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Diverse effects of anti-GluN1 antibodies in hippocampal excitatory synapses

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A thesis submitted in partial fulfilment of the requirements for the degree of
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Abstract

Antibodies are highly specialised immunological proteins produced by our immune system upon exposure to foreign, pathogenic protein material, which interact with specific epitope sites on their respective immunogen. The penetrative ability of antibodies to cross the blood-brain barrier and into the central nervous system has opened new avenues for immunotherapy development in various neurological disorders. We have previously reported neuroprotective and cognitive-enhancing effects of antibodies against the obligatory GluN1 subunit of the NMDA subtype glutamate receptor (NMDAR) in animal immunisation studies. This present study aimed to elucidate the underlying cellular mechanisms of these therapeutic effects and identify possible epitope-dependent responses by examining changes in protein expression as well as excitatory synaptic transmission in the glutamatergic synapse of cultured hippocampal neurons. Anti-GluN1 antibodies which bind to the glycine-binding site of GluN1 (recNR1 IgG) increased the total number of excitatory synapses and NMDAR-containing synapses but not AMPA receptor (AMPAR)-containing synapses in a concentration-dependent manner. This change in receptor expression was paralleled by an increase in NMDAR-mediated but not AMPAR-mediated synaptic response. On the other hand, anti-GluN1 antibodies which bind to the N-terminal domain of GluN1 (NR1.NTD IgG) did not alter the number of excitatory synapses or NMDAR expression, but did increase synaptic AMPAR expression. However, a coordinated increase in AMPAR-mediated synaptic response was not observed following NR1.NTD IgG treatment. Differences in treatment response between recNR1 IgG and NR1.NTD IgG signify possible epitope-dependent effects. Although the precise pharmacological actions of anti-GluN1 antibodies remain unclear, the increased proportion of NMDAR-containing synapses in hippocampal neurons treated with recNR1 IgG suggests an increased presence of morphologically silent synapses, which could be implicated in plasticity changes relating to the improvements in learning and memory previously observed in our animal studies.
Acknowledgements

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<td>AAV</td>
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</tr>
<tr>
<td>ACSF</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AD</td>
<td>artificial cerebrospinal fluid</td>
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<tr>
<td>AMPA</td>
<td>one-way analysis of variance</td>
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<tr>
<td>AMOVA</td>
<td>antigen-presenting cell</td>
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<tr>
<td>APS</td>
<td>ammonium persulphate</td>
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<tr>
<td>ASD</td>
<td>autism spectrum disorders</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid beta</td>
</tr>
<tr>
<td>B</td>
<td>blood-brain barrier</td>
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<tr>
<td>BBB</td>
<td>bovine serum albumin</td>
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<tr>
<td>BSA</td>
<td>carboxyl-</td>
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<tr>
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<td>calcium/calmodulin-dependent serine protein kinase</td>
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<td>CCD</td>
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<tr>
<td>DIV</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>E</td>
<td>excitatory postsynaptic current</td>
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<td>ELISA</td>
<td>actin filament</td>
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<tr>
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<td>green fluorescent protein</td>
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<td>glutamate receptor 3</td>
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<td>polyhistidine</td>
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<td>HIS</td>
<td>horseradish peroxidase</td>
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<td><strong>I</strong></td>
<td>immunoglobulin G</td>
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<td>IPSC</td>
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<tr>
<td><strong>L</strong></td>
<td>Luria-Bertani broth</td>
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<tr>
<td>LB</td>
<td>long-term potentiation</td>
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<tr>
<td>LTP</td>
<td>luciferase</td>
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<tr>
<td><strong>M</strong></td>
<td>membrane-associated guanylate kinase</td>
</tr>
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<td>microtubule-associated protein 2</td>
</tr>
<tr>
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<td>mitogen-associated protein kinase</td>
</tr>
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<td>middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MCAO</td>
<td>minimum essential media</td>
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<td>MEM</td>
<td>miniature excitatory postsynaptic current</td>
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<td>metabolotropic glutamate receptor</td>
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<td>major histocompatibility complex</td>
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<td>MHC</td>
<td>Munc18-1-interacting protein 1</td>
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<td>NGS</td>
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<td><strong>O</strong></td>
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<tr>
<td>OD_{600}</td>
<td>o-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>P</td>
<td>postnatal day</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCP</td>
<td>phencyclidine</td>
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<td>poly-D-lysine</td>
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<tr>
<td>PDZ</td>
<td>postsynaptic density 95/discs large/zona occludens-1 homology</td>
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<td>paraformaldehyde</td>
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<td>protein kinase A</td>
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<td>protein kinase C</td>
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<td>rolling ball radius</td>
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<td>recombinant GluN1 protein</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RRP</td>
<td>readily releasable pool</td>
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<tr>
<td>Rs</td>
<td>series resistance</td>
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<td>RSB</td>
<td>reducing sample buffer</td>
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<td>RT</td>
<td>room temperature</td>
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<td>synapse-associated protein-102</td>
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<td>synapse-associated protein-97</td>
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<td>SAPAP</td>
<td>SAP90/PSD-95-associated protein</td>
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<td>SDS-PAGE</td>
<td>sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SH3</td>
<td>src homology 3</td>
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<tr>
<td>Shank</td>
<td>SH3 and multiple ankyrin repeat domains protein</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor</td>
</tr>
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<td>T</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>U</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>vesicle-associated membrane protein</td>
</tr>
<tr>
<td>VGLUT</td>
<td>vesicular glutamate transporter</td>
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**W**  
WT  
wild type

**Z**  
z-stack  
z-series stack
Chapter 1.

General introduction
1.1. The immune system and antibodies

Antibodies are the first line defenders of the immune system. The innate immune response is first triggered upon exposure to foreign, potentially pathogenic material, followed by an adaptive immune response if infection persists (Kawai & Akira, 2010). Through memory cells, the adaptive immune system provides long-term protection from infections with high specificity and efficiency (Sprent, 1997). In brief, foreign particle is endocytosed by antigen-presenting cells (APCs) in peripheral tissues, which process the antigens and re-express them with major histocompatibility complex (MHC) class II molecule on the cell surface (Erb & Feldmann, 1975). Antigens are subsequently delivered by APCs to the lymph nodes or spleen where antigen-MHC class II complexes are recognised by the antigen-binding T-cell receptors on naïve T-helper cells, a conventional lymphocyte (Reinhardt et al., 2001). Unlike T-helper cells, conventional B-cell lymphocytes are capable of recognising and internalising most antigens on their own by binding to specific epitopes with their B-cell receptors. Internalised antigens in B-cells are degraded and complexed with MHC class II before they are presented to an activated T-helper cells (Mitchison, 1992; Mitchison, 2004). Cytokines are then secreted by activated T-helper cells, which stimulate the proliferation and differentiation of B-cells into antibody-producing plasma cells or long-lived memory cells (Seder & Paul, 1994; Takatsu, 1997). Large quantities of antibodies are produced by plasma B-cells, which enter the circulation to directly neutralise the pathogen or tag them for destruction. The role of memory B-cells in the adaptive immune system is to initiate rapid humoral immune response upon re-encounter of the same antigen (Ahmed & Gray, 1996).

1.2. Antibodies in therapy and diseases

1.2.1. Therapeutic antibodies for disorders of the CNS

The central nervous system (CNS) was once thought as an immunologically privileged environment. However, it has recently come to light that antibodies are able to penetrate the blood-brain barrier (BBB), the continuous monolayer of endothelial cells which lie between the circulatory system and the CNS (Bard et al., 2000). Therefore, there is much potential for the development of therapeutic antibodies to treat disorders of the CNS. One prominent
example is the development of antibodies targeted against amyloid beta (Aβ) as a treatment for Alzheimer’s disease (AD) to drive the removal of amyloid plaques. In a study involving a transgenic mouse model of AD, anti-Aβ antibodies were detected in the cerebrospinal fluid (CSF) of animals within 24 hours of passive transfer (Dodart et al., 2002). Furthermore, deficits in behavioural tests for memory functions were reversed in animals treated with the antibodies. The potential efficacy of anti-Aβ antibodies as a treatment of AD was also investigated in human clinical trials. A monoclonal antibody named solanezumab was developed to target Aβ plaques and has demonstrated safety in humans (Farlow et al., 2012). In a later trial, the same monoclonal antibody exhibited therapeutic efficacy by slowing down cognitive decline in mild AD patients (Valera & Masliah, 2013). However, this effect was not translated to late-stage AD patients with more severe cognitive deficits (Doody et al., 2014). This shows that although the introduction of antibodies to the CNS has now become an option for treatment, the therapeutic outcomes will still depend on careful investigations on the precise action of antibodies of interest.

1.2.2. Autoimmune disorders of the CNS

If therapeutic antibodies can gain access to the CNS through the BBB then so can self-recognising antibodies. In addition to producing antibodies against harmful pathogens for clearance, the immune system can produce antibodies against ‘self’ proteins with detrimental effects if self-tolerance is not maintained (Sakaguchi et al., 2012). Cells in the CNS contain a multitude of potential antibody targets, including surface receptors. The deleterious effects of antibody-receptor interactions have been demonstrated in autoimmune disorders of the CNS. In a study by Rogers et al. (1994), rabbits were immunised with recombinant glutamate receptor 3 (GluR3) proteins, which led to the production of high titres of anti-GluR3 antibodies. These rabbits developed seizures and neuropathology that were characteristic of Rasmussen’s encephalitis, a progressive childhood disease with the presentation of epilepsy, dementia and inflammation (Rogers et al., 1994). Antibodies against the glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors have been identified in cases of anti-AMPA receptor encephalitis in humans (Lai et al., 2009). These specific antibodies against the GluA1 or GluA2 subunits of the AMPA receptor resulted in a pronounced reduction of surface AMPA receptors in cultured hippocampal neurons with no alteration in the expressions of other glutamate receptors (Peng et al., 2015). In addition to AMPA receptors, autoantibodies against the glutamate N-methyl-D-aspartate (NMDA)
receptor have also been identified in various CNS disorders. Anti-NMDA receptor antibodies, specifically against the GluN2 subunit, detected in a subset of patients with systemic lupus erythematosus have been shown to mediate neuronal death through the apoptotic pathway both in vitro and in vivo (DeGiorgio et al., 2001). Patients with anti-NMDA receptor encephalitis are also presented with high titres of circulating anti-NMDA receptor antibodies, which are detectable in their CSF (Dalmau et al., 2008). However, these particular antibodies are targeted against a different subunit of the NMDA receptor, the GluN1 subunit, and a crucial epitope has been identified in the N368/G369 region of the amino (N)-terminal domain (Gleichman et al., 2012). The antibodies presented by anti-NMDA receptor encephalitis patients also have a NMDA receptor subtype preference, with higher binding to GluN1/GluN2B receptors (Dalmau et al., 2008). Cultured hippocampal neurons exhibited a drastic reduction in synaptic NMDA receptor expression but not AMPA receptor expression when they were treated with antibodies from anti-NMDA receptor encephalitis patients (Hughes et al., 2010). Like anti-AMPA receptor encephalitis, the antibody effect was only specific to its target receptor. The studies involving anti-NMDA receptor antibodies demonstrate that differential effects may result from antibody-receptor interactions when they bind to different regions or subunits even if the target receptor is the same.

1.3. The glutamatergic excitatory synapse

Neurons in the brain communicate with each other through highly specialised junctions called synapses where electrochemical information is exchanged in a series of molecular events during synaptic transmission (Jessell & Kandel, 1993; Kandel et al., 2000). Synapses can either be excitatory or inhibitory, as defined by the neurotransmitter released from the presynaptic neuron and the types of receptors available on the postsynaptic neuron. Glutamate is the main excitatory neurotransmitter in the CNS and plays a critical role in learning and memory (Monaghan et al., 1989).

1.3.1. Structure of the excitatory synapse

Most excitatory synapses in the CNS are asymmetrical and characterised by a thick electron-dense, disk-like postsynaptic density (PSD) opposing the presynaptic terminal (Harris & Stevens, 1989). Synaptic vesicles containing glutamate are gathered at highly specialised
sites of the presynaptic terminal called the active zone, where they fuse with the plasma membrane in response to an action potential (Burns & Augustine, 1995). Glutamate is loaded into synaptic vesicles by vesicular glutamate transporters (VGLUTs), which require the driving forces of an electrochemical proton gradient generated by the vacuolar proton adenosine triphosphatase (Südhof, 2004). Each presynaptic bouton contains a total of around 200 vesicles, with approximately 10 of them docked at the active zone (Schikorski & Stevens, 1997). The fusion of docked synaptic vesicles with the presynaptic membrane leads to the release of glutamate into the synaptic cleft, which is a space that is approximately 20 nm wide between the presynaptic terminal and the postsynaptic membrane (Schikorski & Stevens, 1997). Most excitatory synapses terminate on dendritic spines, which are protrusions from a parent dendrite. Dendritic spines are approximately 1 µm in length in pyramidal neurons from the cornu ammonis (CA) 1 region of the hippocampus and are characterised by their large mushroom-shaped spine head supported by a thin neck (Harris & Stevens, 1989). Dendritic spines facilitate synaptic signalling by compartmentalising chemical signals such as calcium into a localised environment, which is functionally important for synaptic plasticity (Svoboda et al., 1996; Yasuda et al., 2003).

