealed a significant impairment in new memory formation in TgNL1 mice \((\text{Latency } F(1,290)=95.74; \ p<0.001; \ \text{Distance } F(1,290)=48.0, \ p<0.001; \ \text{Line crossings } F(1,290)=34.68; \ p<0.001; \ \text{Number of correct tries } F(1,290)=13.43, \ p=0.006; \ \text{mean swimming speed } F(1,290)=0.15, \ p>0.05)\).

After completion of training, a probe trial lacking the platform was performed (Figure 3.3G-J). Surprisingly, both, Wt and TgNL1 mice, showed a significant preference for the area of the former platform location after training \((F(3,79)=21.34, \ p<0.001; \ \text{post hoc } t=7.57, \ p<0.001 \text{ for Wt and } t=2.59, \ p<0.05 \text{ for TgNL1 mice, data not shown})\) as well as after reversal training \((F(3,79)=18.07, \ p<0.001 \text{ post hoc } t=6.18, \ p<0.001 \text{ for Wt and } t=3.97, \ p<0.01 \text{ for TgNL1 mice, data not shown})\) and no difference in the time spent in the former platform area was detected between TgNL1 mice and their littermate controls \((p>0.05; \ \text{Figure 3.3G-J})\), suggesting that despite the poorer learning performance of TgNL1 mice, once learned, the memory for the platform position remains intact. However, TgNL1 mice did show a significant reduction in the number of entries to the former platform area compared to their Wt littermates \((F(1,18)=10.16, \ p=0.005; \ \text{reverse training: } F(1,18)=55.49, \ p<0.001)\), implying the use of a different search strategy or a lack of motivation to continue searching.

Similar results were obtained from the Morris water maze training paradigms (Figure 3.5). While no difference in the swim speed of TgNL1 and Wt mice was detected \((F(1,290)=0.53, \ p>0.05)\), TgNL1 mice show a significant increase in the distance travelled \((F(1,290)=37.56, \ p<0.001)\), the number of lines crossed \((F(1,290)=46.68, \ p<0.001)\), and the escape latency \((F(1,290)=51.62, \ p<0.001)\) when compared to their littermate controls. During reverse training, the trend toward an increase in the number of lines crossed was not significantly different.
Figure 3.5. Similar impairment in memory acquisition in the Morris water maze in mice expressing neuroligin 1. A. Representative track plots from the last training session. S, start. T, target (hidden platform). B. Forward (light bars) and reverse (dark bars) training in the Morris water maze. Each bar displays the mean percent change compared to wildtype animals (Wt, =0%), while the error bars show the confidence interval (95%). *** p<0.001, ns: non-significant. C-E. Probe trial. C. Representative track plots from probe trial. S, start. T, target (hidden platform). Number of entries wildtype animal:12, TgNL1.7:7. D. Forward training. C. Reverse training. C,D. Bars represent the average time spent in the former platform area or the other areas, respectively. P= former platform area. O= other areas.
(\(F_{(1,174)}=2.67, p>0.05\)), while both the distance and escape latency of TgNL1 mice show a significant increase in comparison to Wt control animals (Distance \(F_{(1,174)}=21.68, p<0.001\); Latency \(F_{(1,174)}=54.69, p<0.001\); Figure 3.5A). These findings further confirm the deficits in memory acquisition in TgNL1 mice.

Interestingly, the subsequent probe tests in the Morris water maze (Figure 3.5C-E) revealed that only the Wt mice developed a significant preference for the area of the former platform location, while TgNL1 mice did not show any difference in the time spent in the different quadrants of the Morris water maze during both training paradigms (Training: \(F_{(3,79)}=4.41, p=0.0096\); post hoc \(t=3.64, p<0.01\) for Wt, but \(t=0.10, p>0.05\) for TgNL1 mice; Reversal training: \(F_{(3,79)}=3.07, p=0.0403\); post hoc \(t=3.02, p<0.05\) for Wt, but \(t=0.35, p>0.05\) for TgNL1 mice). These findings might reflect the increased difficulty of the Morris water maze when compared to the plus-shaped maze and this may have more profoundly, and negatively, impacted learning in the TgNL1 mice. TgNL1 mice were also assessed for spatial reference working memory (Figure 3.6) by changing the platform position after 6 (Morris water maze) or 8 (plus shaped water maze) consecutive trials, respectively, in a daily manner. In both mazes, TgNL1 mice as well as their Wt littermates demonstrate a regular learning curve as indicated by a significant effect of training session (Latency (Plus shaped water maze) \(F_{(7,384)}=4.89, p<0.001\); Latency (Morris water maze) \(F_{(5,290)}=5.86, p=0.006\); Distance (Plus shaped water maze) \(F_{(7,384)}=5.07, p<0.001\); Distance (Morris water maze) \(F_{(5,290)}=5.87, p=0.006\). When compared to their Wt littermates, TgNL1 mice show a significant increase in the distance travelled (\(F_{(1,384)}=9.07\) and \(p=0.003\) in the plus shaped water maze and \(F_{(1,290)}=12.08\) and \(p=0.005\) in the Morris water maze) and the latency (\(F_{(1,384)}=46.43\) and \(p<0.001\) in the plus shaped water maze and \(F_{(1,290)}=62.56\) and \(p<0.001\) in the Morris water maze) to escape from the water maze,
Figure 3.6. Working memory impairment and alterations in the search strategy of mice expressing neuroligin 1. A-C. Spatial working memory training in the plus-shaped water maze. A,B. Each data point represents mean of summed results per training session. Error bars indicate confidence interval (95%). *** p<0.001; ** p<0.01. Black symbols represent TgNL1.7 animals, while white symbols indicate Wt mice. C. Representative track plots from the last training session. S, start. T, target (hidden plat form). D-F. Spatial working memory training in the Morris water maze. D,E. Each data point represents mean of summed results per training session. Error bars indicate confidence interval (95%). *** p<0.001; ** p<0.01. Black symbols represent TgNL1.7 animals, while white symbols indicate Wt mice. F. Representative track plots from the last training session. S, start. T, target (hidden plat form). G. Comparison of the search performance of Wt and TgNL1 animals for wall platform positions (dark bars) and center platform positions (light bars). Each bar displays the mean percent change compared to Wt animals, while the error bars show the confidence interval (95%). *** p<0.001; ** p<0.01; * p<0.05, -: not significant. H,I. Representative track plots from a final trial for a center position. S=start. T=target (hidden plat form). Punctate circles: potential platform positions. Note that occasionally, TgNL1 animals, due to the displayed searching pattern, never find the platform if it is hidden at a wall position (data not shown).
reflecting deficits in working memory acquisition in TgNL1 mice. The track plots (Figure 3.6A, D) of the final trial illustrate the lack of spatial reference memory formation in TgNL1 mice and furthermore point to the use of a different search strategy in these mice.

To investigate the possibility of an alternative search strategy, the ability of TgNL1 mice to locate wall or centre platform positions, respectively, was analyzed in the Morris water maze (Figure 3.6G-I). A significant increase in the latency ($F_{(3,23)}=34.18$, $p<0.001$; post hoc $t=8.56$ and $p<0.001$), distance ($F_{(3,23)}=7.75$, $p=0.002$; post hoc $t=4.22$ and $p<0.01$) and number of lines crossed ($F_{(3,23)}=7.61$, $p=0.002$; post hoc $t=4.25$ and $p<0.01$) can be observed during the search for wall positions by TgNL1 mice when compared to their Wt littermates (Figure 3.6I). By contrast, no significant difference ($F_{(3,23)}=7.75$, $p=0.002$; post hoc $t=0.90$ and $p>0.05$) between Wt and TgNL1 mice can be detected in the distance and number of lines crossed to escape from the water maze ($F_{(3,23)}=7.61$, $p=0.002$; post hoc $t=0.62$ and $p>0.05$), when the platform is hidden at one of the centre positions, although the escape latency of TgNL1 mice is still significantly increased ($F_{(3,23)}=34.18$, $p<0.001$; post hoc $t=4.98$ and $p<0.001$) compared to Wt animals. The track plots (Figure 3.6G, H) illustrate that the circular search pattern typically employed by TgNL1 mice favors the detection of a platform hidden at a centre position.

3.3.4. Mice Expressing Neuroligin 1 show Changes in the Expression Levels of Related Synaptic Proteins

Western Blot analysis (Figure 3.7) of protein expression levels showed that increasing the expression of NL1 by 112% results in a significant up-regulation of marker proteins for excitatory (PSD-95: 16 ± 7%; VGluT: 30 ± 16%) and inhibitory (Gephyrin: 26 ± 11%; VGAT: 29 ± 6%) synapses in TgNL1 mice. The expression level of GluR2/3 subunits was significantly
Figure 3.7. Alterations in the expression of synaptic proteins in mice expressing neuroligin 1. A. Representative Western blot strips illustrating expression levels of several synaptic marker proteins from Wt and TgNL1.7 mice. B. Quantification, showing the percent change in expression compared to Wt expression level (middle line). Proteins showing significant changes are indicated by stars: p(NL1)<0.0001, p(NL3)=0.0052, p(Gephyrin)=0.0198, p(PSD-95)=0.0116, p(VGluT)=0.0289, p(VGAT)=0.0026, p(GluR2/3)<0.0001. Synphys: Synaptophysin, Syntag: Synaptotagmin. C. Representative immunofluorescence staining for excitatory (VGluT1+2) and inhibitory (VGAT) presynaptic marker proteins in Wt (left panel) and TgNL1.7 (right panel) hippocampus (CA1). Scale bar: 2 μm. D. Quantification of synaptic areas positive for excitatory or inhibitory synaptic marker proteins, respectively, by confocal analysis of immunofluorescence labeling. White bars: Wt; black bars: TgNL1.7, p<0.0001(***) and p<0.001(**).
A

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C

D

**Puncta Area (pixels)**

- **WT**: 25,000
- **TgNL1**: 22,000

**Significance:**
- ***: p < 0.001
- **: p < 0.01
- : p < 0.05
decreased (-30 ± 10%) in TgNL1 animals, whereas expression of the GluR1 subunit was significantly increased (122% ± 35%), thereby indicating altered AMPA receptor properties and/or numbers at synapses. Further analysis by immunohistochemistry confirmed these findings (GluR1: 146% ± 14%, *p*<0.000; GluR2/3: -58% ± 8%, *p*<0.000). However there were no changes in endogenous NL2, synaptophysin or synaptotagmin expression levels. Using IHC staining for the synaptic marker proteins, similar changes in VGlUT (82 ± 18%) and VGAT (75 ± 24%) were observed (Figure 3.7C, D) in TgNL1.7 mice. These findings are also in line with recent *in vitro* studies showing that NLs can influence both excitatory and inhibitory synapses (Levinson et al., 2005b; Chih et al., 2006).