1.3.2. Glutamate release in the excitatory synapse

Rapid glutamate release from the axon terminal is facilitated by a subset of synaptic vesicles at the active zone that have been primed for exocytotic fusion with the presynaptic membrane in response to action potential (Rizzoli & Betz, 2005). First, synaptic vesicles are docked at the active zone in close proximity to voltage-dependent calcium channels, where they mature and undergo the process of vesicle priming (Harris & Stevens, 1989; Horrigan & Bookman, 1994). Mature, fusion-competent vesicles form the readily releasable pool (RRP) of synaptic vesicles (Augustin et al., 1999). Action potential-mediated depolarisation of the presynaptic membrane causes a brief influx of calcium ions through voltage-dependent calcium channels, which subsequently triggers fusion of synaptic vesicles from the RRP in a series of events (Rosenmund & Stevens, 1996). The release probability is defined as the probability of neurotransmitter release by the stimulation of a single action potential and is proportional to the size of the RRP in small synapses, including those in dissociated cultures of hippocampal neurons (Murthy et al., 2001; Murthy et al., 1997). Calcium ions which enter the presynaptic terminal through voltage-dependent calcium channels bind to the calcium sensor synaptotagmin on the surface of synaptic vesicles and mediates vesicle exocytosis by
promoting interactions between soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) (Brose et al., 1992). SNARE complexes are the core machinery for membrane fusion and are composed of synaptobrevin/vesicle-associated membrane protein (VAMP) in vesicle-associated SNARE on synaptic vesicles as well as syntaxin1 and synaptosomal-associated protein 25 in target SNARE at the plasma membrane (Söllner et al., 1993). Following neurotransmitter release, empty vesicles are endocytosed back into the presynaptic terminal via clathrin-coated pits, where they are replenished with neurotransmitter (Heuser & Reese, 1973). It takes around 30 s for recycled synaptic vesicles to become available for re-release in cultured hippocampal neurons (Ryan et al., 1993).

Synaptic vesicle fusion can also occur spontaneously in the absence of action potentials, leading to the occurrence of miniature excitatory postsynaptic currents (mEPSCs) in the postsynaptic cell. Each mEPSC event is the result of the fusion and release of glutamate from a single vesicle at a synapse. (Murthy & Stevens, 1999). However, the probability of spontaneous vesicle fusion is relatively low and occurs at an approximate rate of 1 to 2 vesicles per min per synapse in cultured hippocampal neurons (Murthy & Stevens, 1999).

1.3.3. Architecture and molecular organisation of the excitatory synapse

Excitatory synapses are composed of a wide array of proteins with roles in maintaining synaptic function (Figure 1.1). The pre- and postsynaptic membranes of the synapse are held together by transsynaptic interactions through cell adhesion molecules (CAMs) including the neurexin-neuroligin complex (Dalva et al., 2007). Neurexin and neuroligin are anchored to the pre- and postsynapse via interactions of their postsynaptic density 95/discs large/zona occludens-1 homology (PDZ)-binding domains with calcium/calmodulin-dependent serine protein kinase (CASK) and postsynaptic density protein (PSD)-95, respectively (Garner et al., 2000). The molecular scaffold within the presynaptic terminal is organised around synaptic vesicles to facilitate and modulate glutamate release through various protein interactions in the active zone (Schoch & Gundelfinger, 2006). Examples of presynaptic proteins which make up the molecular complex include voltage-dependent calcium channels, Piccolo, Bassoon, CASK (Hsueh, 2006) and Munc18-1-interacting protein 1 (Mint1) (Rogelj et al., 2006).
Figure 1.1 Schematic diagram of synaptic proteins located at glutamatergic excitatory synapses

A simplified representation of the excitatory synapse to provide an overview of its molecular composition. Glutamate receptors are shown here in relation to other proteins in the PSD. Glutamate released from the presynaptic terminal binds to and activates glutamate receptors (AMPAR, NMDAR and mGluR) in the postsynaptic membrane. Receptor expression is stabilised by the interactions of various scaffolding proteins in the PSD including PSD-95, Shank and Homer.

AMPAR, AMPA receptor; CAMKII, calmodulin-dependent protein kinase II; CASK, calcium/calmodulin-dependent serine protein kinase; GRIP, glutamate receptor-interacting protein; mGluR, metabotropic glutamate receptor; Mint1, Munc18-interacting protein 1; NMDAR, NMDA receptor; PSD-95, postsynaptic density protein-95; RIM, rab3-interacting molecule; SAP-97, synaptic-associated protein-97; SAPAP, SAP90/PSD-95-associated protein; SV, synaptic vesicle; VAMP, vesicle-associated membrane protein.
Directly aligned with the active zone is the complex protein network in the PSD. The PSD is composed of various proteins which interact with each other through their multiple binding domains, leading to a layered, mesh-like organisation of proteins (Hayashi et al., 2000; Sheng & Hoogenraad, 2007). Glutamate receptors and CAMs are first recruited to the PSD, followed by signal transduction molecules, which are all anchored to the actin-based cytoskeletal network of the spine via cytoplasmic scaffolding proteins (Sheng & Hoogenraad, 2007). The cytoskeleton of the spine is mainly composed of actin filaments (F-actin), which regulate trafficking of proteins and vesicles in addition providing structural framework (Hotulainen & Hoogenraad, 2010).

Scaffolding proteins serving as molecular organisers of the PSD include members of the membrane-associated guanylate kinases (MAGUKs) and src homology 3 (SH3) and multiple ankyrin repeat domains protein (Shank). Members of the Drosophila tumour suppressor protein discs-large subfamily of MAGUKs include synaptic proteins PSD-93, PSD-95, synapse-associated protein (SAP)-97 and SAP-102, all contain three PDZ domains, one SH3 domain and one guanylate kinase domain (Oliva et al., 2012; Woods & Bryant, 1991). MAGUKs are capable of interacting with the carboxyl (C)-terminal domain of various proteins including glutamate receptors (Saras & Heldin, 1996). Through this particular interaction, MAGUKs have been shown to regulate the targeting and trafficking of glutamate receptors at the synapse. PSD-95 binds to NMDA receptor subunits GluN2A and GluN2B (Kornau et al., 1995) but it does not appear to be necessary for the synaptic clustering of NMDA receptors, as demonstrated by mice expressing mutant PSD-95 (Migaud et al., 1998). Interestingly, the mutant mice displayed spatial learning impairments but their hippocampal neurons exhibited normal NMDA receptor expression and function as well as increased synaptic plasticity in the form of long-term potentiation (LTP), signifying additional roles of PSD-95 in learning and memory. Furthermore, overexpression of PSD-95 in cultured hippocampal neurons increased the number of postsynaptic AMPA receptors through protein association with Stargazin but NMDA receptors expression remained unchanged (El-Husseini et al., 2002; Schnell et al., 2002). SAP-97 is another member of the MAGUK family and has been shown to bind to the AMPA receptor subunit GluA1 (Leonard et al., 1998) and the NMDA receptor subunit GluN2A (Gardoni et al., 2003). SAP-97 regulates NMDA receptor trafficking and insertion in a calmodulin-dependent protein kinase II (CaMKII)-dependent manner (Mauceri et al., 2007). The phosphorylation of SAP-97 by CaMKII at residue S39 drives the release of the SAP-97/GluN2A complex from the endoplasmic
reticulum and trafficking to the PSD. Moreover, phosphorylation of SAP-97 at residue S232 results in the insertion of GluN2A into the postsynaptic membrane.

On the other hand, the Shank family of scaffolding proteins is considered a key organiser of the PSD and acts as a ‘scaffold of scaffolds’ due to multiple interactions with other scaffolding proteins (Schoch & Gundelfinger, 2006). The multiple binding domains on Shank allow it to create direct and indirect links with glutamate receptors. Shank binds to PSD-95 through its interaction with SAP90/PSD-95-associated protein (SAPAP), thereby linking it to NMDA receptors (Naisbitt et al., 1999). Furthermore, the proline-rich region of Shank forms a connection with metabotropic glutamate receptors (mGluRs) through another scaffolding protein called Homer (Hayashi et al., 2009; Tu et al., 1999). Shank proteins also contribute to the structural integrity of the PSD by interacting with the F-actin cytoskeletal network through cortactin, an actin cross-linking protein (Du et al., 1998; Wu & Parsons, 1993).

1.4. Glutamate and its receptors

Glutamate in the excitatory synapse activates two distinct families of receptors: ionotropic receptors, which are gated cation channels, and metabotropic receptors, which are coupled to G-protein-mediated intracellular secondary messenger systems (Nakanishi, 1992). Subtypes of the ionotropic glutamate receptor family include AMPA, NMDA, and Kainate receptors.

The focus of the review here will be on AMPA and NMDA receptors, which play profound roles in synaptic plasticity (Derkach et al., 2007; Lau & Zukin, 2007).

1.4.1. Glutamate the neurotransmitter

The excitatory effect of glutamate was discovered in 1954 by Hayashi (1954) long before it was recognised as a neurotransmitter in the 1980’s (Fonnum et al., 1981). As mentioned before, glutamate is the main excitatory neurotransmitter of the CNS. Most of the glutamate in neurons is synthesised from glutamine during glucose metabolism (Hamberger et al., 1979). Following release from the presynaptic terminal, excess glutamate in the extracellular space is taken up by astrocytes through glutamate transporters (Danbolt, 2001). Astrocytes convert glutamate into glutamine through glutamine synthetase and release it into the extracellular space, where it is taken up by neurons to be reconverted into glutamate (Hertz, 1979). Although glutamatergic neurons mediate many vital processes, overactivation of
glutamate receptors has been associated with neuronal cell death in the pathophysiology of various insults to the brain including stroke, epileptic seizures, hypoxia and hypoglycaemia (Kristián & Siesjö, 1998).

### 1.4.2. AMPA receptors

AMPA receptors are heterotetrameric complexes made up of GluA1, GluA2, GluA3 and GluA4 subunits (Keinanen et al., 1990; Shi et al., 2001). The subunit composition of AMPA receptors vary between brain regions and developmental stages. In hippocampal neurons, most AMPA receptors exist as heteromeric GluA1/GluA2 receptors, with fewer heteromeric GluA2/GluA3 receptors, and a very small number of homomeric GluA1 receptors (Wenthold et al., 1996). Each of the GluA1-3 subunits has two splice variants that are called 'flip' and 'flop'. These splice variants show variation in their distribution in different types of cells (Sommer et al., 1990). In the hippocampus, CA3 pyramidal neurons only express the 'flip' variant while granule cells in the dentate gyrus express more 'flop' variants compared to 'flip'. The 'flip' variant has been shown to be more efficient in AMPA receptor activation by glutamate (Sommer et al., 1990). AMPA receptors are activated by AMPA or kainate, which act on the same site on the receptor (Keinanen et al., 1990). The activation of AMPA receptors leads to an excitatory postsynaptic responses with fast kinetics (Colquhoun et al., 1992).

### 1.4.3. NMDA receptors

Similar to AMPA receptors, NMDA receptors are also heterotetrameric complexes. To date, seven different NMDA receptor subunits have been identified and they are classified into three subfamilies based on sequence homology: the GluN1 subunit, four types of GluN2 subunits (GluN2A-2D) and two GluN3 subunits (GluN3A, GluN3B) (Cull-Candy & Leszkiewicz, 2004; Paoletti, 2011; Traynelis et al., 2010). Furthermore, each subunit is encoded by a different gene: GluN1 by GRIN1, GluN2 subtypes by GRIN2 (A-D) and GluN3 subtypes by GRIN3 (A and B) (Andersson et al., 2001; Dingledine et al., 1999). The subunits differ in size with the total number of amino acids ranging between 900 to over 1,400, which can be accounted by differences in the length of their C-terminal domain (Traynelis et al., 2010). NMDA receptor subunits share similar structures (GluN1 shown as an example in Figure 1.2), which consist of four hydrophobic transmembrane domains (TM1-4), an extracellular N-terminus, and an intracellular C-terminus that is involved in trafficking and
intracellular signalling (Chen & Roche, 2007). The agonist binding domain is formed by the S1 domain before TM1, and the S2 domain (also known as M3-M4) between TM3 and TM4 (Moretti et al., 2004).

![Diagram of the NMDA receptor GluN1 subunit](image)

**Figure 1.2 Schematic diagram of the NMDA receptor GluN1 subunit**

### 1.4.3.1. Subunit composition

As heterotetramers, each receptor consists of two obligatory GluN1 subunits and most commonly two GluN2 subunits, but there can also be mixtures of GluN2 or GluN3 (Traynelis et al., 2010). The subunit composition of NMDA receptors can differ between developmental stages, CNS regions, cell types and subcellular localisation. GluN1 is ubiquitously expressed in all areas of the adult rodent brain from early in development (Akazawa et al., 1994; Monyer et al., 1994). On the other hand, the expressions of GluN2 and GluN3 subunits are more variable. For example, GluN2B and GluN2D are the main GluN2 subunits in the brain during embryonic development but GluN2A becomes the most prominently expressed GluN2 subunit after birth and in adulthood (Akazawa et al., 1994; Monyer et al., 1994). NMDA receptor subunit composition differs between hippocampal regions at different stages of development. A study by Ritter et al. (2002) quantified the messenger RNA (mRNA) expressions of NMDA receptor subunits in the developing rat hippocampus. Hippocampal GluN1 expression levels remain high until postnatal day (P)14, and its expression is the highest in the CA2 region but lowest in the dentate gyrus. On the other hand, hippocampal GluN2A expression level gradually increases between P0 and P21, and its expression is the
highest in the CA1 and CA2 but lowest in the dentate gyrus. Furthermore, hippocampal GluN2B and GluN2C expression levels start high early in development but gradually decline to below birth levels at P35. The expressions of GluN2B and GluN2C are the lowest in the CA3 region and dentate gyrus. Hippocampal GluN2D expression level gradually increases from birth but the overall expression is comparatively lower than other subunits. Lastly, hippocampal GluN3A expression peaks at P7 in the CA1 and CA2 regions but gradually declines to birth levels. The spatiotemporal nature of these subunit composition changes suggests a functional role in synaptic development and plasticity in the hippocampus (Collard et al., 1993). In addition to region-specific differences, NMDA receptor subunit composition can also differ between subcellular locations. During synaptic development, NMDA receptors are predominantly extrasynaptic and composed of GluN1 and GluN2B subunits while there is a low expression of GluN2A. As synapses mature, extrasynaptic receptors are reduced in numbers in comparison to the growing number of synaptic receptors, which are mostly GluN2A-containing NMDA receptors (Tovar & Westbrook, 1999). This further emphasises the importance of subunit composition in NMDA receptor function.

1.4.3.2. NMDA receptor activation

Activation of NMDA receptors requires both glutamate and glycine to bind to their respective binding sites (Figure 1.3). The agonist glutamate-binding site is located on the GluN2 subunit while the co-agonist glycine-binding site is located on the GluN1 subunit (Chen et al., 2005). NMDA receptor subunits also contain various modulatory sites which can be activated by zinc ions, protons or polyamines (Paoletti, 2011). Upon binding by glutamate and glycine, the agonist-binding domains undergo a conformational change to prepare for channel opening (Banke & Traynelis, 2003). However, NMDA receptor activation also requires the depolarisation of the postsynaptic membrane to relieve the voltage-dependent magnesium block in the pore of the channel (Shinichi & Dingledine, 1993). This particular feature makes the NMDA receptor a synaptic coincidence detector of both pre- and postsynaptic activities, which is a prerequisite of Hebbian plasticity that implies enhanced synaptic coincidence detection should lead to improved learning and memory (Hebb, 1949). Activation of the receptor leads to an influx of calcium ions, as well as sodium ion influx and potassium ion efflux to some extent (Harvey & McIlwain, 1968; MacDermott et al., 1986). In contrast to AMPA receptors, NMDA receptors exhibit slow electrophysiological kinetics due to the slow unbinding of glutamate from the receptor (Lester et al., 1990).
Figure 1.3 Schematic diagram of NMDA receptor activation

NMDA receptors are activated by the binding of agonist and co-agonist, glutamate and glycine, and the depolarisation of the postsynaptic membrane to remove the magnesium ($\text{Mg}^{2+}$) block from the channel pore. Calcium ion ($\text{Ca}^{2+}$) influx occurs as a result, as well as sodium ion ($\text{Na}^{+}$) influx and potassium ion ($\text{K}^{+}$) efflux to lesser extents.

1.5. Synaptic plasticity in the hippocampus

1.5.1. Structure of the hippocampus

The hippocampus is a part of the limbic system in the CNS known to be involved in learning and memory functions. Much of the characterisation on the anatomy of the hippocampus was carried out by Rafael Lorente de Nó (1934) and Santiago Ramón y Cajal (1968). The hippocampus proper is divided into three regions, the CA1, CA2 and CA3, which contain cells of different morphological characteristics and density (Dam, 1979). The CA1 region contains well distributed pyramidal neurons with distinctive triangular shaped cell bodies. On the other hand, the CA2 region is densely packed with large neurons with oval shaped cell bodies. Lastly, the curved CA3 region also contains pyramidal neurons with triangular shaped cell bodies. Other parts of the hippocampal formation include the dentate gyrus, the subicular complex and the entorhinal cortex (Lorente de Nó, 1934; y Cajal, 1968). Granule cells, which have small oval shaped cell bodies, are the main type of cell in the dentate gyrus (Seri et al., 2004). The hippocampal network arises from axonal projections between different areas of the hippocampal formation (Figure 1.4). Intrahippocampal circuitry can be divided into two
pathways (Duvernoy, 2005). Firstly, the polysynaptic intrahippocampal pathway involves cells from the entorhinal cortex, the dentate gyrus, CA3, CA1 and the subiculum. The perfortant path originates from the posterior parietal association cortex and projects to the entorhinal cortex. The path is continued by the synaptic input of layer II entorhinal cortical neurons onto the dentate gyrus. Granule cells from the dentate gyrus then project their axons (mossy fibres) to pyramidal cells in the CA3 region. Next, CA3 pyramidal neurons project their axons (Schaffer collaterals) to the CA1 region, where projections output to the subiculum and the entorhinal cortex. Secondly, the direct pathway involves cells from the entorhinal cortex, CA1 and the subiculum. The pathway originates from the inferior temporal association cortex and projects to the entorhinal cortex. Layer III entorhinal cortical neurons project directly to CA1 pyramidal neurons, where projections output to the subiculum and the entorhinal cortex. The polysynaptic pathway is mainly involved in episodic and spatial memory while the direct pathway has a role in semantic memory (Duvernoy, 2005).

Figure 1.4 Structure of the hippocampal network

The perforant path originates from the entorhinal cortex and projects first to the dentate gyrus (DG) then to the field CA3 and CA1 of the hippocampus proper. The axons of the granule cells in the DG (mossy fibres) project to pyramidal cells in CA3 while the axons in CA3 pyramidal cells (Schaffer collaterals) project to CA1 pyramidal cells. Projections from CA1 go to subiculum and then to the entorhinal cortex.
1.5.2. NMDAR-dependent long-term plasticity

There are many forms of synaptic plasticity but in this review we will focus on NMDAR-dependent LTP. Most research on NMDAR-dependent LTP has been focused on synapses in the CA1 region due to the implication of the hippocampus in learning and memory (Bliss & Collingridge, 1993). LTP is defined as a persistent enhancement of postsynaptic response in a single cell or a population of neurons, and is typically induced by high frequency tetanic stimulation of the presynaptic neuron (Larson et al., 1986). As mentioned before, the voltage-dependent magnesium block allows NMDA receptor to behave as a synaptic coincidence detector, which is ideal for LTP induction. LTP is said to have three properties: cooperativity, associativity and input-specificity (Bliss & Collingridge, 1993). The cooperativity of LTP occurs when a greater potentiation in response results from the stimulation of a higher number of fibres in the direct input (Kelso et al., 1986). The associativity of LTP is demonstrated by the induction of weaker synaptic pathways as a result of the direct stimulation of fibres in an adjacent stronger pathway. Lastly, the input-specificity of LTP is the requirement for sufficient presynaptic terminal release of glutamate at the same time as the stimulation to elicit adequate postsynaptic NMDA receptor activation.

The mechanism of NMDAR-dependent LTP is thought to rely on the activation of downstream protein kinase pathways, which include protein kinase A (PKA), protein kinase C (PKC), CaMKII and mitogen-associated protein kinase (MAPK). However, studies have yielded opposing results. The inhibition of specific isoforms of PKCs has been shown to eliminate LTP, but only under specific induction protocols and it does not have a global effect on all sites of LTP (Abeliovich et al., 1993). In addition, the use of PKA inhibitors has produced mixed results in the inhibition of early or late forms of LTP (Bortolotto & Collingridge, 2000; Huang & Kandel, 1994; Otmaikova et al., 2000). On the other hand, the contribution of CAMKII to LTP is better characterised. The two isoforms of CaMKII containing α- or β-subunits are thought to have the most important roles in the CaMKII signalling cascade (Coultrap & Bayer, 2012). Upon activation, CaMKII moves to the PSD where its interaction with the GluN2B subunit of NMDA receptors is thought to be critical for LTP induction (Barria & Malinow, 2005). Mobile CaMKII has been shown to bind to AMPA receptors through Stargazin, which results in AMPA receptor translocation to the PSD (Shen & Meyer, 1999). In addition, the phosphorylation of the GluA1 subunit of AMPA receptors by activated αCaMKII isoforms in the PSD has been shown to potentiate postsynaptic response (Lisman et al., 2012). Moreover, binding of CaMKII to GluN2B has...
been shown to mediate the phosphorylation of AMPA receptors and potentiate synaptic response (Halt et al., 2012). The role of CaMKII is further supported by the observation of memory impairments and lack of LTP in αCaMKII knockout mice (Silva, Paylor, et al., 1992; Silva, Stevens, et al., 1992). The MAPK signalling cascade, which lies downstream of NMDA receptor activation, also plays a role in LTP expression. Activation of the MAPK cascade leads to hippocampal LTP in an isoform-dependent manner where p42 MAPK, but not p44 MAPK, results in a NMDAR-dependent synaptic potentiation (English & Sweatt, 1996). However, no alterations in LTP expression were observed in the neurons of mice lacking MAPK3 even though the animals exhibited impairments in learning functions (Selcher et al., 2001).

1.6. NMDA receptor as a therapeutic target

Since the activation of NMDA receptors has an important role in synaptic plasticity, NMDA receptor enhancers may be useful in treating cognitive dysfunction. The hypofunction of NMDA receptors has been suggested in cognitive disorders such as schizophrenia. NMDA receptor blockers such as phencyclidine (PCP) or ketamine have been shown to disrupt memory formation and cause schizophrenia-like symptoms in humans (Tsai & Coyle, 2002). Furthermore, transgenic mice with reduced NMDA receptor expression or function exhibit schizophrenic-like behaviour (Mohn et al., 1999). However, in another study, the behaviour of transgenic mice with reduced GluN1 expression was interpreted to be more similar to the behavioural manifestations of autism spectrum disorders (ASDs) rather than schizophrenia (Gandal et al., 2012). Therefore, NMDA receptor enhancers may potentially prove to be beneficial in the treatment of ASD.

In addition to being a crucial player in synaptic transmission and plasticity, NMDA receptors are also a key mediator of excitotoxicity in neurons (Cull-Candy & Leszkiewicz, 2004). Excitotoxicity usually occurs as a result of excess levels of glutamate in the extracellular space. The glutamate activates large numbers of AMPA receptors, which in turn causes the depolarisation of the postsynaptic membrane and subsequent activation of NMDA receptors and calcium ion influx (Lynch & Guttmann, 2002). The increased intracellular levels of calcium ion activates multiple processes that are potentially toxic to the cell, including the production of nitric oxide, reactive oxygen species, as well as the activation of calpain, a
protein involved in apoptosis (Brorson et al., 1995). Excitotoxicity has been implicated in multiple CNS disorders including epilepsy, Parkinson’s disease, Huntington’s disease, hypoxia and ischemia (Lynch & Guttmann, 2002), which calls for the development of NMDA receptor neuroprotective agents such as antagonists. This approach appears simple in theory but has proved difficult in practise.

1.6.1. NMDA receptor-targeting treatments

There are many NMDA receptor antagonists currently in existence and they vary in their mechanisms of action. However, the side effects that accompany their use have hampered their clinical development. Most NMDA receptor antagonists target a specific site on the NMDA receptor complex and can be categorised as allosteric modulators or channel blockers (Popescu et al., 2010) (Figure 1.5). One example of an ion channel blocker is memantine, which is a non-selective antagonist that binds to all NMDA receptor subtypes (Johnson & Kotermanski, 2006). Memantine is currently in use for treating moderate to severe AD. However, its inability to discriminate between NMDA receptor subtypes can lead to unwanted side effects such hallucinations at high doses (Monastero et al., 2007). On the other hand, GluN2B-selective NMDA receptor antagonists such as ifenprodil has been shown to not induce side effects seen with non-selective antagonists, which provides an added advantage (Carter et al., 1989). Furthermore, zinc has demonstrated high affinity binding to GluN2A subunits at the same N-terminal domain region where ifenprodil binds to GluN2B (Karakas et al., 2009). Selective antagonism may be the answer to the successful development of an NMDA receptor antagonist without adverse effects.

NMDA receptor enhancers have had less development compared to antagonists. Due to the risk of excitotoxicity from the direct use of glutamate, attention has been focused on other agents to increase NMDA receptor function. Examples include NMDA receptor glycine site agonists such as D-serine and D-cycloserine, which have demonstrated therapeutic efficacy in treating cognitive deficits in schizophrenia (Heresco-Levy et al., 2002). There is potential for further development of other NMDA receptor enhancers to make use of the many binding sites that are available for targeting by allosteric modulators (Popescu et al., 2010). However, complete antagonism or activation of NMDA receptor function can interrupt normal physiological processes in the CNS. Therefore, NMDA receptors activity has to be carefully modulated in order for benefits to outweigh adverse effects.
Figure 1.5 Binding sites on the NMDA receptor

Schematic diagram of a GluN1/GluN2 receptor showing its various binding sites. The extracellular region of each subunit is made up of two globular domains, the N-terminal domain (NTD) and the ligand/agonist binding domain (ABD). The ABD is connected to the transmembrane domain (TMD), which is made up of three membrane-spanning helices (TM1, TM2 and TM4) as well as a re-entrant loop (M2). The intracellular region of each subunit consists mostly the C-terminal domain (CTD).