### 3.3.5. Alterations in Spine and Synapse Morphology in Mice Expressing Neuroligin 1

Due to prior *in vitro* results showing that NL expression increases synapse maturation and stabilization (Scheiffele et al., 2000; Graf et al., 2004; Prange et al., 2004; Chih et al., 2005; Levinson et al., 2005; Nam and Chen, 2005) we wanted to assess synapse morphology in TgNL1 mice. Assessment of spine morphology using Golgi impregnation revealed an increase in the head size (21 ± 4.4%, *p*<0.001) of mushroom shaped spines, with a decrease in the neck length (-26 ± 8.8%, *p*<0.001) in CA1 of TgNL1.7 (Figure 3.8A, B). No change was observed in the overall length of these spines in TgNL1.7 mice (n=4 animals) compared with littermate controls (n=4 animals; Figure 3.8B). It was also observed using Golgi impregnation that the spines of CA1 pyramidal neurons in the TgNL1.7 mice had complex or lobed morphologies (Figure 3.8A, arrow heads).
Figure 3.8. Changes in the morphology of spines and synapses in mice expressing neuroligin 1. A. Representative images of dendritic spines in the CA1 region of the hippocampus of Wt and TgNL1.7 mice. Scale bar: 3 µm. B. Quantification of spine morphology in the CA1 region of the hippocampus. Bars show percent change compared to Wt for total spine length, head and neck size, ns=not significant, p<0.001 (**). C. Representative micrographs of asymmetric synapses in Wt and TgNL1 stratum radiatum. Scale bar: 500 nm. D. Quantification of the average length of the postsynaptic density and length of contact between pre and postsynaptic compartments. E. Quantification of the curvature of synaptic contacts, calculated by dividing the length of contact by the straight line length across the contact. F. Quantification of the average number of docked and reserve pool vesicles of asymmetric synapses in Wt, TgNL1.6 and TgNL1.7 mice. Straight line indicates overall significance, whereas brackets indicate significance from post-hoc tests.
The dramatic changes observed in spine morphology prompted a more detailed assessment of synapse morphology via EM. Quantitative assessment of synaptic elements (Figure 3.8D) revealed a significant increase in the average length (nm) of asymmetric synaptic contacts (Wt=649.73±40.01, TgNL1.6=785.40±46.77, TgNL1.7=878.39±74.22; ANOVA F(2,121)=4.21 p=.017; LSD post hoc: p=0.048 Wt vs TgNL1.6, p=0.005 Wt vs TgNL1.7) in TgNL1.6 and TgNL1.7 stratum radiatum (Figure 3.8F), with no change in the average length (nm) of the postsynaptic density (Figure 3.8E; Wt=363.47±20.75, TgNL1.6=36.83±14.89, TgNL1.7=394.26±20.67; ANOVA F(2,121)=0.797 p=0.453). The increased synaptic contact length seen in TgNL1.6 and TgNL1.7 mice is in agreement with the Golgi findings that these spines have increased head size. We also assessed the curvature of synaptic contacts by dividing the total contact length by the straight line length across the contact (Figure 3.8D). This analysis revealed an increase in the curvature of synaptic contacts (Wt=1.11±0.03, TgNL1.6=1.44±0.09, TgNL1.7=1.64±0.11; ANOVA F(2,121)=10.16 p<.001; LSD post hoc: p=.006 Wt vs TgNL1.6, p<.001 Wt vs TgNL1.7) in both TgNL1.6 and TgNL1.7 synapses compared to Wt controls (Figure 3.8F). It is unclear why we were able to observe an increase in PSD-95 expression via WB while no change in the length of the PSD was detected using EM. The change observed via WB may instead reflect the increase in the number of excitatory synapses. It is also interesting to consider the lack of change in PSD length in light of the change observed in synaptic contact length, as increases in synapse size commonly parallel one another. This may suggest that although expression of NL1 can result in altered synapse morphology, NL1 may not be sufficient to recruit the full complement of proteins that make up the PSD. However, since increased head to neck ratio, synaptic contact length, and curvature of synapses are hallmarks of stable or mature spines (Marrone and Petit,
2002; Bourne and Harris, 2007), together these findings suggest that TgNL1 mice display a greater proportion of spines with a mature morphology compared to Wt littermates.

3.3.6. Mice Expressing Neuroligin 1 show Synaptic Changes Suggestive of a Shift in the Ratio of Excitation to Inhibition

Because NLs have been indicated in regulating the ratio of excitatory to inhibitory synapses (E/I ratio; (Prange et al., 2004a; Levinson et al., 2005b; Nam and Chen, 2005; Chih et al., 2006; Graf et al., 2006; Kang et al., 2008), we wanted to examine in more detail whether a change in the E/I ratio can be observed in TgNL1 mice. Using Golgi impregnation, we observed that hippocampal dendrites of CA1 in TgNL1.7 mice show an increased density of spines (27%, ±8.4%, p=0.0303) compared to dendrites of littermate controls (Figure 3.9A,B). We did not observe a change in the overall branching of dendrites (-8%, ±15%, p=0.730) when comparing TgNL1.7 with littermate controls.

Since the increase in the number of spines observed in TgNL1 CA1 may suggest an increase in synapses, we wanted to assess the number of synapses in the stratum radiatum of the hippocampus, adjacent to CA1, using EM (Figure 3.9C). EM assessments revealed an increase in the number of asymmetric (typically excitatory) synapses (Wt=26.74±2.15, TgNL1.6=45.28±3.34, TgNL1.7=49.55±4.74; ANOVA F(2,44)=11.55  p<0.001; LSD post hoc: p=0.001 Wt vs TgNL1.6, p<0.001 Wt vs TgNL1.7) in both TgNL1.6 and TgNL1.7 compared to Wt controls. This finding is consistent with the increased spine density, and parallels increases in excitatory synaptic markers (VGluT and PSD-95) observed using WB. In contrast to asymmetric synapses, no change was observed in the number of symmetric (typically inhibitory; Wt=7.14±1.70, TgNL1.6=8.56±1.54, TgNL1.7=7.84±1.94; ANOVA F(2,44)=0.168  p=0.846)
Figure 3.9. Mice expressing neuroligin 1 show increased spine and synapse number, and a shift in the ratio of excitation to inhibition. A. Representative images of dendrites in the CA1 region of the hippocampus of Wt and TgNL1.7 mice. Scale bar: 5µm. B. Quantification of dendritic spine number (spines/µm) in the CA1 region of the hippocampus. White bar: Wt animals and black bar: TgNL1.7 mice, p(number)=0.0293 (*). C. Quantification of the number of asymmetric (typically excitatory) and symmetric (typically inhibitory) synapses (per 100 µm²) using EM in the stratum radiatum of Wt, TgNL1.6 and TgNL1.7 mice. D. Calculation of the number of excitatory (asymmetric) to inhibitory (symmetric) synapses in Wt, TgNL1.6 and TgNL1.7 mice. Straight line indicates overall significance, whereas brackets indicate significance from post-hoc tests.
synapses. It is unclear why the increases in the inhibitory markers VGAT and Gephyrin observed are not paralleled by an increase in symmetric synapses. It is possible that protein expression is increased as a means of compensation for the dramatic increase observed in excitatory synapses, but the formation and alteration of symmetric synapses might fail in vivo due to a lack of regulatory mechanisms. To obtain an estimate of the excitatory to inhibitory ratio, we divided the number of asymmetric synapses (excitatory) by the number of symmetric synapses (inhibitory; Figure 3.9D). This calculation reveals a shift in the ratio toward increased excitation (Wt=4.95±0.86, TgNL1.6=8.55±0.77, TgNL1.7=8.11±0.82; ANOVA F(2,44)=5.87 p=0.008; LSD post hoc: p=0.004 Wt vs TgNL1.6, p=0.013 Wt vs TgNL1.7) in stratum radiatum of both TgNL1.6 and TgNL1.7.

3.3.7. Impairments in the Induction of Long Term Potentiation in the Hippocampus of Mice Expressing Neuroligin 1

To further elucidate how these structural alterations might alter synaptic communication, and thus exert their effects on behaviour, we also examined synaptic plasticity in the CA1 subfield of the hippocampus (Figure 3.10). Paired-pulse analyses were conducted to test for obvious alterations in presynaptic transmitter release. A significant difference in paired-pulse facilitation in the stratum radiatum was not observed between genotypes (TgNL1: 15.98±1.04%; n=10; Wt: 17.54±1.45%; n=9; p=0.36), suggesting that NL1 overexpression does not induce an obvious alteration in presynaptic transmitter release. However, when 100 Hz conditioning stimuli were applied, the TgNL1 animals showed significantly (P<0.05) less short-term potentiation (STP: 22.22±9.15%, n=11) and long-term potentiation (LTP: 13.99 + 4.69%; n=11) than their Wt littermates (STP: 115.5+17.5%; LTP: 50.1 + 5.71; n=8; Figure 3.10A). This
Figure 3.10. Long-term potentiation in the CA1 region is significantly impaired in mice expressing neuroligin 1. A. Application of high frequency stimuli (100 Hz) reliably induces long-term potentiation of synaptic efficacy in Wt animals (open circles). These same stimuli are far less efficacious when applied to hippocampal slices taken from mice over-expressing NL1 (closed circles). Insets show example evoked responses taken from Wt (left) and TgNL1 (right) CA1 subfields. Note that the initial size of evoked responses in both animals is equivalent, but that only the TgNL1 response fails to enhance. B. Comparison of the percentage change in the EPSP slope at one hour post-conditioning for Wt animals (white bar at left); TgNL1 animals administered one bout of HFS (Black bar in centre); and TgNL1 administered three bouts of HFS (grey bar at right). In all instances, the TgNL1 animals exhibited significantly less LTP than Wt animals. (*) = p<0.05.
reduction in synaptic efficacy did not appear to be due to a reduction in the threshold for LTP induction, as even administering the conditioning stimuli multiple times (3X), did not induce significant LTP in TgNL1 animals (1.05+20.7%; n=9; Figure 3.10B) even though they did exhibit robust STP (97.47+36.56%). Thus, over expression of NL1 reduces the capacity of the CA1 region of the hippocampus to exhibit long-lasting alterations in synaptic efficacy.