1.6.2. Antibody treatment against NMDA receptors

Our laboratory has previously described a novel oral genetic GluN1 vaccine which has demonstrated therapeutic efficacy in animal models of stroke and epilepsy (During et al., 2000). The study utilised recombinant adeno-associated viral (AAV) vectors for gene delivery in rats, which resulted in long-term transgene expression in cells residing in the lamina propria of the intestine that lasted for at least 5 months. The expression of GluN1 led to the production of high titres of autoantibodies against the native GluN1 subunit. Animals treated with the oral vaccine were challenged with kainate to induce seizures or subjected to a middle cerebral artery occlusion (MCAO) as the ischemic insult. The GluN1 treatment reduced kainate-induced seizure in animals and offered neuroprotection from seizure-induced injuries. In addition, the infarct volume following MCAO was significantly smaller in treated animals.
Other studies have also investigated the effects of anti-GluN1 antibodies in the context of stroke. Benchenane et al. (2007) generated specific anti-GluN1 antibodies which target the N-terminal tissue plasminogen activator cleavage site in mice by immunisation with recombinant GluN1 N-terminal domain. The treatment was shown to reduce infarct lesion size following MCAO. Similar results were reported in two later studies which described neuroprotection in mice expressing antibodies against the GluN1 N-terminal domain in a MCAO model as well as a focal cerebral ischemia model of stroke (Macrez et al., 2010; Macrez et al., 2011).

In addition to the neuroprotective properties of anti-GluN1 antibodies, our laboratory has also observed cognitive-enhancing effects from the antibody treatment in recent studies. Rats immunised with a recombinant GluN1 protein (recNR1), which consists of the two extracellular domains, produced high titres of antibodies against the GluN1 subunit and performed better in learning and memory behavioural tests including the novel object recognition test and the step-through passive avoidance test (Chen, 2010). In a later study, immunoglobulin G (IgG) was purified from sera collected from rats in the first study and used for passive transfer in mice (Chen, 2015). Mice treated with IgG from recNR1-immunised animals also showed improved performance in the same battery of behavioural tests. These results indicate that our anti-GluN1 antibodies possess both neuroprotective and cognitive-enhancing properties, which are ideal qualities of an NMDA receptor modulator, and may suggest a partial agonist-like effect. However, the cellular mechanisms that mediate these behavioural changes are unknown and require characterisation.

1.7. Autism spectrum disorder

ASDs are a range of neurodevelopmental disorders characterised by severe impairments in social interaction, communication skills, and the presence of stereotypical, repetitive behaviours (Wing & Gould, 1979). Cognitive impairments, seizures, and bowel disorders are frequent comorbidities that accompany ASD, in addition to hyperactivity, attention deficits, anxiety, and obsessive-compulsive impulses (Bauman & Kemper, 2005). The prevalence of ASD has risen over time and is now estimated to be approximately 1 in 68 individuals (Centers for Disease Control and Prevention, 2016). Although genetic evidence from twin studies have shown ASD to be highly heritable (Rosenberg et al., 2009; Taniai et al., 2008),
the identification of genes associated with susceptibility have been difficult due to the heterogeneous nature of the syndrome.

1.7.1. Excitatory/inhibitory imbalance in ASD

Based on extensive evidence of glutamate- and gamma-aminobutyric acid (GABA)-related abnormalities found in the brains of ASD individuals, a recently proposed hypothesis of ASD etiology describes an imbalance of the excitatory and inhibitory systems (Rubenstein & Merzenich, 2003). Findings from a number of studies are in support of this hypothesis. Approximately 30% of ASD individuals experience epileptic seizures at some point in their lives and around 60% of individuals display abnormal epileptiform electroencephalogram activity during sleep (Chez et al., 2007; Spence & Schneider, 2009). In addition, an underdevelopment of inhibitory neurons in certain brain regions of ASD individuals has been suggested (Courchesne & Pierce, 2005). Other alterations in the inhibitory system include low levels of glutamate decarboxylase levels as well as reduced GABA-A and GABA-B receptor numbers in brain regions that are associated with ASD behavioural phenotypes (Oblak et al., 2010; Oblak et al., 2011). Furthermore, increased levels of glutamate have been detected in the sera of ASD individuals, which positively correlate with levels in the brain and social scores (Hassan et al., 2013; Shinohe et al., 2006). The results from these studies suggest that the CNS environment in ASD individuals is hyperglutamatergic. Most preclinical and clinical studies for NMDA receptor modulatory treatments in ASD have focused on the use of partial agonists (Yang & Chang, 2014). Depending on the level of receptor activation, partial agonists can behave as agonists or antagonists (Watson et al., 1990), which may be a suitable treatment approach for ASD where the excitatory and inhibitory systems require rebalancing.

1.7.2. Shank3 and ASD

As mentioned before, Shank proteins are key molecular scaffolding proteins in the PSD which facilitate signalling of glutamate receptors including NMDA receptors. Structurally, Shank proteins are characterised by the expression of the following binding domains: multiple ankyrin repeat domain in the N-terminus, SH3 domain, PDZ domain, proline-rich region containing Homer and cortactin binding sites, and the sterile alpha motile domain (Sheng & Kim, 2000). Three Shank isoforms have been identified and they vary in their expressions of protein binding domains (Figure 1.6). Mutations have been identified in all
three Shank isoforms but Shank3 mutations have been the most extensively studied (Jiang & Ehlers, 2013). Disruption in the Shank3 gene is a major genetic cause of ASD, with mutations found in approximately 1% of individuals (Moessner et al., 2007). Shank3 mutations have been shown to result in various changes in synaptic protein expression, ASD-like behavioural phenotype, and altered synaptic plasticity both in vitro and in vivo. Mice with haploinsufficiency in Shank3 expression exhibited impairments in basal synaptic transmission and LTP induction (Bozdagi et al., 2010). Moreover, mice with disrupted expression of Shank3 displayed ASD-like behavioural deficits with impairments in LTP induction but had normal synaptic transmission (Wang et al., 2011). Another study deleted specific isoforms of Shank3 in mice, which resulted in various ASD-like behavioural phenotypes such as anxiety-like behaviours, reduced social interaction, and excessing grooming (Peça et al., 2011). Also, deletion of the Shank3 Homer binding domain-containing C-terminus in mice resulted in deficits in hippocampal-dependent learning and reduced LTP in the hippocampus (Kouser et al., 2013). On the other hand, Shank3 mutations and knockdown in cultured hippocampal neurons are associated with decreased AMPA and NMDAR-mediated currents and excitatory synapse expression (Arons et al., 2012). Shank3 deficiency in cultured cortical neurons as a result of knockdown reduced both NMDAR-mediated currents and surface expression of GluN1 (Duffney et al., 2013).

Figure 1.6 Shank protein isoforms
1.8. Thesis outline

The overall aim of the research presented in this thesis was to elucidate the underlying mechanisms for the effects of anti-GluN1 IgG previously reported by our laboratory, which include neuroprotective properties in animal models of stroke and epilepsy (During et al., 2000) and cognitive-enhancing properties in GluN1 protein-immunised and passively transferred animals (Chen, 2010; Chen, 2015). However, these beneficial observations are in stark contrast to the detrimental effects of GluN1 N-terminal-targeting anti-NMDA receptor encephalitis patient antibodies (Dalmau et al., 2008; Gleichman et al., 2012; Hughes et al., 2010). Therefore, we hypothesised that anti-GluN1 IgG treatment may modulate NMDA receptors to cause changes in excitatory synaptic protein expression and function, and that epitope-dependent effects may arise from anti-GluN1 IgG targeted against different extracellular regions of the GluN1 subunit. Based on our past findings of improvements in learning and memory, we also decided to investigate the potential therapeutic application of anti-GluN1 IgG treatment in ASD, where cognitive deficits are presented at varying degrees of severity. We chose to examine the effects of anti-GluN1 IgG in neurons with Shank3 dysfunctions associated with ASD due to strong evidence of impairments in synaptic transmission and protein expression, including that of NMDA receptors both in vitro and in vivo (Arons et al., 2012; Bozdagi et al., 2010; Kouser et al., 2013; Peça et al., 2011; Wang et al., 2011).

In order to test our hypotheses, the specific aims of this thesis were:

1) To produce recombinant GluN1 proteins for immunisation in rats to generate anti-GluN1 antibodies with different sets of epitopes, and to validate purified IgG preparations prior to use in primary rat hippocampal neuronal cultures;

2) To investigate the effects of anti-GluN1 IgG treatment at the cellular level in primary hippocampal neurons, specifically on changes in protein expression in the excitatory glutamatergic synapse;

3) To determine if changes in glutamate receptor expression following anti-GluN1 IgG treatment is translated to coordinated functional modifications in excitatory synaptic transmission in primary hippocampal neurons;
4) To examine the therapeutic potential of anti-GluN1 IgG treatment in primary hippocampal neurons expressing ASD-associated Shank3 mutations or with Shank3 knockdown in a pilot study to determine whether it is capable of normalising deficits in the excitatory synapse.
Chapter 2.
Production of recombinant proteins and generation of anti-GluN1 IgG
2.1. Introduction

The primary aim of this chapter was to produce recombinant proteins and use them to generate IgG against the GluN1 subunit of the NMDA receptor in rats by immunisation. IgG purified from rat sera were thoroughly validated prior to use in the primary cell culture experiments detailed in subsequent chapters.

Firstly, three recombinant proteins were produced in this chapter. Firefly luciferase (Luc) was chosen as a control for the work in this thesis because it is a well-characterised protein commonly used as a reporter. It is a foreign protein that is non-toxic and has previously demonstrated immunogenicity in rats (Chen, 2010; Dicker, 2010). Furthermore, anti-Luc antibodies are unlikely to target or alter endogenous protein expressions. In addition to Luc protein, two different recombinant GluN1 proteins were produced to investigate potential epitope-dependent effects of anti-GluN1 antibodies. The first protein was recNR1, which contained the two GluN1 extracellular domains connected by a peptide bridging sequence. The second protein was NR1extra2, which consisted of the extracellular loop between M3 and M4.

The pET and pTriEx 1.1 expression systems we used for large-scale recombinant protein production both contained the T7 promoter, which forms the basis of the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible protein expression platform in an E. coli host (Baneyx, 1999). The expression constructs used for the three recombinant proteins also contained a polyhistidine (HIS) tag which enabled purification via immobilised metal affinity chromatography (IMAC) (Bornhorst & Falke, 2000). HIS-tagged proteins can be rapidly purified and eluted from the affinity purification column using buffers containing high concentrations of imidazole.

Purified protein preparations were used to immunise rats and stimulate generation of antibodies against their respective immunogen over the period of three months. The main goal of the immunisation regimen was to acquire control and anti-GluN1 IgG samples for characterisation. Although Luc and recNR1 proteins have been used in previous studies by our laboratory to assess alterations in animal behaviour and CNS protein expressions following immunisation (Chen, 2010; Dicker, 2010), this thesis was the first to immunise animals with the NR1extra2 protein. Observations made during welfare checks after
immunisations indicated a potential difference in animal behaviour between treatment groups, which prompted an open field test to be conducted before the final blood collection.

Following immunisation in rats, we utilised the caprylic acid precipitation method to purify IgG from collected sera as an alternative to conventional methods. The caprylic acid method is quick, inexpensive, and only requires a basic set up. It has been shown to produce better yields of IgG compared to ammonium sulphate precipitation, ion exchange chromatography and high pressure liquid chromatography (Page & Thorpe, 1996; Russo et al., 1983; Temponi et al., 1989). However, IgG isolated by the caprylic acid method may be of lower purity depending on the quality of the starting material and concentration of caprylic acid used.
2.2. Materials and methods

2.2.1. Recombinant protein production in *E. coli* host

2.2.1.1. Plasmids

Plasmid constructs used for the work in this chapter were previously cloned and verified by former members of our laboratory. All constructs used for protein production contained an ampicillin resistance gene for bacterial selection. Within each construct, a HIS-tag was fused to the complementary DNA (cDNA) of the protein of interest, which was required for denaturing IMAC purification using the Profinia™ Protein Purification System (Bio-Rad). The pET-Luc construct (Figure 2.1 A) contained the cDNA for the full-length 65 kDa recombinant firefly luciferase protein (Luc), cloned into the pET100/D-TOPO vector by Dr Jerry Wong. The pTriEx-recNR1 construct (Figure 2.1 B) contained the cDNA sequence encoding the 83 kDa recombinant mouse recNR1 protein, which comprised of the two extracellular domains of GluN1 joined by a bridging sequence (Figure 2.2). The N-terminus and the loop between TM3 and TM4 (M3-M4) were fused via a linker sequence and cloned into the pTriEx 1.1 vector by Dr Kevin C.E. Little. The pET-NR1extra2 construct (Figure 2.1 C) contained the cDNA sequence encoding the 20 kDa recombinant mouse NR1extra2 protein, which was the GluN1 M3-M4 extracellular loop (Figure 2.2). The sequence was cloned into the pET3 vector by Ms Claudia B. Klugmann. Plasmid maps were generated with DNA sequence data using Vector NTI software (InforMax).
Figure 2.1 Plasmid constructs used for recombinant protein production

Plasmid maps of pET-Luc (A), pTriEx-recNR1 (B) and pET-NR1extra2 (C). Full maps are shown for the pET-Luc and pTriEx-recNR1 plasmids while a partial map is shown for pET-NR1extra2. Each map depicts the location of various restriction enzyme cleavage sites, as well as the size and position of the cDNA sequence for the protein of interest. The position of the HIS-tag in each construct is also shown, specifically, at the N-terminus for the Luc cDNA sequence, and the C-termini for the recNR1 and NR1extra2 cDNA sequences.
Figure 2.2 Schematic diagram of the GluN1 subunit and the boundaries of recombinant GluN1 (recNR1 and NR1extra2) proteins

The diagram shows the structure of GluN1 subunit in the plasma membrane and its protein domains. The subunit is made up of three hydrophobic transmembrane domains (TM1, TM3, TM4) and a re-entry loop (TM2). The N-terminal domain and ligand-binding domain are located extracellularly while the C-terminal domain is located intracellularly. Two separate extracellular regions (S1 and S2 domains) form the ligand-binding domain, which binds the co-agonist glycine. The diagram also depicts the amino acid boundaries of the recombinant recNR1 and NR1extra2 proteins in the extracellular domains. The recNR1 protein spans both GluN1 extracellular domains, encompassing amino acids 1-562 and 654-812. The NR1extra2 protein only contains the second extracellular loop, which comprises the amino acids 654-812.
2.2.1.2. Bacterial transformation

*E. coli* BL21 (DE3) strain competent cells (Novagen) were used because they contain the phage T7 RNA polymerase gene under the control of an inducible *lac*UV5 promoter which can be activated by IPTG. An aliquot (25 µL) of BL21 (DE3) competent cells was removed from -80 °C storage and thawed on ice. Next, 0.5-1 µg of plasmid DNA was added to the cells and mixed gently by tapping. Cells were incubated on ice for 30 min before heat shock transformation was performed at 42 °C for 1 min to allow for DNA uptake. Cells were then immediately placed on ice to allow for membrane reformation and 100 µL of Miller’s Luria-Bertani broth (LB) (Invitrogen, 12795-027) without ampicillin was added. The suspension was incubated at 37 °C with shaking at 220 RPM for 1 hour. LB agar plates were prepared by autoclaving 37 g of Miller’s LB Agar (Millipore, 1102830500) in 1 L of MilliQ water, adding 50 µg/mL ampicillin (Fisher BioReagents, BP1760-25) when the agar has cooled down to 56 °C, and pouring 20 mL in each sterile Petri dish. After the agar sets, plates were stored at 4 °C until use. Following incubation, transformed cells were spread onto an LB agar plate. The bacterial culture was then incubated at 37 °C overnight for up to 16 hours.

2.2.1.3. Bacterial culture

Starter culture medium for 1 L of general culture was prepared by adding 4 mL of filter sterile LB containing 10% (w/v) glucose and 120 µL of 25 mg/mL ampicillin to 36 mL of sterile LB. The 40 mL of LB containing 1% (w/v) glucose and 75 µg/mL ampicillin was inoculated with 3-5 transformant colonies from the LB agar plate and cultured at 37 °C with shaking at 220 RPM overnight. A general culture was grown from the starter culture following overnight incubation. General culture medium was prepared by adding 50 mL of LB containing 10% (w/v) glucose and 1.5 mL of 25 mg/mL ampicillin to 450 mL of sterile LB in a 2 L conical flask to ensure adequate aeration. A small volume of general culture medium was extracted to use as a blank in the spectrophotometer. The 500 mL of LB containing 1% (w/v) glucose and 75 µg/mL ampicillin was inoculated with 20 mL of starter culture and grown at 37 °C with shaking at 220 RPM until the optical density measurement at 600 nm (OD\textsubscript{600}) has reached 0.6. The OD\textsubscript{600} was measured using a GeneQuant™ pro RNA/DNA Calculator spectrometer (Biochrom). The rate of growth may be improved by preheating the sterile LB without glucose and ampicillin in the incubator the previous night.
2.2.1.4. Induction of protein expression

Upon reaching 0.6 OD\textsubscript{600}, production of recombinant protein was induced by adding 1.25 mL of 400 mM filter sterile IPTG (AppliChem, A4773.0005) to 500 mL of general culture medium to obtain a final concentration of 1 mM. The induced culture was grown for a further 3 hours under the same condition. Cells were then harvested by centrifugation at 6,000 x g, 4 °C for 10 min. Bacterial pellets were stored at -20 °C or lysed immediately.

2.2.1.5. Bacterial lysis

Cell pellet from 1 L of culture was resuspended in 80 mL of bacterial lysis buffer (50 mM KCl, 50 mM KH\textsubscript{2}PO\textsubscript{4}, pH 8.0) by vigorous pipetting. The cell suspension was divided into 20 mL aliquots in 50 mL centrifuge tubes. To lyse bacterial cells, 12.5 µL of 300 U/µL rLysozyme (Novagen, 71110-3) was added to each tube. Luc preparation lysate was incubated at room temperature (RT) with gentle shaking for 20 min, whereas recNR1 and NR1extra2 preparation lysates were incubated on ice with gentle shaking for 1 hour. Lysates were then sonicated (Misonix XL-2020 Sonicator) on ice at output level 4, 1 min pulsar on, 10 sec pulsar off, for a total processing time of 10 min. Following sonication, lysates were centrifuged at 6,000 x g, 4 °C for 15 min to pellet insoluble inclusion bodies. The supernatant was discarded and the pellet was resuspended in 20 mL of Denaturing IMAC Wash Buffer #1 (300 mM KCl, 50 mM KH\textsubscript{2}PO\textsubscript{4}, 5 mM Imidazole, 6 M Urea, pH 8.0) by vigorous pipetting and vortexing. The suspension was then sonicated on ice using the aforementioned settings for 10 min to solubilise inclusions before it was centrifuged at 6,000 x g, 4 °C for 45 min. The supernatant was retained and stored at 4 or -20 °C until purification.

2.2.1.6. Purification of recombinant proteins

The Profinia\textsuperscript{TM} instrument, an automated chromatography system, was used for the purification of HIS-tagged recombinant proteins from bacterial lysate. Lysates were filtered through 5 µm pore size Acrodisc\textsuperscript{®} 32 mm Syringe Filters (Pall, 4650) prior to loading to prevent clogging of the Profinia cartridge. Bio-Scale Mini Profinia IMAC cartridges (5 mL) (Bio-Rad, #732-4614) were used in conjunction with the preprogrammed Denaturing IMAC purification method on the Profinia system. The preset low sample loading flow rate was used to improve the yield of low-expression proteins. Also, extended cartridge wash time was selected for the protocol to improve protein purity. For each purification run, ~40 mL of filtered lysate was loaded into the cartridge to allow binding. In brief, flowthrough containing
unbound protein was first flushed out of the cartridge followed by a wash with 30 mL of Denaturing IMAC Wash Buffer #1. The cartridge was then washed for a second time with 30 mL of Denaturing IMAC Wash Buffer #2 (300 mM KCl, 50 mM KH₂PO₄, 10 mM Imidazole, 6 M Urea, pH 8.0). Following the wash steps, column-bound proteins were eluted with 15 mL of Denaturing IMAC Elution Buffer (300 mM KCl, 50 mM KH₂PO₄, 250 mM Imidazole, 6 M Urea, pH 8.0) and collected into a 50 mL centrifuge tube. Eluates of purified recombinant protein were stored at 4 or -20 °C until concentration and dialysis. The Profinia system generates a chromatogram which shows the sample loading, wash steps and protein elution ultraviolet (UV) absorbance profile for each purification run. These were saved and accessed using the Profinia Software Version 1.0. All solutions required for the Profinia Denaturing IMAC purification method are detailed in the Appendix.

2.2.1.7. Concentration of recombinant proteins

To concentrate purified protein solutions, eluates of the same recombinant protein were pooled and spun down using 15 mL Amicon (Ultra-15) centrifugal filter devices with an appropriate molecular weight cut-off (MWCO) pore size: 30K MWCO (Millipore, UFC903008) for Luc and recNR1 proteins; 3K MWCO (Millipore, UFC900308) for NR1extra2 protein. Each protein sample was loaded to the maximum volume of 12 mL for fixed angle rotors. Tubes were then centrifuged at 5,000 x g, 4 °C, for 15-20 min each time, until a final total volume of 20-25 mL was obtained for each protein.

2.2.1.8. Dialysis of recombinant proteins

Purified protein solutions were dialysed against phosphate-buffered saline (PBS) solution (1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 137 mM NaCl, 1.5 mM KCl, pH 7.4) to remove urea following protein concentration. Slide-A-Lyzer dialysis cassettes with a MWCO of 10K and 12-30 mL capacity (Thermo Scientific, #66830) were used for dialysis. Cassettes were first hydrated in PBS, which increases membrane flexibility and allows safe sample loading. Each protein sample was loaded into a separate cassette by using a 30 mL syringe and an 18 gauge 1 inch bevelled needle. The syringe was filled up with the protein sample and a small amount of air. The needle was then inserted into one of the ports located at the top of the cassette. Approximately half of the sample volume was slowly injected to prevent foaming, followed by the withdrawal of some air from the cassette cavity. The rest of the sample was injected and the remaining air was removed from the cavity to compress the membranes, thereby
maximising the surface area contacting the dialysing PBS solution. The needle was carefully retracted from the cassette, sealing the gasket. The sample was dialysed in 800 volumes of PBS (20 L per 25 mL of sample) at 4 °C for at least 2 hours with constant stirring in a large container. The buffer was then replaced with fresh PBS and dialysis was conducted for a further 2 hours before a final buffer change. Overnight dialysis was carried out and the protein sample was retrieved from the cassette the next day with a syringe and needle. A small amount of air was gently injected into the cassette first to inflate and separate the membranes before sample retrieval. Dialysed protein samples were stored in 5 mL aliquots at -20 °C.

2.2.2. Analysis of recombinant protein preparations

2.2.2.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Samples of recombinant protein purification fractions were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for analysis. Samples were mixed 1:1 with 2X reducing sample buffer (RSB) (125 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 20% (v/v) Glycerol, 0.02% (w/v) Bromophenol blue, 5% (v/v) β-mercaptoethanol) before heated at 95 °C for 5 min to denature proteins. A 12% running acrylamide gel (375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) Ammonium persulphate (APS), 12% (w/v) Acrylamide, 0.04% (v/v) TEMED) was cast using a Mini-PROTEAN 3 casting stand (Bio-Rad) and ~0.5 mL 50% (v/v) isopropanol was applied along the top of the gel to flatten the running gel. The isopropanol was poured off after approximately 30 min when the running gel has set. An acrylamide stacking gel (125 mM Tris-HCl, pH 6.7, 0.1% (w/v) SDS, 0.1% (w/v) APS, 3.75% (w/v) Acrylamide, 0.1% (v/v) TEMED) was poured on top of the polymerised running gel until it reaches the brim of the glass template, after which a 10-well comb was inserted. Once the stacking gel has set, the comb was removed and the gel cassette was inserted into a Mini-PROTEAN 3 Cell (Bio-Rad) tank containing SDS-PAGE electrophoresis tank buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS). Samples were loaded at equal volumes (10 µL) per well, alongside a well loaded with Broad Range SDS-PAGE standards (Bio-Rad) for determining molecular weights. The gel was first electrophoresed at a constant voltage of 150 V until samples were compressed through the stacking gel. A constant voltage of 200 V was
then used for 50 min or until the dye front has reached the bottom of the gel. The gel was carefully removed from the template following electrophoresis.

### 2.2.2.2. Coomassie blue staining

The SDS-PAGE gel was placed into fixative solution (40% (v/v) Methanol, 10% (v/v) Acetic acid) with gentle shaking for 5 min, briefly washed with distilled water (dH$_2$O), and incubated in Coomassie blue staining solution (0.05% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad, #161-0400), 50% (v/v) Methanol, 10% (v/v) Acetic acid) with gentle shaking for no more than 30 min. Excess dye was removed from the gel by incubating in destaining solution (5% (v/v) Methanol, 7% (v/v) Acetic acid) with a piece of paper towel.