3.3.8. Increased Basal Excitation in the Hippocampus of Mice Expressing Neuroligin 1

To address the functional relationship between excitation and inhibition we examined the response of the population of cells to the removal of inhibition using bicuculline methiodide and examined the evoked excitatory and inhibitory responses of individual pyramidal neurons. Population spikes were recorded in the pyramidal cell layer and induced by stimulation of the Schaffer’s collaterals (Pavlov et al., 2004). After a minimum of 15 minutes stable baseline responses, bicuculline methiodide (5 μM) was applied for 30 minutes. Following blockade of inhibition, the population spike increased significantly less in TgNL1 mice (152+18%; n=5) compared to controls (515+116%; n=5) (Figure 3.11A,B). This may suggest that the hippocampus is basally more excitable due to an increase in excitation or a decrease in inhibition as a result of NL1 expression (consistent with our observation that population spikes were more easily obtained from TgNL1 mice). To test this more directly, we performed whole cell recordings and evoked EPSCs and IPSCs at increasing stimulation strengths. We observed larger magnitude EPSCs (evident at multiple stimulation strengths) in TgNL1 mice (-151.2 pA; n=4) relative to controls (-59.6; n=5) (Figure 3.11C,D). In contrast we did not observe a difference in the magnitude of IPSCs between genotypes (TgNL1: +84.8 pA; n=4) (Wt: +61.0
Figure 3.11. Mice expressing neuroligin 1 show an increase in excitation and the ratio of excitation to inhibition. A. Representative traces showing population spikes obtained from the stratum pyramidale in response to stratum radiatum stimulation in age-matched Wt and TgNL1 slices. Traces obtained in normal ACSF shown in black, while those obtained with inhibition blocked are shown in red. B. Time course of changes in population spike amplitude in field recordings obtained following bath application of bicuculline methiodide. C. Increasing evoked synaptic stimulation (pulse width) to the Schaffer’s collaterals induces larger excitatory postsynaptic currents (EPSCs) in mice expressing Neuroligin 1 (TgNL1; closed circles) relative to wild-type (Wt; open circles) mice. D. Averaged traces from one set of IO EPSCs from Wt mice (grey lines) and TgNL1+ mice (black lines). E. Increasing evoked synaptic stimulation (pulse width) to the Schaffer’s collaterals induces similar inhibitory postsynaptic currents (IPSCs) in mice expressing Neuroligin 1 (TgNL1; closed circles) relative to wild-type (Wt; open circles) mice. F. Averaged traces from one set of IO IPSCs from Wt mice (grey lines) and TgNL1+ mice (black lines). Vertical scale bar represents 50 pA and horizontal scale bar represents 10 ms. Scale bars are identical for D and F.
pA; n=5) (Figure 3.11E, F). At a functional level, it appears that overexpression of NL1 leads to an increase in basal excitation in the hippocampus without altering inhibition.

3.4. Discussion

The present experiments focused on NL1, a postsynaptic cell-adhesion molecule preferentially localized at excitatory synapses, and examined NL1’s function in synapse development, synaptic plasticity and learning in vivo. Using mice over-expressing NL1 as a model, we found that increased NL1 expression leads to striking deficits in memory acquisition, paralleled by impairment in the induction of LTP, altered spine morphology, and enhanced excitatory synaptic transmission.

NLs have been primarily implicated in autism spectrum disorders (ASD) as mutations in the genes encoding NL1, NL3 and NL4 have been associated with some rare cases of inherited ASD (Jamain et al., 2003a; Talebizadeh et al., 2005; Yan et al., 2005a; Ylisaukko-oja et al., 2005a; Hines et al., 2008; Jamain et al., 2008; Lawson-Yuen et al., 2008; Hines, in press). While the involvement of NLs in synapse formation has also been addressed in several studies (Lise and El-Husseini, 2006; Craig and Kang, 2007), a role for NLs in synaptic plasticity and memory formation is currently being established. Tabouchi and colleges (2007) found that the Arg451>Cys451 substitution in NL3 that has been linked to ASD, not only induced social deficits in transgenic mice, but also increased their spatial learning performance. However, as a deletion of NL3 did not cause corresponding changes, these findings represent a specific gain of function mutation. By contrast, NL1 has just recently been demonstrated to be necessary for the expression of long-term potentiation in the amygdala as well as the development of
associative fear memory in adult animals (Kim et al., 2008). In line with these findings, our data now indicate that NL1 expression levels can also influence spatial learning and that, moreover, increased expression levels result in the blockage of long-term potentiation in the hippocampus. Importantly, neither of the TgNL1 strains displayed deficits in basic sensory, reflexive and motor function or exploratory motivation, thereby confirming TgNL1 mice as candidates for detailed behavioural characterization.

In the plus shaped and Morris water maze tasks, TgNL1 mice showed a striking reduction of their performance during memory acquisition training. However, as indicated by the swim paths and the strategy analysis, TgNL1 mice also developed an alternative search strategy, which might contribute to their improvement in performance. Interestingly, the probe tests conducted after memory acquisition revealed different results in the plus shaped and the Morris water maze, respectively: While TgNL1 mice displayed a significant preference for the area of the former platform location in the plus shaped water maze, they failed to do so in the Morris water maze. These results indicate that the ability of TgNL1 mice to develop true spatial memory is limited. In this context, the observed reduction in the number of entries into the former platform area by TgNL1 mice might reflect the use of a different search strategy or indicate a lack of motivation to continue searching after the platform is not found. On the other hand, these results also suggest that the formation and/or employment of true spatial memory by TgNL1 mice is limited as the increased difficulty mice experience in the Morris water maze, which was in contrast to the plus shaped maze originally designed for rats, is sufficient to prevent TgNL1 mice from the formation of true spatial memory, but not their Wt litter mates.
Memory acquisition in the water maze is thought to be largely determined by the ability of mice to develop appropriate strategies. Therefore, acquisition performance is thought to reflect not only the capacity for an animal to learn, but also the degree of behavioural flexibility. The circular, or chaining response strategy displayed by TgNL1 mice, usually a transitional search behaviour, has been described before (Janus, 2004; Brody and Holtzman, 2006), but is less commonly found than a thigmotactic search pattern, which is often indicative of increased anxiety (Sakic et al., 1993; Oitzl et al., 1997; Gass et al., 1998; Bjorklund et al., 1999; Champagne et al., 2002; Lang et al., 2003; Schmitt et al., 2006; Wilcoxon et al., 2007; Zhu et al., 2007). Interestingly, the chaining strategy was not a useful alternative for our mice as it fails to detect 5 out of 9 possible positions. While more efficient strategies can be adopted by the Wt mice if chaining does not provide a solution (Brody and Holtzman, 2006), TgNL1 mice failed to adapt their strategy, indicating impaired procedural learning and a lack of flexibility. Similar results have been obtained with rats when the septo-hippocampal cholinergic system was impaired (Sutherland et al., 1982).

Western blot and immunohistochemistry revealed an increased expression of marker proteins for excitatory and inhibitory synapses. Closer examination using EM revealed an increase in the number of asymmetric (excitatory), but no change in the number of symmetric (inhibitory) synapses. Further, we observed an increase in the contact length of asymmetric synapses that was not observed with symmetric synapses. This data suggests that in vivo, NL1 expression mainly enhances excitatory synapse formation, although it also elevates the expression of proteins resident at inhibitory synapses. The finding that excitatory activity was not enhanced as much in the TgNL mice as the Wt when inhibition was blocked, indicates that inhibition is playing a lesser role in these animals. This could be because (1) there is already greater
excitatory input at activated synapses, so removing inhibition has less of an effect, or (2) because there are fewer inhibitory synapses in TgNL1 mice. As no differences were observed in inhibitory synapse numbers, it seems likely that there has been a shift towards excitation.

Analogously, a previous paper has shown that NL2 expression results in an increase in the maturation and transmission of inhibitory synapses, with a minor change observed in excitatory synapse morphology (Hines et al., 2008). Thus, in vivo studies of NL1 and NL2 function suggest that activity of specific NLs is not fully restricted to effects on either excitatory or inhibitory synapses, but may act more subtly to sway the E/I ratio. These findings also hint to some redundant functions in the NL family, which is supported by data showing that knock-out of NL 1-3 is lethal, whereas all single and double knock-out combinations are viable (Varoqueaux et al., 2006). Furthermore, these two papers taken together demonstrate the influence of altering the E/I ratio on brain function and behaviour, as the two strains of mice (expressing either NL1 or NL2) display differences in the behavioural phenotype observed.

The hypothesis that the physical substrate of memory in the brain resides in alterations of synaptic efficacy has been proposed frequently (Bliss and Collingridge, 1993; Bliss et al., 2007). The most common long term alteration in synaptic efficacy applied to study memory is LTP. LTP is an activity-dependent strengthening of synaptic connections induced by calcium influx through NMDA receptors leading to insertion of AMPA receptors (Andrasfalvy and Magee, 2004; Ehrlich and Malinow, 2004). LTP has also been reported to be accompanied by alterations in spine morphology (Marrone and Petit, 2002; Bourne and Harris, 2007).

Interestingly, TgNL1 mice show a shift toward spines with a more mature appearance, which is paralleled by impairment in LTP induction and learning, but not memory deficits. This supports
the idea that larger spines are less capable of adaptation in response to synaptic activity, and that maintaining a population of more flexible, thin spines, which can form, disappear or reshape more rapidly (Zuo et al., 2005; Holtmaat et al., 2006), may be essential for plasticity and learning (Kasai et al., 2003; Bourne and Harris, 2007). In addition, spine morphology can also regulate calcium handling (Rusakov et al., 1996; Majewska et al., 2000a; Holthoff and Tsay, 2002; Noguchi et al., 2005), with spines with longer or narrower necks retaining more calcium in their heads following activation. Furthermore, it has also been shown that protein diffusion is regulated by spine morphology (Bloodgood and Sabatini, 2005). In particular, proteins such as inositol 1,4,5-triphosphate (Santamaria et al., 2006) and PSD-95 (Gray et al., 2006) can be more effectively trapped in spines with narrower or longer necks, further impacting calcium dynamics and/or synaptic efficacy. Therefore, it is possible that the larger spines with shorter necks observed in the hippocampus of TgNL1 mice may demonstrate aberrant calcium handling and thus impairments in signalling cascades that follow synaptic activation.

Under normal conditions, the volume of the spine-head is directly proportional to the number of postsynaptic receptors (Nusser et al., 1998; Matsuzaki et al., 2001) and the number of docked vesicles in the presynaptic bouton (Schikorski and Stevens, 1999). While Golgi and EM analysis showed an increase in the head size of spines/synapses in TgNL1 mice, EM studies revealed no change in the number of docked vesicles or the size of the PSD. This may suggest that the morphologically mature-appearing spines in TgNL1 mice are in fact lacking corresponding alterations in functional components, resulting in a discrepancy between morphological properties and functional capabilities of asymmetric synapses. This possibility would also indicate that NL1 is not sufficient to induce the formation of fully functional synapses in vivo, but instead might influence the composition and/or numbers of AMPA
receptors as indicated by WB analysis. This suggests that the coordinated action of multiple proteins or pathways is required to build fully functional synapses. Taken together, our data suggests that neither excessively thin nor excessively large spines are adaptive, and that spine morphology and functional signaling machinery must be appropriately balanced for plasticity and learning.

In conclusion, we have detailed synapse and behavioural abnormalities in mice with altered expression of NL1 that differ from those observed with mice expressing NL2 (Hines et al., 2008). These data demonstrate the overlapping, yet distinct roles for specific NL family members, and further sheds light on the importance of the E/I ratio for brain function and behaviour. Our new findings demonstrate the influence of NL1 expression in vivo on synapse morphology, LTP, and acquisition of spatial learning in the water maze. Further, these findings draw a link between aberrant synapse morphology, impaired synaptic plasticity and deficits in learning.
3.5. Bibliography


4. CONCLUDING CHAPTER

4.1. Summary of Findings

Previous studies have identified multiple mutations in NLs and related proteins in populations with autism (Mariner et al., 1986; Konstantareas and Homatidis, 1999; Risch et al., 1999; Thomas et al., 1999; Auranen et al., 2002; Auranen et al., 2003; Jamain et al., 2003b; Laumonnier et al., 2004; Yan et al., 2004; Sebat et al., 2007; Szatmari et al., 2007; Kim et al., 2008; Marshall et al., 2008; Yan et al., 2008a; Yan et al., 2008b). Further it has been suggested that autism, amongst other CNS disorders, is caused by deregulation of the E/I ratio (Rubenstein and Merzenich, 2003). At the intersection of these two ideas lie the findings suggesting that NL proteins are key regulators of synapse maturation and the excitatory to inhibitory synaptic ratio (Prange et al., 2004b; Chih et al., 2005; Cline, 2005; Levinson et al., 2005a; Levinson and El-Husseini, 2005b, a; Chubykin et al., 2007; Craig and Kang, 2007).

Based on this background research, the objective of the current thesis work was to determine:

(1) The influence that expression of NL1 and NL2 in vivo would have on the expression of binding partners and related synaptic proteins.

(2) The influence that expression of NL1 or NL2 in vivo would have on excitatory and inhibitory synapse number and/or structure and in turn, the excitatory to inhibitory ratio.

(3) The result of in vivo alterations to NL1 and NL2 expression levels on mouse behaviour. In particular, the role of NL induced alteration of the excitatory to inhibitory ratio in CNS dysfunction, focusing on autism relevance, and development of potential animal models.
We demonstrate that increased expression of NL1 or NL2 can alter the expression of related synaptic proteins, shift the E/I ratio, thereby influencing the function of the CNS and exerting effects on behaviour.

4.2. The Role of Neuroligins in Synapse Formation

A large collection of studies has lead to the realization that it is unlikely that a single molecule or family of molecules is sufficient for the assembly and maturation of synaptic contacts. In particular, it has been shown that several different families of adhesion molecules and scaffolding proteins cooperate in the stages of synapse formation (Brose, 1999; Zhang and Benson, 2000; Garner et al., 2002; Goda, 2002; Togashi et al., 2002; Kim and Sheng, 2004; Gerrow and El-Husseini, 2006; Varoqueaux et al., 2006; Kang et al., 2008). Furthermore, despite some overlap in function, each group of molecules possesses unique structural features, and spatiotemporal separation, which helps in defining their most appropriate tasks.

The diverse connectivity of NL and Nrx adhesion proteins make them good candidates for central organizing molecules with the task of stabilizing protein networks in pre and post synaptic compartments, and across the synaptic cleft (Ichtchenko et al., 1996; Irie et al., 1997; Bolliger et al., 2001; Graf et al., 2004; Meyer et al., 2004; Boucard et al., 2005; Levinson et al., 2005a). Through NL interaction with scaffolding proteins such as PSD-95, the NL-Nrx complex is indirectly linked to neurotransmitter receptors and signalling proteins in the postsynaptic compartment (Irie et al., 1997; Bolliger et al., 2001; Meyer et al., 2004; Levinson et al., 2005a). In the presynaptic compartment, Nrx binds directly to synaptotagmin, as well as the presynaptic scaffold CASK (Zhang et al., 2001), which serves to link the NL-Nrx complex to
Calcium channels and proteins key for synaptic vesicle exocytosis (O'Connor et al., 1993; Perin, 1994; Butz et al., 1998). Thus, in addition to the physical link between pre and post, the direct and indirect interactions of NL-Nrx serve to functionally link all of the key components required for calcium activated vesicle fusion, neurotransmitter release, receptor activation and downstream signalling. In agreement with this, our results demonstrate changes in the expression of several related synaptic proteins as a result of enhanced NL expression. In TgNL2 mice a significant increase was observed in the expression of syntaxin, while a significant decrease was observed in NL3 expression. Interestingly, TgNL1 mice showed differing alterations in related protein expression levels, with significant increases in both NL3 and PSD-95. In addition, alterations were observed in synapse morphology and size, including increases in the size of synaptic vesicle reserve pools, indicating that expression of NLs can influence aspects of synapse formation such as protein recruitment and maturation, with changes reaching the presynaptic compartment.

4.3. The Role of Neuroligins in the Excitatory to Inhibitory Ratio

In addition to their possible role as central organizers, the specific enrichment of different NL-Nrx family members to excitatory versus inhibitory synaptic contacts makes them candidates for the task of specification of excitatory versus inhibitory synaptic protein recruitment. Since it is well established that a carefully established E/I ratio is critical for normal brain function (Berretta et al., 2001; Cline, 2005; Levinson and El-Husseini, 2005b, a; Schutz, 2005; Kumar and Buckmaster, 2006; Yang et al., 2007; Avramescu and Timofeev, 2008; Medrihan et al., 2008;
Belichenko et al., 2009), a role for NLs in determination of synapse fate and the E/I ratio is of great interest.

An example of the importance of E/I regulation can be found in the highly recurrent cortical networks, which are based on extensive positive feedback connections between excitatory pyramidal neurons, which in turn are modulated by complex networks of interneurons exerting feedback and feedforward inhibition (Figure 4.1). A consequence of this delicately balanced cortical network is that even small changes in receptor distribution, quantal amplitude, and synapse density can result in runaway excitation (Chagnac-Amitai and Connors, 1989a, b) or impaired plasticity (Hensch et al., 1998; Huang et al., 1999; Hensch and Stryker, 2004). Studies in cultured cortical neurons have demonstrated that under conditions of activity blockade, excitation between pyramidal neurons is increased while feedback inhibition is dampened, resulting in increased firing rates of pyramidal neurons (Ramakers et al., 1990; Rutherford et al., 1997; Turrigiano et al., 1998). In contrast, when activity is too high, excitation between pyramidal cells is reduced, while excitation onto interneurons and inhibitory inputs onto pyramidal cells are increased, providing an increase in feedback inhibition (Turrigiano et al., 1998). All of these modifications lead to a reduction in the activity of pyramidal neurons. This example illustrates how coordinated changes are made in networks to regulate activity levels toward a hypothetical “set point” of glutamatergic/GABAergic signalling, or maintain a functional E/I ratio. It is important to note that consideration of the E/I ratio in terms of glutamate and GABA is a simplified construct that does not take into account mixed excitatory/inhibitory signals such as serotonin, norepinephrine, dopamine and acetylcholine, or extra neuronal influences on excitatory or inhibitory signalling such as glial cells.
Figure 4.1. Homeostatic regulation of the excitation–inhibition balance in cortical networks. The recurrent activity of cortical networks is strongly affected by feedback excitation and inhibition. Pyramidal neurons (blue) make excitatory outputs (triangles) onto other pyramidal neurons, and also onto inhibitory interneurons (red). These inhibitory neurons in turn feed inhibition (red circles) back onto the pyramidal neurons. In cortical cultures, raising activity for two days produces a coordinated set of changes in synaptic strength that result in reduced feedback excitation and increased feedback inhibition onto pyramidal neurons (lower left). Conversely, blocking activity for two days increases the gain of excitatory feedback and decreases inhibitory feedback. Similar changes in the cortical excitation–inhibition balance are induced by sensory deprivation. Turrigiano and Nelson. 2004. Homeostatic plasticity in the developing nervous system. Nature Reviews Neuroscience 5(2):97-107.
Multiple studies have suggested that NL proteins are key regulators of the both glutamatergic and GABAergic synapses and the E/I ratio (Prange et al., 2004b; Chih et al., 2005; Cline, 2005; Levinson et al., 2005a; Levinson and El-Husseini, 2005b, a; Chubykin et al., 2007; Craig and Kang, 2007). In the present thesis we observed predominant increases in the expression of the excitatory synaptic proteins PSD-95 and VGlut with transgenic expression of NL1, whereas transgenic expression of NL2 resulted predominantly in increases in the inhibitory synaptic protein VGAT. We also observed increases in the number and size of excitatory synapses in mice expressing NL1, whereas mice expressing NL2 showed increases in the size and number of inhibitory synapses. In support of these molecular and structural findings, electrophysiology studies demonstrated functional changes in excitation and inhibition in mice expressing NLs. These results demonstrate that, in spite of extensive homeostatic mechanisms (Turrigiano and Nelson, 2004), expression of NLs in vivo can influence synapse maturation and excitatory / inhibitory fate determination.