### 2.2.2.3. Quantification of protein concentration

The concentration of each purified recombinant protein preparation was determined using bovine serum albumin (BSA) standards. Duplicate samples of 0.5, 1.0 and 2.0 µg of BSA (Gibco, 30036578) were run alongside duplicate samples of the recombinant protein preparation with a known volume in an SDS-PAGE gel (see Section 2.2.2.1.). The gel was subsequently stained with Coomassie blue (see Section 2.2.2.2). A scanner was used to digitally capture the image of the gel onto the computer, where it was analysed using the Multi-Gauge Software Version 3.0 (Fuji Film). A linear standard curve was generated by plotting the integrated density of each BSA standard band against the amount of protein present (Figure 2.3). Protein concentration of the full-length protein was determined from the most prominent band at the expected molecular weight.
Figure 2.3 Quantification of protein concentration using Multi-Gauge Software

An example image of an SDS-PAGE gel stained with Coomassie blue used for protein concentration quantification (top). BSA standards at 0.5 µg (lanes 1, 2), 1.0 µg (lanes 3, 4) and 2.0 µg (lanes 5, 6) were loaded in duplicate and ran alongside the protein of interest (lanes 7, 8). Protein bands were highlighted using Multi-Gauge Software Version 3.0 (Fuji Film) and a linear standard curve was generated (bottom) to determine the concentration of the full-length recombinant protein.
2.2.2.4. Western blotting

Western blots were performed to assess the purity of each recombinant protein preparation. Acrylamide gels (12%) were cast and run as described in Section 2.2.2.1. Recombinant proteins preparations were loaded at 1 µg per lane. While the SDS-PAGE is running, two pieces of extra thick blot filter paper (Bio-Rad, 1703960), and one piece of Amersham Hybond™ ECL™ nitrocellulose membrane (GE Healthcare) were cut into sizes suitable for the Mini-PROTEAN gel and soaked in transfer buffer (48 mM Tris, 39 mM Glycine, 20% (v/v) Methanol, 0.0375% (w/v) SDS) for at least 15 min at RT. Following electrophoresis, the gel was removed and equilibrated in two exchanges of transfer buffer, for 5 min each. A ‘sandwich’ was assembled in a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad) in the following order:

<table>
<thead>
<tr>
<th>Blot paper</th>
<th>Gel</th>
<th>Nitrocellulose membrane</th>
<th>Blot paper</th>
</tr>
</thead>
</table>

Protein transfer was carried out at 15 V for 45-60 min. The ‘sandwich’ was disassembled and the nitrocellulose membrane was incubated in Ponceau S solution (Sigma, P7170) for 5 min with gentle shaking to visualise proteins and confirm proper transfer. After a brief wash in dH₂O, the Broad Range standard bands were marked using an oil-based ballpoint pen. The membrane was then fully destained in tris-buffered saline (TBS)-T (20 mM Tris, 500 mM NaCl, pH 7.5, 0.1% (v/v) Tween 20) for 5 min before the blot was blocked with TBS-T containing 5% (w/v) skim milk powder for 1 hour at RT with shaking. Following the blocking step, the membrane was incubated in primary antibody diluted in TBS-T containing 1% (w/v) skim milk powder (see Table 2.1 for primary antibodies and dilutions) at 4 °C overnight with shaking. The membrane was washed three times with TBS-T, for 5 min each, and then incubated in horseradish peroxidase (HRP)-conjugated secondary antibody of the appropriate target species diluted in TBS-T (see Table 2.2 for secondary antibodies and dilutions) for 2 hours at RT with shaking. Once again, the membrane was washed three times with TBS-T, for 5 min each, before the blot was developed. The Amersham ECL™ Prime Western Blotting Detection chemiluminescence reagent (GE Healthcare) was applied to the membrane, using ~1 mL of reagent mix per full-sized membrane. Protein bands were
visualised and images were acquired using the ChemiDoc™ MP Imaging System (Bio-Rad) and its accompanying software Image Lab™ Software Version 4.1 (Bio-Rad).

**Table 2.1 List of primary antibodies used for Western blotting with recombinant protein preparations**

Details of primary antibodies used to detect presence of recombinant Luc, recNR1 and NR1extra2 proteins including the host species, antibody source and the working dilution.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Company</th>
<th>Catalogue no.</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Luciferase</td>
<td>Rabbit</td>
<td>Fitzgerald</td>
<td>70C-CR2029RAP</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Anti-GluN1 (N-term)</td>
<td>Mouse</td>
<td>Neuromab</td>
<td>75-272</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Anti-GluN1 (M3-M4)</td>
<td>Mouse</td>
<td>BD Pharmingen</td>
<td>556308</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>

**Table 2.2 List of secondary antibodies used for Western blotting with recombinant protein preparations**

Details of secondary antibodies used to detect presence of recombinant Luc, recNR1 and NR1extra2 proteins including the host species, antibody source and the working dilution.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Company</th>
<th>Catalogue no.</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP-conjugated Anti-rabbit IgG</td>
<td>Goat</td>
<td>Santa Cruz</td>
<td>sc-2004</td>
<td>1:5,000</td>
</tr>
<tr>
<td>HRP-conjugated Anti-mouse IgG</td>
<td>Goat</td>
<td>Santa Cruz</td>
<td>sc-2005</td>
<td>1:5,000</td>
</tr>
</tbody>
</table>
2.2.3. Recombinant protein immunisation in rats

2.2.3.1. Animals

Adult male Sprague Dawley rats weighing 200-250 g were obtained from the Vernon Jansen Unit (Faculty of Medical and Health Sciences, University of Auckland). All animal experiments were carried out in accordance with the guidelines for animal care, under the approval of the University of Auckland Animal Ethics Committee (AEC number 1038). Animals were housed in the Vernon Jansen Unit with regulated humidity and temperature, a 12/12-hour light/dark cycle and had *ad libitum* access to rodent diet (Harlan Teklad 2018 diet, Harlan) and water.

2.2.3.2. Immunisation and blood collection schedule

Fifteen rats were randomly allocated to the following experimental groups: PBS (*n* = 2), Luc (*n* = 3), recNR1 (*n* = 5) and NR1extra2 (*n* = 5). In addition to the Luc protein control, a PBS treatment group was included as an immunisation control. Prior to the initial priming shot, a pre-immune blood sample was collected from each animal. Rats were then administered with a subcutaneous injection of 100 µg recombinant protein or an equivalent volume of PBS treatment solution. All animals received a total of six boosting shots following the priming shot, which were 50 µg protein each and injected once every fortnight. Blood sampling also took place before each boosting shot. Terminal intracardiac blood collection was carried out on Week 13, one week after the final boost.
Figure 2.4 Timeline of the immunisation schedule

The immunisation schedule included pre-immune blood sampling and the priming shot (100 µg protein) at Week 0, followed by blood sampling and boosting shots (50 µg protein) every two weeks until Week 12 when an open field test was also conducted, and the final intracardiac blood collection at Week 13.

2.2.3.3. Preparation of recombinant protein preparations for immunisation

All recombinant protein preparations were diluted to the same concentration as the least concentrated protein determined in Section 2.2.2.3, thereby ensuring the treatments received by all animals were of equal volume. Protein preparations were diluted in PBS and mixed 1:1 with Pierce Imject Alum adjuvant (Thermo Scientific, 77161) by dropwise addition with constant stirring in a 50 mL centrifuge tube. Similarly, PBS treatment solution was prepared by mixing one volume of PBS with an equal volume of adjuvant. Each immunogen/alum mixture was left to stir for a further 30 min to facilitate adsorption of the protein immunogen before it was ready to be used for immunisation.
2.2.3.4. Blood sampling and processing

Blood samples were collected from each animal via the tail vein before they received the priming shot (pre-immune) and each subsequent boosting shot. Each rat was carefully and lightly restrained while its tail was immersed in warm water for around 2 min to encourage vasodilation. The tail was then removed from the water, quickly dried and wiped with 70% ethanol before a small 1-2 mm incision was made on the tail vein with an 18 gauge needle or a scalpel blade. Gentle rubbing of the tail was applied to stimulate blood flow and aid blood collection. Approximately 0.3 mL of blood was collected into a 1.5 mL microfuge tube. When collection was completed, pressure was applied to the area of incision to stem the bleeding. Blood samples were allowed to clot for 1 hour at RT in an upright position before they were centrifuged at 17,900 x g for 10 min at RT. The serum supernatant was then transferred into new 1.5 mL microfuge tubes and stored at -20 °C.

2.2.3.5. Subcutaneous injections

Immunogen/alum mixtures prepared in Section 2.2.3.3 were delivered via the subcutaneous route. Each rat was firmly restrained around the head and hip areas and gently pressed against the bench surface. The loose skin between the shoulders was lifted to form a ‘tent’. For injections, 25 gauge needles and 1 mL syringes were used. The needle was inserted in a parallel manner with the bevel facing up at the base of the ‘tent’, close to the animal’s body. Prior to injecting, the syringe was gently aspirated to check that the needle did not enter a blood vessel. Once confirmed, the immunogen/alum mixture was injected and the needle was removed while gently pressing on the injection site to seal it.

2.2.3.6. Monitoring IgG production

Serum samples obtained from animals injected with protein were used to monitor the production of IgG against their respective immunogen over the course of the immunisation period. Western blotting was performed as described in Section 2.2.3.4 with a few adjustments. A 15-well 12% acrylamide gel was used to resolve proteins. After proteins were transferred from the SDS-PAGE gel to the nitrocellulose membrane and stained with Ponceau S solution, lanes were labelled with an oil-based ballpoint pen and carefully cut into individual strips using a clean razor blade. This allowed multiple serum samples to be screened simultaneously by incubating and washing membrane strips in compartmented containers. Rat serum diluted in TBS-T containing 1% (w/v) skim milk powder was used as
the primary antibody, with Luc and recNR1 sera at 1:10,000 and NR1extra2 serum at 1:5,000. Goat HRP-conjugated anti-rat IgG antibody (Santa Cruz, sc-2006) diluted in TBS-T was used as the secondary antibody at 1:5,000.

### 2.2.3.7. General health checks and behaviour monitoring

General health checks were performed on animals by recording body weights and assessing the condition of injection sites. Notable changes in animal behaviour were also recorded. This was done for three consecutive days following immunisation and on a weekly basis throughout the course of the immunisation period.

### 2.2.3.8. Open field test

#### 2.2.3.8.1. Set up

An open field test was conducted at 12 weeks into the immunisation schedule to assess spontaneous locomotor activity, exploration and innate anxiety (Walsh & Cummins, 1976). Animals were only tested once, in a randomised order within a single day during ‘light’ cycle hours. Rats were moved to individual cages and into the behavioural testing room 1 hour prior to the start of the test to allow acclimatisation to the new environment. Data was acquired using the EthoVision video tracking system (Noldus). The open field test was conducted in a 1 m (width) x 1 m (length) x 50 cm (height) black square arena. The arena was segmented into 16 zones of equal area by the software (Figure 2.5). Recordings were made under dim red-lighting condition. Experimenters exited the behavioural testing room after placing the animal in the arena and starting the recording. Rats were allowed to freely explore the arena for the 20 min duration of the test. Upon completion, rats were returned to their original cages. The arena was cleaned with 70% ethanol in between testing animals. Parameters measured by the software included total distance moved, velocity and total time spent in the inner zone. Manual analysis of the frequency of rearing, latency to first inner zone entry and the frequency of inner zone entry were also performed while blinded to the treatment groups.
Figure 2.5 A schematic diagram and a video screenshot of the open field testing arena

The schematic diagram shows 16 equally-sized squares which correspond to the testing arena. The area covered by the four inner squares (6, 7, 10, 11) defined the inner zone of the arena while the remaining squares made up its periphery. The screenshot from a video recorded by the Noldus EthoVision software shows a rat in the centre of the inner zone, with small white spots on the physical arena marking the inner zone boundary.

2.2.3.8.2. Statistical analysis

Rats treated with PBS or Luc protein were combined into one sample group (PBS/Luc) in order to obtain a group size of \( n = 5 \) for the purpose of statistical testing.

Statistical analysis was performed using GraphPad Prism Software Version 6. All data were presented as mean ± standard error of the mean (SEM). The Kolmogorov-Smirnov test for normality was used to determine if data sets were parametric. To test for equality of variances, Bartlett’s test and Levene's test were conducted for parametric and non-parametric data, respectively. Analysis to determine statistical differences between treatment groups were performed by one-way analysis of variance (ANOVA) or, if assumptions of the parametric method were not satisfied, the non-parametric Kruskal-Wallis test was applied. A difference was considered statistically significant when the \( p \)-value was less than 0.05.

2.2.3.9. Intracardiac blood collection

Rats were euthanised via an intraperitoneal injection of sodium pentobarbitone (90 mg/kg) for the terminal procedure of intracardiac blood collection. The animal was first placed on its back in dorsal recumbency and the position of the heart was determined by placing a finger
on the chest to feel for the heartbeat. A 21 gauge needle attached to a 30 mL syringe was used to collect blood from the heart. Blood samples were transferred into 15 mL centrifuge tubes and left to clot for 1 hour at RT in an upright position. Tubes were then centrifuged using a swing-bucket rotor at 2,000 x g, 4 °C for 15 min.

2.2.4. Characterisation of IgG in rat serum

2.2.4.1. Assessment of IgG cross-reactivity

Individual serum samples from the best responding rats at the end of the immunisation period were tested for cross-reactivity to recombinant protein preparations other than their immunogen. Samples from animals in the PBS treatment group were also assessed. Screening was performed according to Section 2.2.3.6, with the addition of PBS serum used at 1:10,000 dilution.