4.3.1. How Could Neuroligins Influence the Excitatory to Inhibitory Ratio?

It is easy to conceive of multiple ways that NLs could influence the homeostatic set point and disturb the E/I ratio. Firstly, NLs could alter the clustering or surface distribution of neurotransmitter receptors via NL interaction with synaptic scaffolding proteins. Studies on cultured neurons have shown that NL1 expression can result in enhanced clustering of glutamate receptors (Fu et al., 2003; Chih et al., 2005; Nam and Chen, 2005; Chubykin et al., 2007), while expression of NL2 can result in enhanced GABA receptor accumulation (Graf et al., 2004; Kang et al., 2008), thus it is conceivable that in vivo expression could result in similar
accumulation of receptors at synapses expressing increased NL. Our studies demonstrated an increase in the excitatory synaptic scaffold PSD-95, as well as the GluR1 subunit of AMPARs in mice expressing NL1. However, in contrast, a decrease was found in GluR2/3 expression levels in TgNL1 mice, suggesting a shift in AMPAR subunit composition and function (Hollmann et al., 1991). Examination of related proteins in TgNL2 mice did not reveal an increase in the expression of the inhibitory scaffold gephyrin, but showed predominant effects on presynaptic proteins. This data suggests that NLs may not be capable of inducing recruitment of all synaptic proteins. Further studies are required to examine the full complement of proteins at excitatory and inhibitory synapses to determine the extent of NL1 or 2 effects.

A second possibility is that NL could influence neurotransmitter release and thereby influence the function of the specific synapses at which they are expressed. Studies on cultured neurons have shown that NLs, via interaction with Nrx, can induce presynaptic vesicle accumulation (Scheiffele et al., 2000; Dean et al., 2003). Our studies provide further support for these findings since expression of NLs in vivo increased the number of synaptic vesicles in the reserve pool. Both scaffolding protein and receptor accumulation, as well as alterations in transmitter release are consistent with NLs ability to influence recruitment and maturation of synapses.

In addition to altering functional components of synapses, specific NLs may differentially alter the number of either excitatory or inhibitory synaptic contacts, and thereby influence the E/I ratio. Since NLs can influence the maturation and stabilization of synaptic contacts, it is possible that increasing NL expression would increase the number of synapses by preventing their elimination. Indeed we did observe a specific increase in the number of excitatory synaptic contacts in mice expressing NL1, while an increase in inhibitory synaptic contacts was
observed in mice expressing NL2. Related to this possible influence on synapse stability and elimination, NLs may also exert effects on the E/I ratio in our model by virtue of the fact that transgenes under the Thy1 promoter are not expressed uniformly in all cells (Feng et al., 2000). Enrichment of NL in specific cells and their synapses may alter synapse competition, and block the elimination phase of synapse formation resulting in a bias of network connectivity to cells with increased NL. Preliminary results from our lab have shown that expression of NLs in culture can increase the stability of dendritic filopodia, considered to be a precursor of synapses, suggesting that NL containing synapses may be more likely to be stabilized into maturity. Relating to this idea of competitive stabilization or elimination, it is interesting to note that synapses assessed in mice expressing NL1 show an inverted curve of synaptic contact size distribution (Figure 4.2). The disproportionate number of very small and very large synapses in these mice could support the idea that NL over-expressing sites are capable of out competing those with normal expression levels. To confirm this phenomena it will be important to assess if larger synapses are predominantly HA-NL1 positive.

It is possible that NLs may influence receptor distribution, vesicle release, and synapse density in parallel, and further analysis is required to determine which specific alterations have meaningful effects on the physiology and behaviour of mice expressing NLs. It is also important to acknowledge in this section that NLs must act in concert with several other molecules to effect these changes in synaptic network connectivity. In order for the E/I ratio to be altered by changes in synapse density, the new synapses must be appropriately equipped, requiring the cooperative action of receptors, synaptic vesicle proteins, and effector proteins, all with binding partners important for the proper localization and connectivity required for signalling. The orchestration of molecules and processes required for the formation of synapses, and
Figure 4.2. Frequency distribution of synapse size in mice expressing neuroligin 1 compared to controls. Note that the normal distribution in wildtype mice (light grey) follows a bell shape curve, whereas the distribution in TgNL1 mice (dark grey) shows an inverted shape.
requirement of exponentially more complex synaptic networks, sheds light on disorders that result from synaptic alterations.

### 4.4. Synapse Abnormalities in Autism, and the Role of Neuroligins & Related Synaptic Proteins

Alteration in synapse structure or function are predicted to be the underlying cause of neurodevelopmental disorders, yet no unifying pathology or etiology for this complex set of disorders has been defined. The idea that disorders such as autism, Fragile X syndrome, and Rett syndrome are caused by alterations in synaptic connectivity is based on several pieces of information. Firstly, the onset of these disorders, which typically manifests as a halt or regression in development after a period of normal development, occurs at a time point when the vast majority of neurogenesis, neuronal migration, and maturation have already occurred (Zoghbi, 2003; Belmonte et al., 2004). The case for neurodevelopmental disorders as synapse pathologies also stems from findings in post-mortem tissue from individuals with autism, Fragile X or Rett syndrome (Zoghbi, 2003; Belmonte et al., 2004). The synaptic pathology of Fragile X syndrome has perhaps been best defined, with observations of abnormally long, thin, and tortuous dendritic spines being observed in both human post-mortem tissue and the Fragile X knock-out mice (Beckel-Mitchener and Greenough, 2004). Similar results have been obtained from post-mortem samples from a subset of autistic individuals with low IQ (Pickett and London, 2005). Reductions in the dendritic arbour of select cell populations in post-mortem tissue from patients with autism (Raymond et al., 1996) and Rett syndrome (Armstrong et al., 1995) have also been observed. Recent support for synapse alterations in
the pathology of neurodevelopmental disorders has come from a number of genetic studies indicating a link between autism and alterations in synaptic proteins.

Rett syndrome, Fragile X, and autism are all thought of as having a genetic basis, yet display varying degrees of genetic complexity. Both Rett and Fragile X have been linked to alterations in single genes, MECP2 (Amir et al., 1999; Shahbazian et al., 2002) and FMR1 (Pieretti et al., 1991) respectively; however, these genes encode proteins that regulate the expression of several downstream genes ranging in function. The task of determining specific genetic changes that increase the risk of autism is exceptionally more complex due to heterogeneity of underlying genes, as well as in the variations effecting these genes (Abrahams and Geschwind, 2008). Autism has been linked to highly penetrant mutations in rare cases, discrete changes in single genes, chromosomal translocations and inversions, large deletions and duplications, as well as small losses and gains referred to as copy number variations (Sutcliffe, 2008).

Further complication has been added since some individual families with autism have been strongly linked to single gene alterations, which have been inconsistently replicated in association studies from larger populations. The case of NL mutations is an example of this scenario, where mutations in NL3 and NL4 have been identified in specific pedigrees displaying a variable phenotype, including autism, mental retardation and Asperger syndromes (Jamain et al., 2003b; Laumonnier et al., 2004), and have only been identified sporadically in association studies, which is not substantial enough to yield significance (Gauthier et al., 2004; Vincent et al., 2004; Ylisaukko-oja et al., 2005b). However, the synaptic protein link has been bolstered by the identification of several related proteins in cases of autism, including the NL binding proteins Nrx1 (Kim et al., 2008; Marshall et al., 2008; Yan et al., 2008a; Yan et al., 2008b) and
PSD-95 (Risch et al., 1999), the postsynaptic scaffold proteins Shank3 (Durand et al., 2007; Moessner et al., 2007; Gauthier et al., 2008; Marshall et al., 2008) and GABARAP (Risch et al., 1999), the GABA receptor subunit GABRB3 (Cook et al., 1998; Buxbaum et al., 2002; Samaco et al., 2005), and the Nrx superfamily member CNTNAP2 (Abrahams et al., 2007; Alarcon et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008).

Additional support for the link between synaptic proteins and neurodevelopmental disorders can be drawn from contemplation of the downstream genes regulated by MeCP2 and FMRP affected in Rett syndrome and Fragile X respectively. In particular, it has been shown that FMRP can control the translation of the postsynaptic scaffold proteins PSD-95 (Todd et al., 2003; Zalfa et al., 2007) and GKAP, the presynaptic active zone proteins MUNC13 and Mint, the axon guidance cue semaphorin 3F, and multiple kinases (Kaytor and Orr, 2001), and the immediate early gene Arc (Park et al., 2008). Specific targets regulated by the transcriptional repressor MeCP2 have been harder to identify, and the major effects of loss of MeCP2 function remain largely unclear (Colantuoni et al., 2001; Traynor et al., 2002; Tudor et al., 2002; Renieri et al., 2003). Identified candidates for MeCP2 regulation include BDNF (Chen et al., 2003b), the GABA receptor subunit GABRB3, and the ubiquitin ligaseUBE3A (Samaco et al., 2005).

The synaptic role of several of the genes altered in neurodevelopmental disorders is strongly suggestive of a common pathology of altered synapse formation and synaptic connectivity. Further, many of the genes identified have specific roles at either excitatory, inhibitory, or both types of synapses such as members of the NL/Nrx families, PSD-95, BDNF, and GABA receptor subunits and their interacting proteins. Actions on both excitatory and inhibitory synapses may seem contradictory upon first examination, but in fact, all of the genes identified could be seen
as participants in establishing the homostatic set point of neural connectivity and the E/I ratio. Using examples of extremes, Belmonte and Bourgeron (2006) describe that either a completely connected or completely unconnected network could have the same net effect on signal detection. In the case of over-connectivity, the system contains so much noise that the signal is masked, whereas in a non-connected network, the signal can not be propagated to overcome the noise, demonstrating that only in the optimal range of connectivity can network activity be optimized for proper signal detection (Belmonte et al., 2004; Belmonte and Bourgeron, 2006). The same extremes apply to a completely excitatory or a completely inhibitory network.