2.2.4.2. Epitope mapping of rat sera against GluN1 peptides

Final serum samples from rats determined as best responders were pooled at equal volumes for each treatment group. Epitope mapping of pooled sera was carried out by enzyme-linked immunosorbent assay (ELISA) against a panel of 16-mer overlapping synthetic peptides spanning the two extracellular domains of mouse GluN1 (Mimotopes, PepSets™) (see Appendix for peptide sequences). All peptides were biotinylated at the N-terminus via a tetrapeptide linker SGSG spacer sequence. The range of peptides (#41 - 68) encompassing the proposed ligand binding site was based on the ligand-binding residues identified in a 3D homology model of the glycine-binding site of GluN1 (Moretti et al., 2004). Nunc MaxiSorp® flat-bottom 96-well plates (Nunc, 442404) were used for all ELISAs conducted. Plates were sealed with parafilm during incubation steps to prevent evaporation and contamination. A 2 mg/mL stock solution of Streptavidin (Prospec, PRO-283) was prepared in MilliQ water. Streptavidin was diluted to a working concentration of 5 µg/mL with PBS and 50 µL was used to coat each well. Plates were then incubated at 37 °C for 4 hours. After coating with Streptavidin, plates were washed three times with 300 µL of PBS containing 0.05% (v/v) Tween 20 (PBS-Tw) per well for 3 min each time with shaking. Plates were then blocked with 300 µL of PBS-Tw containing 5% (w/v) skim milk powder per well, at 4 °C overnight with shaking. The next day, plates were again washed three times with 300 µL of
PBS-Tw per well for 3 min each time with shaking. Pure peptides were dissolved in dimethyl sulfoxide to give 5 mM stock solutions, which were stored at -80 °C until use. Peptides were diluted to 5 µM in PBS and 50 µL was added to each well for a total amount of 250 pmol. Plates were coated with peptides for 3 hours with shaking at RT and then washed three times with 300 µL of PBS-Tw per well, for 3 min each time with shaking. Pooled serum samples were diluted 1:200 in PBS-Tw containing 5% (w/v) skim milk powder, applied at 50 µL per well, and incubated at 4 °C overnight with shaking. The following day, plates were washed three times with 300 µL of PBS-Tw per well for 3 min each time with shaking. Rabbit peroxidase-conjugated anti-rat IgG antibody (Sigma, A5795) was diluted 1:10,000 in PBS-Tw, applied at 50 µL per well, and incubated at RT for 3 hours with shaking. A final wash step was carried out with the plates washed three times with 300 µL of PBS-Tw per well for 3 min each time with shaking. To develop the assay, a 0.4 mg/mL substrate solution was prepared by dissolving one 5 mg o-phenylenediamine dihydrochloride (OPD) tablet (Sigma, P6912) in 12.5 mL 0.05 M phosphate-citrate buffer (51.4 mM Na₂HPO₄, 24.3 mM Citric acid, pH 5.0) and 5 µL H₂O₂. The OPD substrate solution was applied at 75 µL per well for 30 min at RT while shielded from light. The reaction was stopped by applying 50 µL of 1 N sulphuric acid to each well. Absorbance was measured at 492 nm with a BioTek Synergy 2 plate reader and Gen5™ Software (BioTek Instruments). Specificity was expressed as a z-score calculated by subtracting the mean absorbance of the PBS serum, no-peptide blank from the mean absorbance of the sample and divided by the standard deviation of the blank. The threshold for reactivity was set to a z-score of 30.

\[
Specificity \text{ z-score} = \frac{(Mean \ of \ sample - Mean \ of \ blank)}{SD \ of \ blank}
\]

2.2.5. Purification of IgG from rat serum

2.2.5.1. Caprylic acid precipitation method of IgG purification

Caprylic acid, also known as octanoic acid, was used to crudely purify IgG from rat serum by precipitating non-IgG proteins under mildly acidic conditions. For IgG purification, final serum samples from the best responding rats were pooled at equal volumes for each treatment group. Sera from both animals in the PBS treatment group were pooled for purification. In a small beaker, one volume of pooled rat serum (3 mL) was mixed with two volumes of 60 mM
sodium acetate (pH 4.0) (6 mL) with constant stirring. The pH of the serum/sodium acetate mixture was adjusted to 4.85-4.88 with 1 M NaOH. Octanoic acid (Sigma, C2875) was added dropwise to the mixture to attain a final concentration of 5% (v/v) (0.475 mL for a 9 mL serum/sodium acetate mixture), and left to stir for a further 30 min at RT to allow precipitate formation. The suspension was then transferred to a 15 mL centrifuge tube and spun at 10,000 x g, 4 °C for 30 min to pellet precipitated proteins. The supernatant was transferred to another 15 mL tube and centrifuged again at 10,000 x g, 4 °C for 10 min to pellet residual precipitates. After the second spin, the IgG-containing supernatant was carefully aspirated and diluted in filter sterile PBS to a total volume of 20 times the volume of serum used (60 mL).

2.2.5.2. Concentration and desalting of IgG

The purified IgG solution was concentrated and desalted using 100K MWCO Amicon (Ultra-15) centrifugal filter devices (Millipore, UFC910024). IgG solution was loaded into the sample reservoir of the filter device at the maximum volume of 12 mL and centrifuged at 3,500 x g, 4°C for 10-20 min. Spin times differed between IgG purification runs and was adjusted accordingly to prevent overspinning. The filtrate was discarded following each centrifugation step and the sample reservoir was topped up with more IgG solution up to 12 mL. This process was repeated until the IgG solution was concentrated down to a final volume of ~500 µL retentate, which should appear pale blue in colour. Concentrated IgG solutions were divided into 100 µL aliquots and stored at -20 °C.

2.2.5.3. Quantification of IgG concentration

The concentration of purified IgG solution was quantified by the Bio-Rad (Bradford) Protein Assay (Bio-Rad, #500-0006) microtitre plate procedure with BSA standards. Working dye reagent solution was made by diluting one volume of Dye Reagent Concentrate with four volumes of MilliQ water. BSA standards at 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 µg/µL were prepared by diluting stock BSA solution (10 µg/µL) in PBS. IgG samples were diluted to 1:20, 1:50, and 1:80 in PBS. All solutions were assayed in triplicate in a flat bottom 96-well plate (BD Falcon, 353072) and 10 µL of standard or sample solution was mixed with 200 µL of dye reagent per well. The plate was incubated at RT for at least 5 min but no more than 1 hour before absorbance was measured at 595 nm with a BioTek Synergy 2 plate reader and
Gen5™ Software (BioTek Instruments). The unknown sample concentrations were derived from a standard curve generated by the software.

2.2.6. Quality analysis of purified rat IgG

2.2.6.1. Assessment of rat IgG purity

The purity of IgG solutions were assessed by Coomassie blue staining and Western blot. Procedures from Sections 2.2.2.1, 2.2.2.2 and 2.2.2.4 were followed with a few adjustments. Acrylamide gels (12%) were loaded with 4 µg IgG per lane for Coomassie blue staining or 0.5 µg IgG per lane for Western blotting. Following protein transfer, the nitrocellulose membrane was incubated with goat HRP-conjugated anti-rat IgG antibody (Santa Cruz, sc-2006) (1:5,000) for 3 hours at RT with shaking before washing and blot development.

2.2.6.2. Examination of IgG reactivity against recombinant proteins

Purified IgG solutions were screened for reactivity against their respective protein immunogen and comparisons were made with commercial antibodies. Western blot was performed according to Section 2.2.2.4 with a few adjustments. Recombinant proteins were resolved on a 15-well 12% acrylamide gel. Following protein transfer onto nitrocellulose cellulose membrane, lanes were cut into separate strips. Primary antibodies used were purified IgG solutions at 4 µg/mL and the commercial antibodies specified in Table 2.1. Secondary antibodies included goat HRP-conjugated anti-rat IgG antibody (Santa Cruz, sc-2006) (1:5,000) and other antibodies listed in Table 2.2.

2.2.6.3. Examination of IgG reactivity against rat brain lysate

2.2.6.3.1. Preparation of rat hippocampal tissue lysate

Fresh frozen brain tissue from naïve adult male Sprague Dawley rats was used to prepare hippocampal lysates. The brains were removed from -80 °C storage, thawed on ice, and hippocampi dissected out. Isolated hippocampi were transferred into a 2 mL microfuge tube and ice-cold tissue lysis buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.05% (v/v) Triton X-100) containing cOmplete™ Mini Protease Inhibitor Cocktail (Roche, # 11836153001) was added at 20 mL per g of tissue. Samples were sonicated on ice (Misonix XL-2020
Sonicator) at output level 4, 1 min pulsar on, 30 s pulsar off, and 1 min pulsar on. Samples were then centrifuged at 1,000 x g for 15 min at 4°C to pellet cell debris. Supernatants were transferred to new 1.5 mL microfuge tubes and stored at -20 °C until use.

2.2.6.3.2. Quantification of protein concentration in tissue lysate

The protein concentration of hippocampal tissue lysate was quantified by the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, 23227) with BSA standards according to manufacturer’s instructions. BSA standards at 0, 125, 250, 500, 750, 1,000 and 1,500 µg/mL used for the generation of a standard curve were prepared by diluting stock BSA solution (2 mg/mL) in tissue lysis buffer. A 1:5 dilution hippocampal lysate sample was prepared by adding tissue lysis buffer and solubilising the tissue in 2% (w/v) SDS. All solutions were assayed in triplicate in a flat bottom 96-well plate plate (BD Falcon, 353072). The BCA working reagent was mixed and 200 µL was added to each well. Samples were incubated at 37 °C for 30 min before absorbance was measured at 595 nm using a BioTek Synergy 2 plate reader and Gen5™ Software (BioTek Instruments). Protein concentrations were determined using the standard curve acquired from the software.

2.2.6.3.3. Western blotting against rat brain lysate

Purified IgG solutions were screened for reactivity, specifically against GluN1, in rat hippocampal lysate and comparisons were made with commercial antibodies. Western blot was performed as described in Section 2.2.2.4 with some adjustments. A 15-well 12% acrylamide gel was cast and 20 µg of rat hippocampal tissue lysate per lane was resolved by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane and stained with Ponceau S solution before lanes were cut into individual strips. Purified IgG solutions (4 µg/mL) and commercial anti-GluN1 antibodies (Table 2.1) were applied as primary antibodies. Goat HRP-conjugated anti-rat IgG (Santa Cruz, sc-2006) and anti-mouse IgG (Santa Cruz, sc-2005) antibodies (1:5,000) were used as secondary antibodies.

2.2.7. IgG preabsorption

2.2.7.1. Preabsorption of IgG with recombinant proteins

Purified recNR1 IgG solution was preabsorbed with Luc or recNR1 protein preparations. IgG solution at 1 µg/µL was added to each recombinant protein preparation in a 1.5 mL
microfuge tube at a molar ratio of 1:10. Molecular weights in kDa (numerically equal to kg/mol) were used to calculate the masses of IgG and protein required in the mixture (see Table 2.3). The IgG/protein mixture was incubated overnight at 4 °C and constantly mixed using a microfuge tube rotator. Following overnight incubation, the IgG/protein mixture was centrifuged at 1,000 x g for 10 min at 4 °C to pellet insoluble recombinant protein. The supernatant was then concentrated with a 10K MWCO Microcon (Ultracel) Centrifugal Filter Device (Millipore, 42407) by centrifuging at 10,000 x g for 30 min at 4 °C. The filter device reservoir was refilled with more solution and centrifugation was repeated until the starting volume prior to preabsorption was attained. The reservoir was then placed upside down in a new centrifugal filter device tube and centrifuged at 1,000 x g for 3 min at 4 °C to retrieve the concentrated IgG sample. Bradford protein assay for quantifying IgG concentration was performed as described in Section 2.2.5.3.

Table 2.3 Amount of recNR1 IgG, Luc and recNR1 proteins used for preabsorption

The molecular weight of recNR1 IgG, Luc and recNR1 proteins were used to calculate the mass of each protein required for IgG preabsorption.

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<td>100</td>
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<tr>
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2.2.7.2. Comparison of IgG binding capacity

The binding capacities of preabsorbed recNR1 IgG solutions were compared with non-preabsorbed recNR1 IgG by Western blot, performed according to Section 2.2.2.4 with some adjustments. recNR1 protein preparation was resolved by SDS-PAGE in a 15-well 12% acrylamide gel at 1 µg per lane. Lanes were cut into individual strips following protein transfer onto nitrocellulose membrane and Ponceau S staining. To generate a standard curve,
non-preabsorbed recNR1 IgG was applied to strips at concentrations of 0.25, 0.5, 1.0, 2.0 or 4.0 µg/mL. recNR1 IgG solutions preabsorbed against Luc or recNR1 proteins were both assumed a concentration of 1.0 µg/µL and applied to membrane strips at 2.0 µg/mL. Goat HRP-conjugated anti-rat IgG antibody (Santa Cruz, sc-2006) (1:5,000) was used as the secondary antibody. After the blot was developed and captured using the ChemiDoc™ MP Imaging System (Bio-Rad), the volume or integrated density values for the two major protein species in the recNR1 protein preparation at 83 kDa and ~32 kDa were obtained using Image Lab™ Software Version 4.1 (Bio-Rad) and plotted against IgG concentration. Using the least squares method, the data for the two bands were fitted with second order polynomial regressions to determine the equivalent binding concentrations of the preabsorbed recNR1 IgG solutions.
2.3. Results

2.3.1. Recombinant Luc, recNR1 and NR1extra2 protein production

To produce recombinant Luc, recNR1 and NR1extra2 proteins, *E. coli* BL21 (DE3) cultures were transformed with pET-Luc, pTriEx-recNR1 and pET-NR1extra2 plasmid constructs, respectively. Cultures inoculated with transformed colonies were propagated until they reached mid log phase at approximately 0.6 OD$_{600}$ when protein production was induced by the addition of IPTG to culture medium. Typically, it took Luc and NR1extra2 cultures 2 to 3 hours to grow to mid log phase. However, recNR1 cultures took much longer to achieve 0.6 OD$_{600}$ and required at least 5 hours. Bacterial cells were then allowed to grow for three additional hours to produce proteins before they were harvested into pellets and lysed. Insoluble inclusion bodies were isolated by centrifugation and used for protein purification. In total, 4 L of bacterial culture were grown for each recombinant protein.