Furthermore, the variety of genes implicated in autism, and the possible variation these genes would cause in the function of different networks or systems, may explain the range of dysfunction observed in patients (Figure 4.3). For example, genetic alterations that cause over-connection or disconnection of neuronal networks may result in a syndrome characterized by cognitive impairment or mental retardation (Belmonte et al., 2004; Belmonte and Bourgeron, 2006). In other populations, different genetic alterations may cause a more specific bias toward over-excited or over-inhibited circuits and result in a syndrome with accessory symptoms of anxiety or seizure disorder (Belmonte and Bourgeron, 2006). The results from our studies of mice expressing NLs support a role for synaptic dysfunction in the pathology of neurodevelopmental disorders, since TgNL2 mice recapitulated multiple aspects of these disorders. Further, we observed that mice expressing NL1 and NL2 show contrasting alterations in the E/I ratio, and result in different impairments centered on mental retardation and autism-like features respectively.
Figure 4.3. Modifications of neuronal networks that increase susceptibility to autism. 1. Alterations in the number and/or morphology of synapses may lead to a too weakly or too strongly connected network. Such abnormal synaptic connectivity is frequently observed in mental retardation, which is present in 75% of individuals with autism. 2. A shift in the E/I ratio may lead to an abnormal network signalling associated with epilepsy, an accessory symptom of autism occurring in 30% of individuals with autism. Adapted from Belmonte and Bourgeron. 2006. Fragile X syndrome and autism at the intersection of genetic and neural networks. Nature Neuroscience 9(10):1221-5.
Neuronal Network

Rett
MeCP2

Fragile X
FMRF

BDNF
GABARB3
PSD-95
mGluR
CaMKIIα
Arc

NL1-4
Nrx1
PSD-95
Shank3
GABARB3
GABARAP
CNTNAP2

1. Abnormalities in the shape and/or number of synapses
2. Altered ratio of excitation to inhibition

Low Network Connectivity

High Network Inhibition

Mental Retardation
Anxiety or Epilepsy

Autism Spectrum Disorders
Important information on neurodevelopmental disorders could be gained from a concerted effort to conduct large scale assessments of brain protein expression on post-mortem human tissue that has been carefully categorized according to specific symptoms, such as mental retardation or seizure susceptibility. In addition, it will be beneficial to assess brain protein expression in available transgenic mouse models. These strategies will highlight commonalities and differences between populations, and contribute to the development of a “neurodevelopmental proteome” used to understand the pathology of these syndromes.

4.5. The Role of the Excitatory to Inhibitory Ratio in Autism & Other CNS Disorders

Alterations in the E/I ratio have been hypothesised to underlie neurodevelopmental disorders (Rubenstein and Merzenich, 2003; Cline, 2005; Rippon et al., 2007). The key paper building this hypothesis proposes that autism is likely to result from disproportionately high levels of excitation in systems that mediate language and social behaviours (Rubenstein and Merzenich, 2003). They further conjecture that more excitable cortex is functionally undifferentiated, leading to perceptual, cognitive, and motor abnormalities. In support of their proposal, it is also noted that a high proportion of individuals with autism have seizures (30%; (Gillberg and Billstedt, 2000)) and/or sharp spike activity during sleep (50-70%; (Lewine et al., 1999; Wheless et al., 2002; Orekhova et al., 2007), which is thought to reflect a noisy or hyperexcitable cortex. Further, a substantial body of work has linked Fragile X to excessive metabotropic glutamate receptor signalling, specifically mGluR5 (Antar et al., 2004; Bear et al., 2004; Aschrafi et al.,
2005; Yan et al., 2005b; Nakamoto et al., 2007; Dolen and Bear, 2008), also linking neurodevelopmental disorders to increased excitation.

Despite the indications of increased excitation, some models characterized to this point have show increases in inhibition, as opposed to the increased excitation or decreased inhibition proposed. For example, mouse models of Rett syndrome (Dani et al., 2005), and mice expressing an autism-related mutation of NL3 (NL3R451C; (Tabuchi et al., 2007)) both show increases in inhibition. In support of this, our studies demonstrate that mice expressing NL2 show a shift toward increased inhibition in the prefrontal cortex. In light of increased prefrontal cortex inhibition, is particularly interesting that TgNL2 mice show frontoparietal seizure-like spiking since seizure activity is most commonly associated with hypersynchronous neuronal activity and increased E/I ratios (Kofke et al., 1997; Stief et al., 2007). Recent reports have also shown that focal seizure activity can result from desynchronization of firing caused by increased inhibitory feedback (Netoff and Schiff, 2002; Mormann et al., 2003b; Mormann et al., 2003a; Klaassen et al., 2006).

Although several models have shown increased inhibition, the Fmr1 knock-out model of Fragile X syndrome shows downregulation of tonic GABA currents in the subiculum (Curia et al., 2008), and impairment in local feedback inhibition in cortex, leading to hyperexcitability and decreased synchrony (Gibson et al., 2008). In parallel with this model, we have demonstrated that mice expressing NL1 show increased excitation in hippocampal circuits, accompanied by impairments in the induction of long term synaptic plasticity.

Contrasting findings in the developed mouse models for neurodevelopmental disorders may reflect that specific networks or systems may show increased excitation, while others show
increased inhibition. Alternatively, these contrasting findings may reflect differences in the underlying synaptic abnormalities and resulting behavioural profile of the different mouse strains, and agrees with the ideas that Belmonte and Bourgeron (2006) propose. Specifically, both TgNL1 and Fmr1 knock-out mouse models show impairments in water maze training (D'Hooge et al., 1997; Dobkin et al., 2000; Van Dam et al., 2000), thought to mirror mental retardation, in combination with other deficits. TgNL2 mice, NL3R451C mutant mice (Tabuchi et al., 2007), and MeCP2 knock-out mice (Kerr et al., 2008) all display impairments in social behaviour. To test this possibility, these mouse models will have to be compared systematically using the same behavioural and physiological measures, since in many cases the assessments used can vary vastly from one laboratory to another.

4.6. Possible Therapeutic Strategies for Autism

In order to begin assessment of therapeutic interventions for neurodevelopmental disorders, animal models with a high degree of similarity to multiple aspects of autism are required, which is also referred to as validity (Robbins and Sahakian, 1979). A number of genetic models have been developed and many recapitulate certain aspects of autism (Table 4.1). While the recently generated models resemble aspects of autistic disorders, most models either show only one or two relevant traits, or have not yet been systematically subjected to rigorous testing (Andres, 2002; Crawley, 2007; Tordjman et al., 2007; Chadman et al., 2008). The field of study focusing on autism relevant behaviours is wrought with further difficulty, since universal tests and protocols have not been applied; making results from different labs difficult to interpret. The ability to define a mouse model with high validity is also influenced by the
limitations in assessing autism-relevant features due to their complex cognitive nature (Andres, 2002; Tordjman et al., 2007), these points underscore the importance of developing and thoroughly testing multi-trait models (Chadman et al., 2008). The mouse model we generated using increased expression of NL2 recapitulates multiple core features of autism, as well as accessory symptoms. Because of the high validity of this model, it may be possible to evaluate therapeutic interventions for autism using the TgNL2 strain.

Table 4.1. Proposed genetic mouse models relevant to autism, and their performance on behavioural assessments relevant to autism core and accessory symptoms. Cells shaded in light grey highlight autism relevant findings.

<table>
<thead>
<tr>
<th>Model</th>
<th>Reference</th>
<th>Stereotypies</th>
<th>Social Impairments</th>
<th>Communication Deficits</th>
<th>Other Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgNL1</td>
<td>Dahlhaus et al.</td>
<td>Not noted/ tested</td>
<td>Not noted/ tested</td>
<td>Not noted/ tested</td>
<td>Cognitive deficits</td>
</tr>
<tr>
<td>TgNL2</td>
<td>Hines et al., 2008</td>
<td>Spontaneous jumping</td>
<td>Approach, Reciprocal interaction</td>
<td>Not noted/ tested</td>
<td>Anxiety, Seizure-like spiking</td>
</tr>
<tr>
<td>NL3R451C</td>
<td>1. Tabuchi et al., 2007; 2. Chadman et al., 2008</td>
<td>Not noted/ tested</td>
<td>1. Reciprocal interaction 2. Not impaired</td>
<td>Not noted/ tested</td>
<td>1. Enhanced spatial learning 2. Reduced sensitivity to stim</td>
</tr>
<tr>
<td>NL4 KO</td>
<td>Jamain et al., 2008</td>
<td>Not noted/ tested</td>
<td>Reciprocal interaction</td>
<td>Ultrasonic vocalizations</td>
<td>Not noted/ tested</td>
</tr>
<tr>
<td>MeCP2^{lox/y}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.Fmr1KO 2.Fxr2KO 3.DoubKO</td>
<td>Spencer et al., 1. 2005, 2. 2006</td>
<td>Not noted/ tested</td>
<td>Reduced dominance, Suggested social anxiety</td>
<td>Not noted/ tested</td>
<td>Hyperactivity, Acoustic startle, Cognitive deficits</td>
</tr>
<tr>
<td>BTBR T+tf/J (inbred strain)</td>
<td>McFarlane et al., 2008</td>
<td>High self-grooming</td>
<td>Approach, Reciprocal interaction, Juvenile play</td>
<td>Social transmission food preference</td>
<td>None noted</td>
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237
Specific therapies that may be explored in the treatment of autism and other neurodevelopmental disorders may target the E/I ratio. If a case is supported for increased excitation, then pharmacological agents that reduce neuronal activity, such as benzodiazepines and anticonvulsants, may be of use in autism treatment (Rubenstein and Merzenich, 2003). In support of this, studies have shown improvement in some aspects of autism upon treatment with anticonvulsants (Belsito et al., 2001; Di Martino and Tuchman, 2001; Rugino and Samsock, 2002). Benzodiazepines have shown promise in a small number of studies (Malek-Ahmadi and Simonds, 1998; Fido and Al-Saad, 2008), however the possible adverse effects of this treatment have limited its testing (Rubenstein and Merzenich, 2003). In contrast, if a case is supported for increased inhibition, pharmacological agents that increase neuronal activity may be useful in the treatment of autism. Beneficial effects have been observed in a pilot study using treatment of autistic subjects with flumazenil, a competitive inhibitor at the benzodiazepine site of GABA<sub>A</sub> receptors (Wray et al., 2000). However, since it has not been clearly established that autism results from exclusive over-excitation or over-inhibition, these approaches may lack in specificity.

Increased specificity may be achieved using gene or peptide strategies, where therapies can be developed to target the genes or proteins indicated in neurodevelopmental disorders. An example of this type of strategy is in early investigation in Rett syndrome models treated with expression of BDNF. One group has shown that conditional BDNF overexpression can extend the lifespan, improve locomotor defects, and reverse electrophysiological abnormalities in MeCP2 mutant mice (Chang et al., 2006). In addition, other groups have shown that BDNF expression can rescue the dendritic changes observed with loss of MeCP2 function (Jugloff et
al., 2005; Larimore et al., 2009). Similarly, a recent paper has shown that treatment of MeCP2 mutant mice with an active peptide fragment of Insulin-like GroWth Factor 1 extends life span, improves locomotor function, reduces irregularity in heart rate and breathing patterns, partially restores synapse and spine defects, and stabilizes cortical plasticity (Tropea et al., 2009). Preliminary experiments in our lab have begun investigating the efficacy of cell permeable peptides that disrupt NL binding to its interacting partners as a means of modulating NL function and the E/I ratio. Preliminary assessment in heterologous cells and cultured neurons suggest that these peptides are indeed capable of interfering with NL interactions, and work is ongoing to assess the use of these peptides in TgNL mice. Strategies such as these with high specificity may prove to be beneficial in cases of autism.

### 4.7. Limitations of the Present Findings

One potential pitfall of the current work relates to the overexpression of NL1 and NL2 transgenes. With any case of overexpression there is risk of inducing an artificial state where the expressed proteins become mislocalized. We were able to determine that expressed NL1 and NL2 appropriately targeted to excitatory versus inhibitory synapses using co-staining with the postsynaptic markers PSD-95 and gephyrin. However, we are unable to determine if these are also sites enriched with endogenous NL2, since the NL2 antibody recognises both transgene and endogenous.

In addition, no study has detected increased expression of either NL1 or NL2 in diseased brain tissue. In particular, most mutations in NLs associated with autism are thought to be caused by a loss or reduction in functioning of these proteins. However, in this regard, a gain of NL2
function may result in a shift toward increased inhibition, mimicking the reduced function of the mutant forms of other NLs which act predominantly at excitatory synapses and are most commonly associated with autism. It is likely that for many genes, the “dose-response curve” would resemble that typical of pharmacological agents, following a normal distribution, where either too high or too low of a dose would result in disrupted function (Belmonte and Bourgeron, 2006). This gene dose-response effect has been observed in models of Rett syndrome, where both reduced and enhanced expression of affected genes may recapitulate pathological aspects of the disease (Gemelli et al., 2006; Collins et al., 2004). In further support of the idea that both loss and gain of function of the same gene can lead to similar pathology, a recent paper by Blundell et al., (2009) showed that knock-out of NL2 in mice also results in anxiety-like behaviour, similar to that observed with increased expression of NL2 in the TgNL2 mice.

4.8. Final Conclusions and Significance

The data from this thesis demonstrates that altered expression of members of the NL family in vivo leads to altered synapse number and morphology, which potentially underlies the profound behavioural changes we observe in these mice. Specifically, NL2 expression induces a predominant effect on inhibitory synapses, while NL1 primarily influences excitatory synapses, supporting the idea that NLs act to regulate the E/I ratio. In addition this data may provide insight into the pathology and behavioural profile of human neurodevelopmental disorders such as autism, which have been linked to alterations in NL amongst other synaptic proteins, and are thought to be caused by synaptic dysfunction. These studies have also provided an
important animal model system which will be invaluable in the assessment of potential therapies for autism and related disorders.
4.9. Bibliography


5. APPENDIX A: GENERATION AND CHARACTERIZATION OF TRANSGENIC MOUSE STRAINS

5.1. Preparation of DNA Fragments for Pronuclear Injection

Thy1.2 plasmids (Caroni, 1997; Feng et al., 2000) containing NL1 (1AB splice variant) and NL2 (Chih et al., 2006) were prepared using sequential digestion in Pmel and PvuI (NEB biolabs) to remove the majority of the bacterial backbone. Following digestion, samples were run on low melting point agarose gels, and the 10KDa band was excised for gel purification (Qiagen). Samples were then dialyzed into low EDTA injection buffer overnight, with a change of buffer after 3 hours. A small aliquot of each cut and purified sample was then run on an agarose gel with standard samples to quantify DNA concentration. Before the constructs were sent for pronuclear injection at the Centre for Molecular Medicine Transgenic Core Facility, they were transfected into cultured neurons to verify their expression (Figure 5.1).

5.2. Genotyping and Screening Founders

Several sets of primers were designed (Table 5.1) and tested (Table 5.2) for optimal detection of the transgenes using PCR on genomic DNA isolated from tail samples. The primers that gave the most robust and consistent PCR amplification from the genomic DNA spanned a region from within the Thy1.2 promoter region into the NL signal sequence, and as such were capable of recognising both the NL1 and NL2 transgenes. In addition, a consistent set of primers specifically for detection of NL1 was also used to ensure that we could differentiate TgNL1 DNA from TgNL2 DNA (Figure 5.2). A typical genotyping gel is shown in Figure 5.3.
Figure 5.1. Expression of neuroligin 1 HA Thy1.2 and neuroligin 2 HA Thy1.2 in cultured hippocampal neurons. Antibodies specific for the hemaglutinin (HA) epitope tag were used to detect the NL1 or NL2 (both in green). Transfected hippocampal neurons were costained with the excitatory synaptic marker VGlut (blue) and the inhibitory synaptic marker VGAT (red).
Table 5.1. Sequences of primers designed to detect neuroligin 1 and neuroligin 2 transgenes.

**Primers for Genotyping TgNL Mice:**

1. Thy1-5' for: TAGGCTCCCACTTCTTGCC
2. NLG1/2-5' rev: GGACCCACATGCCCATAGC
3. NLG1-3' for: CATACATTCAACACATTACTGG
4. Thy1-3' rev: GGATGATGGCATGCAGCACTG
5. NLG2-3' for: CCTGACCCTGCTGCCACGTGG
6. NLG1-5': CATCTCCTGGTCTGACATC
   rev (to order): GATGTCAGACCAGGGAGATG
7. NLG1-5': CATTGATGGCAGATTGCCTGAAG
   rev (to order): CTTCAGGCGCGTGATAGAAG
8. NLG2-5': CGACGCTCAATCCGCCAGACA
   rev (to order): TGTCTGGCGGATTGAGCGTCG
9. NLG1 exon for: CCAAGACCAGAGTGAAGACTGTC
10. NLG1 exon rev: CTTCTTGAGCACAGGGGATCAGG
    rev (to order): CCTGATCCCCTGTGCTCAAGAAG
11. NLG1 intron rev: GGAACCTGTGTTCTCGTATGGG
    rev (to order): CCCATA CGAGAACACAGGTTCC
12. NLG1 intron for: GCAGTCTCTAGATGGTCCATAC
13. NLG2 intron span for: GAACATGTTCGACGGCTCAG
14. NLG2 intron span rev: CCTGTGTCAACTTGCTGATCC
    rev (to order): GGATCAGCAAGTTGACACAGG
15. NLG1/2-5': GCTTGTTGGGATGTTTGC
    rev (to order): GCAAACATCCCAACAAGC
16. NLG1/2-5': GCTTGTTGGGATGTTTGC

Table 5.2. Details of primer combinations used, the size of PCR product produced and their intended and actual specificity. ns= not specific

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<tr>
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Figure 5.2. Genotyping of mice expressing neuroligin 1 or neuroligin 2 using PCR amplification from genomic DNA. Two primer sets are used to firstly detect mice transgenic for NL (primer set 1+2), and second to differentiate TgNL1 mice from TgNL2 mice (primer set 1+6). The band at 300 bp represents a region of Thy1 spanning into the NL signal sequence, common to both NL1 and NL2 transgenes used, while the band at 590 bp recognises only the NL1 transgene. The lowest, and most faint bands are likely primer-dimer bands as they occur in all samples, including the negative control sample, which contains no DNA.
Figure 5.3. Example of a typical agarose gel run to observe the PCR genotyping results from mouse genomic DNA. Offspring positive for either the NL1 or NL2 transgenes show a higher band on the gel (300bp).
5.3. Catalogue of Founders Generated

From four rounds of injection of cut and purified DNA, 113 potential founders were screened (Table 5.3) using the above described PCR genotyping from DNA extracted from tail cuttings. Of the potential founders screened, seven were determined as positives for the NL2 transgene (termed TgNL2), while eight were positive for the NL1 transgene (termed TgNL1). A brief summary of each founder, including sex, reproductive success and basic observations is listed in Table 5.4. In general, an obvious phenotype was noted in TgNL2 mice, which showed marked reductions in body weight and size (Figure 5.4), as well as pronounced hindlimb clasping when suspended by the tail (Figure 5.5). One TgNL2 strain did not show obvious reduction in body weight/size, and was later determined to have a much lower level of transgene expression compared to other founders. Although a slight reduction in body weight could be noted in TgNL1 mice with weighing, no obvious phenotype was detected. In addition it was quickly noted that female TgNL2 founders were not successful at rearing young, although seemed capable of reproduction since litters were found dead. Consequently, both nanny and foster strategies were attempted. Nannies used were 8-10 week old female 129 mice that had previously raised at least one litter. 129 mice were used so that pups resulting from a nanny mating could be distinguished from founder pups.

Table 5.3. Offspring generated from pronuclear injection of NL1 HA Thy1.2 or NL2 HA Thy1.2 DNA fragments, and screening results based on PCR genotyping. Rows shaded in blue are identified male founders, while those in pink are identified female founders.

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<td>♂️</td>
<td>17/12/2005</td>
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Table 5.4. Summary table of founders generated and used to develop independent strains. The characteristics noted for each strain are also listed. Shaded rows denote the primary strains used in subsequent experiments.

<table>
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<tr>
<th>Founder ID</th>
<th>Sex</th>
<th>Offspring</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgNL2.1</td>
<td>Male</td>
<td>None</td>
<td>• Extreme run &lt;br&gt;• Hindlimb clasping</td>
</tr>
<tr>
<td>TgNL2.2</td>
<td>Male</td>
<td>9 generations</td>
<td>• Subtle reduction in body weight &lt;br&gt;• Low expression level</td>
</tr>
<tr>
<td>TgNL2.3</td>
<td>Female</td>
<td>None</td>
<td>• Runt &lt;br&gt;• Hindlimb clasping &lt;br&gt;• Attempted nanny &lt;br&gt;• Attempted foster</td>
</tr>
<tr>
<td>TgNL2.4</td>
<td>Female</td>
<td>None</td>
<td>• Extreme run &lt;br&gt;• Severe hindlimb clasping &lt;br&gt;Developed anal fistula (sacrificed)</td>
</tr>
<tr>
<td>TgNL2.5</td>
<td>Female</td>
<td>None</td>
<td>• Extreme run &lt;br&gt;• Severe hindlimb clasping &lt;br&gt;• Attempted nanny &lt;br&gt;• Attempted foster</td>
</tr>
<tr>
<td>TgNL2.6</td>
<td>Male</td>
<td>9 generations</td>
<td>• Runt &lt;br&gt;• Hindlimb clasping</td>
</tr>
<tr>
<td>TgNL2.7</td>
<td>Male</td>
<td>3 litters</td>
<td>• Extreme run &lt;br&gt;• Severe hindlimb clasping &lt;br&gt;• Only positive offspring to survive are female, superovulation failed, subsequently females used for ovary transplants (3 generations)</td>
</tr>
<tr>
<td>TgNL1.1</td>
<td>Male</td>
<td>Mating stopped</td>
<td>• No positive offspring</td>
</tr>
<tr>
<td>TgNL1.2</td>
<td>Male</td>
<td>Mating stopped</td>
<td>• Low expression level</td>
</tr>
<tr>
<td>TgNL1.3</td>
<td>Male</td>
<td>Mating stopped</td>
<td>• No positive offspring</td>
</tr>
<tr>
<td>TgNL1.4</td>
<td>Male</td>
<td>Mating stopped</td>
<td>• Only positive offspring are male, all males positive, sex linked insertion of transgene?</td>
</tr>
<tr>
<td>Founder ID</td>
<td>Sex</td>
<td>Offspring</td>
<td>Observations</td>
</tr>
<tr>
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<td>---------</td>
<td>---------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TgNL1.5</td>
<td>Female</td>
<td>Mating stopped</td>
<td>• No positive offspring</td>
</tr>
<tr>
<td>TgNL1.6</td>
<td>Male</td>
<td>6 generations</td>
<td>• Subtle reduction in body weight</td>
</tr>
<tr>
<td>TgNL1.7</td>
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<td>• Subtle reduction in body weight</td>
</tr>
<tr>
<td>TgNL1.8</td>
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</table>

5.4. Preliminary Behaviour Screen

A significant development in the field of mouse genetics came with initiatives to generate sets of standardized protocols by which these important research tools can be evaluated. Standardized protocols will enable the comparison of mouse strains within and between different laboratories, as well as be reliable over time (Rogers et al., 1997). For example, if two independent labs are unable to consistently assess mouse models affecting the same gene, then any similarities or differences between strains developed in independent labs will be difficult to interpret, and consequently affect our understanding of gene function. The requirement for standardized protocols led to the development of the SHIRPA procedure designed to characterize the phenotype of mice using standard methods (Rogers et al., 1997; Nolan et al., 2000b; Nolan et al., 2000a).

The SHIRPA was divided into three stages with the first providing a basic behavioural and functional profile. The secondary screen involved more comprehensive behavioural and pathological assessment, while the tertiary stage was tailored to the assessment of models of neurological disease, or the assessment of phenotypic variability. While multiple laboratories tested the validity of the SHIRPA screen and found it to be reliable in detecting subtle
Figure 5.4. Comparison of the body size and weight observed in littermates from neuroligin 2 HA Thy1.2 pronuclear injection offspring. The mouse on the left did not test positive for the NL2 transgene (wildtype) with genotyping whereas the mouse on the right did (founder TgNL2.6).
Figure 5.5. Observation of hindlimb clasping in founder mice expressing neuroligin 2. When TgNL2 mice are suspended by the tail, the majority show clasping of hind- and often forelimbs, providing the first indications of a neurological phenotype in these animals.
phenotypes (Rafael et al., 2000; Lalonde et al., 2005), other laboratories found the need for additional assessments or modifications (Hossain et al., 2004; Masuya et al., 2005) including dark testing. While the process for evaluating these protocols is ongoing, and further refinement over time is likely, they provide a valuable tool for phenotype assessments. The details of the tests used and methods employed in preliminary phenotyping of the TgNL1 and TgNL2 mouse strains are listed below.

5.5. Behaviour Screen Rating Scales

I. Viability
Viability of founder
Normal lifespan
1 = Die in early adulthood
2 = Die during development, adolescence
3 = Die early postnatal
4 = Die embryonically

Viability of offspring
Normal number of offspring are viable
1 = Increased number of offspring found dead
2 = High number of offspring found dead
3 = No viable offspring found

II. Animal observed during uninterrupted activity in the home cage
Body position
Normal: sitting or standing
1 = Occasional hunching of back (kyphosis)
2 = Intermittent but pronounced kyphosis
3 = Lying on side, or prone

Basal activity (square crossings)
Normal: vigorous scratching, grooming, moderate movement
1 = Vigorous, rapid/darting movement
2 = Repeated rearing on hind legs and/or repeated vertical leaping

Palpebral Closure
Normal: eyes open
1 = Eyes 1/2 closed
2 = Eyes closed

Piloerection
None
1 = Coat stands on end

III. Transfer arousal
Normal: brief freeze followed by active movement
1 = Prolonged freeze, then slight movement
2 = No freeze, immediate movement
3 = Extremely excited ("manic")

IV. Animal observed in open arena with manipulation
Body Weight
Normal body weight
1 = Mild body weight reduction (70-90% the body weight of controls)
2 = Moderate body weight reduction (50-70% the body weight of controls)
3 = Severe body weight reduction (less than 50% the body weight of controls)

Startle response to a 90dB click from 30cm above arena
Normal preyer reflex (backwards flick of pinnae)
1 = Jump less than 1 cm
2 = Jump more than 1 cm
3 = None

Tail position during forward motion
Normal: horizontally extended
1 = Dragging
2 = Elevated / Straub Tail

Touch escape from finger stroke on back from above
Normal: moderate (rapid response to light stroke)
1 = Vigorous (escape response to approach)
2 = No response

Limb clasping (during tail suspension)
Normal: absent
1 = Rare (less than 10% of trials)
2 = Infrequent (20-50% of trials)
3 = Common (50-70% of trials)
4 = Frequent (greater than 70% of trials)

Visual placing of forelimbs (when animal lowered by base of tail from a height of approximately 15cm above wire grid)
Normal: before vibrissae contact (18mm)
1 = Upon vibrissae contact
2 = Upon nose contact
3 = None (occurs when animal is clasping both hind and forelimbs, when not clasping these animals place normally)

Pinna reflex (to touch of the proximal part of the inner canthus)
Normal: active retraction, moderately brisk flick
1 = Hyperactive, repetitive flick
2 = None

Corneal reflex (to light touch of the cornea)
Normal: active single eye blink
1 = Multiple eye blink
2 = None

Buried food retrieval (conducted on a separate set of mice trained with the food item (chocolate chips) and given limited access to food for a 24 hr period preceding testing)
Normal: retrieval of food within 10 seconds of entering the arena
1 = slow retrieval (10-60 seconds)
2 = long latency to retrieval (60 seconds – 3 minutes)
3 = no retrieval of food item within 5 minutes

Body tone (resistance to finger press on body cavity)
Normal: resistance
1 = Flaccid, no return of cavity to normal
2 = Extreme resistance, board like

Limb tone (limb resistance to gentle finger tip pressure on hind paw)
Normal: resistance
1 = Extreme resistance
2 = No resistance

Contact righting reflex: animal response to release from supine position
Normal: Present (animal rights itself immediately)
1 = Absent
5.6. Bibliography


6. APPENDIX B: OTHER CONTRIBUTIONS

6.1. Published Contributions


For this paper I completed retroactive immunohistochemistry of lesion sites, and assisted with figure preparation.


For this publication, I assisted with immunohistochemistry and confocal imaging, with particular focus on staining of endogenous ERBB4 in tissue sections.


For this project I assisted with constructs, cell cultures, and fluorescence imaging in heterologous cell lines. I also completed confocal imaging of expressed constructs in oocytes.

For this project I assisted with constructs, cell cultures, and fluorescence imaging in heterologous cell lines. I also completed confocal imaging of expressed constructs in oocytes.


For this project I assisted with constructs, cell cultures, and fluorescence imaging in heterologous cell lines. I also completed confocal imaging of expressed constructs in oocytes. In addition, I quantified and analysed the imaging data, generated figures and contributed to the text of the manuscript.


For this publication, I assisted with immunohistochemistry and confocal imaging, with particular focus on staining of endogenous Myosin V in tissue sections. I was involved in figure preparation and writing portions of the text.
6.2. Contributions under Review


For this publication I completed electron microscopy in the striata of YAC128 and wildtype mice to assess changes in peri-synaptic astrocyte contact. I also contributed to manuscript preparation by generating a figure and writing sections for the manuscript text.

Goytain, A., Hines, R.M., & Quamme, G.A. The golgi-specific DHHC zinc finger protein, GODZ, mediates Ca2+ transport. Submitted to JBC.

For this project I assisted with constructs, cell cultures, and fluorescence imaging in heterologous cell lines. I also completed confocal imaging of expressed constructs in oocytes, and assisted with site-directed mutagenesis of GODZ. In addition, assisted with generation of figures and contributed to the text of the manuscript.

For this publication I completed electron microscopy in the striata of HIP14-/-, YAC128, and HIP14+/+, wildtype mice to assess changes in cell and synapse morphology and number. I also assisted with immunohistochemistry of synaptic proteins in tissue sections. I also contributed to manuscript preparation by generating figures and writing sections for the manuscript text.


For this project I completed co-immunoprecipitation of PMCA1 and PMCAIP in heterologous cells. I also prepared manuscript figures and contributed to the text.
7. APPENDIX C: ETHICS BOARD CERTIFICATES
ANIMAL CARE CERTIFICATE

Application Number: A05-1580

Investigator or Course Director: Steven R. Vincent

Department: Psychiatry

Animals:

- Mice Transgenic NLG2 250
- Mice C57/Bl6 30
- Rats 50
- Mice Transgenic NLG1 250
- Mice Transgenic NLG2 125
- Mice C57/Bl6 30

Start Date: April 1, 2001

Approval Date: February 27, 2008

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Postsynaptic mechanisms that regulate synapse development

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Excitatory and inhibitory synaptic imbalance in neurodevelopmental disorders

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Protein Palmitoylation and regulation of synaptic protein trafficking and function

Funding Agency: High Q Foundation
Funding Title: TREAT-HD: Translational Research on Excitotoxicity to Accelerate Therapies for Huntington's Disease

Funding Source: NeuroScience Canada Foundation

https://rise.ubc.ca/rise/Doc/0/1262F1O8B7VKD8DMGFH5IS1RFA/fromString.html 17/03/2008
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<th>Synaptic repair by peptides interfering with protein-protein interactions critical for synaptic plasticity and maturation: A rational pharmacology for restoring cognitive and emotional function</th>
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The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093