Bacterial lysates were fed into the Profinia™ Protein Purification system set up with a Bio-Scale Mini Profinia IMAC cartridge to bind HIS-tagged recombinant proteins. The UV absorbance readings of eluted solutions were recorded by the Profinia instrument in real-time during the purification process (Figure 2.6). Elution peaks for the three proteins were all relatively sharp, which was indicative of a fast kinetics exchange within the column with bound proteins quickly displaced by the high imidazole concentration of the elution buffer. Protein yields were fairly consistent between purification runs of the same recombinant protein. Similarities in the Coomassie blue staining profiles of the starting bacterial lysate and initial purification flowthrough for all recombinant proteins showed that most non-specific proteins had failed to bind to columns and were eluted in the flowthrough (Figure 2.7). Weakly-bound proteins were further eluted from the column during the two wash steps when the imidazole concentration of the wash buffer solution was increased. Elution buffer containing a high concentration (250 mM) of imidazole was used to elute remaining proteins in columns. Final protein eluates were relatively clean compared to the starting bacterial lysates but varied in purity as shown by the observation of multiple secondary bands in the Luc and recNR1 protein eluates (Figure 2.7 A, B). On the other hand, only a single visible band was observed in the NR1extra2 protein eluate (Figure 2.7 C). The most prominent band in each protein eluate was observed at the expected molecular weight of the full-length protein, which was 65 kDa for Luc, 83 kDa for recNR1 and 20 kDa for NR1extra2.
When recombinant proteins were purified from all available bacterial lysates, protein eluates from individual purification runs were pooled and concentrated down to volumes of 20 to 25 mL for dialysis in Slide-A-Lyzer dialysis cassettes. The protein solutions expanded slightly in volume and white-coloured protein precipitates were observed in solution following extensive dialysis in PBS. The concentration of each recombinant protein preparation was quantified by SDS-PAGE analysis by measuring the integrated density of the full-length protein band relative to a standard curve generated with known amounts of BSA using the Multi-Gauge image analysis software (Figure 2.8 A, C, E). The final concentrations were determined to be 1.066, 0.373 and 0.749 µg/µL for Luc, recNR1 and NR1extra2, respectively. The SDS-PAGE method was chosen over the use of protein assays because the preparations contained precipitated proteins which would require the use of a detergent-compatible assay. Also, SDS-PAGE analysis allowed the quantification of a specific protein band as opposed to measuring the concentration of total protein content.

Western blots were conducted using commercial antibodies to validate the protein species in each recombinant protein preparation and to assess purity (Figure 2.8 B, D, F). Signals were absent in all secondary antibody-only control lanes. Commercial anti-Luc antibody was able to detect the major protein species in the Luc protein preparation at 65 kDa, which confirmed its identity as the full-length Luc protein (Figure 2.8 B). In addition to the full-length protein, anti-Luc antibody also reacted to several secondary bands of lower molecular weights. Commercial anti-GluN1 antibodies targeting the N-terminus or the M3-M4 loop were used to probe both recNR1 and NR1extra2 protein preparations. For the recNR1 protein preparation, both anti-GluN1 N-term and anti-GluN1 M3-M4 antibodies were able to detect one of the two main protein species at 83 kDa, thereby confirming its identity as the full-length recNR1 protein which possessed both GluN1 extracellular domains (Figure 2.8 D). The two anti-GluN1 antibodies also reacted to numerous secondary bands which were likely degradation products of the full-length recNR1. The other main protein species at ~32 kDa in the recNR1 protein preparation was only detected by the M3-M4 antibody and not the N-term antibody. This suggested that it is a degradation product of recNR1 that mostly contained amino acids from the M3-M4 region. As for the NR1extra2 protein preparation, the anti-GluN1 M3-M4 antibody reacted with the major protein species at 20 kDa, which identified it as the full-length NR1extra2 protein (Figure 2.8 F). No secondary bands were observed with the anti-GluN1 M3-M4 antibody and, as expected, no positive signals were detected when the NR1extra2 protein preparation was incubated with anti-GluN1 N-term antibody.
The yields of the three recombinant proteins were very different despite being purified from the same total volume of bacterial culture. The final yields in mass of full-length protein were 31.98, 7.83 and 17.53 mg for Luc, recNR1 and NR1extra2, respectively. The amount of protein produced was confirmed to be sufficient for the subsequent immunisation in rats to generate antibodies against each recombinant protein.
Figure 2.6 Denaturing IMAC protein purification elution profiles

Example Profinia system chromatograms showing UV absorbance profiles of protein flowthrough in red from the denaturing IMAC purification of Luc (A), recNR1 (B) and NR1extra2 (C) proteins. The elution peak of HIS-tagged recombinant protein from the column is shown within the gray-shaded region. Purification runs of all three proteins consistently yielded sharp elution peaks.
Starting bacterial lysate and purification fraction samples from denaturing IMAC purification of Luc (A), recNR1 (B) and NR1extra2 (C) proteins on the Profinia system were resolved on a 12 % acrylamide gel and stained with Coomassie blue. The gels shown here are examples of a single purification run of each protein. The starting bacterial lysate, flowthrough, wash 1, wash 2, and the protein eluate were loaded at equal volumes (10 µL). For each protein eluate, the most prominent band was observed at the expected molecular weight of the full-length protein (arrows), with Luc at 65 kDa, recNR1 at 83 kDa and NR1extra2 at 20 kDa.

L, lysate; FT, flowthrough; W1, wash 1; W2, wash 2; P, protein eluate.
Figure 2.8 Quantification of purified recombinant protein concentration and assessment of purity

Following concentration and dialysis, final recombinant Luc (A), recNR1 (C) and NR1extra2 (E) protein preparations (at known volumes) were resolved alongside 0.5, 1.0 and 2.0 µg of BSA standard on 12 % acrylamide gels, in duplicate, and stained with Coomassie blue. Full-length protein bands (arrows) were used to quantify protein concentration with Multi-Gauge Software. Many secondary bands were visible in the Luc and recNR1 samples while the NR1extra2 sample was visibly free of additional bands. Western blots were performed on recombinant protein preparations using respective commercial antibodies to examine the purity of specific protein. Lane 1 of all blots were secondary antibody-only control lanes.

Commercial anti-Luc antibody reacted with full-length Luc protein at 65 kDa, which was the major protein species in the preparation, as well as a few secondary bands (B, lane 2). Both commercial anti-GluN1 N-term (D, lane 2) and anti-GluN1 M3-M4 (D, lane 3) antibodies detected the full-length recNR1 protein at 83 kDa, one of two main protein species in the preparation. Both anti-GluN1 antibodies also reacted to several secondary bands but only the M3-M4 antibody bound to the second main protein species at ~32 kDa. No signals were visible from probing NR1extra2 protein preparation with anti-GluN1 N-term antibody (F, lane 2) but the full-length protein was detected by the anti-GluN1 M3-M4 antibody at 20 kDa (F, lane 3). Purification runs for each recombinant protein produced comparable yields of protein eluate.
Figure 2.8

A. Luc

B. Luc

C. recNR1

D. recNR1

E. NR1extra2

F. NR1extra2
2.3.2. Immunisation of rats with recombinant protein preparations

2.3.2.1. Development of IgG against Luc, recNR1 and NR1extra2 proteins in rats

Recombinant protein preparations were used to immunise rats with the primary aim of generating antibodies against Luc, recNR1, and NR1extra2. Protein preparations were diluted in PBS to the same concentration as the least concentrated recombinant protein, which was determined to be recNR1 at 0.373 µg/µL. Doing so ensured animals in all treatment groups were injected with the same volume. Pierce Imject Alum adjuvant was used in injections to promote a strong, sustained immune response against the immunogen. A small number of adult male Sprague Dawley rats were immunised with a 1:1 mixture of adjuvant and one of the three recombinant protein preparations (Luc, $n = 3$; recNR1, $n = 5$; NR1extra2, $n = 5$). An equivalent volume of 1:1 adjuvant and PBS mixture was injected into rats in the PBS treatment group ($n = 2$), which was included as an immunisation control. As planned, each rat received a subcutaneous priming shot of 100 µg protein and six 50 µg boosting shots, which were administered every two weeks.

Blood samples were collected from rats prior to all injections, from which sera were isolated and used to screen for the presence of IgG against their respective protein immunogen. IgG production in animals immunised with protein was monitored by Western blot over the course of the immunisation period (Figure 2.9). As expected, pre-immune serum samples obtained from Luc-, recNR1- and NR1extra2-immunised animals before priming shots at Week 0 were free of IgG against their immunogens. In general, the expression of IgG against NR1extra2 protein preparation was comparatively lower than Luc and recNR1. This was demonstrated by the required use of a lower serum dilution (1:5,000 instead of 1:10,000) when screening of NR1extra2 sera was performed. IgG against recombinant protein preparations were detectable in Luc- and recNR1-immunised animals from Week 2. Immune response to recombinant protein immunisation appeared delayed in NR1extra2 animals as IgG was only detectable from Week 4 onwards. IgG expression against full-length Luc and recNR1 proteins in immunised animals reached a plateau between Weeks 8 and 10 in most animals and persisted at an elevated level until the end of the immunisation period. In contrast, IgG expression in NR1extra2-immunised animals followed a completely different pattern. All five rats successfully developed IgG against the full-length NR1extra2 protein but the expression peaked early at Week 6 and was followed by a gradual decline until the end of the immunisation period at Week 13. Concurrently, NR1extra2-immunised animals
also developed IgG against an unknown protein species at ~28 kD, of which expression was relatively stable compared to IgG specific for the full-length NR1extra2 protein. The expression of IgG against the ~28 kDa protein peaked at Week 10 and remained elevated until the end of the immunisation in most animals.
Serum samples collected from rats prior to each injection and at the end of the immunisation period were used to monitor production of IgG against their respective protein immunogen over time. Western blots were performed with recombinant protein preparations and probed with Luc, recNR1 or NR1extra2 rat sera. Example IgG expression profiles from animals which responded well to immunisations with Luc (A), recNR1 (B) or NR1extra2 (C) protein are shown. Full-length recombinant proteins and the recNR1 protein species at ~32 kDa are indicated by arrows. IgG against recombinant proteins were absent in all pre-immune serum samples collected at Week 0. For Luc- and recNR1-immunised animals, IgG expression generally plateaued between Weeks 8 and 10 and remained stable until the end of the immunisation period. NR1extra2-immunised animals developed specific IgG against the full-length NR1extra2 protein but the expression peaked at Week 6 and steadily decreased until the end of the immunisation period. In addition to the NR1extra2 protein band, reactivity against an unknown protein species at ~28 kDa was also observed. Expression of IgG against this unknown protein eventually surpassed that of NR1extra2 and plateaued at Week 10.
Figure 2.9

A

Luc

Week  0  2  4  6  8  10  12  13

kDa
118.3
97.4
66.2
45.0
31.0
21.5

B

recNR1

Week  0  2  4  6  8  10  12  13

kDa
116.3
97.4
66.2
45.0
31.0
21.5

C

NR1extra2

Week  0  2  4  6  8  10  12  13

kDa
116.3
97.4
66.2
45.0
31.0
21.5
2.3.2.2. General animal health and behaviour

Health checks and behavioural observations were carried out on animals during the immunisation period. Rats were weighed for three consecutive days after each injection and on a weekly basis. Animals in all treatment groups were in good health and steadily gained weight over 13 weeks (Figure 2.10). Weights were very similar between treatment groups and there were no signs of adverse reaction to immunisation, indicated by the absence of weight loss immediately following each injection. Although no differences in animal health were observed, NR1extra2-immunised animals appeared more hyperactive and excitable compared to other treatment groups when they were handled between Weeks 8 and 10. This particular observation prompted the decision to perform an open field test on the animals at Week 12, one week before terminal blood collection was scheduled.

2.3.2.3. Locomotion, exploration and anxiety in the open field test

Following the observation of a possible difference in the behavioural phenotype between treatment groups, an open field test was conducted on all animals at Week 12. Rats were tested in the open field arena in a randomised order for 20 min each. Animals in the PBS and Luc treatment groups were combined into a single group (PBS/Luc) to match the sample size of recNR1 and NR1extra2 treatment groups (all $n = 5$) for the purpose of statistical analysis. No significant differences were observed in total distance travelled (Kruskal-Wallis test, $H = 0.180, p = 0.925$) (Figure 2.11 A) and velocity (Kruskal-Wallis test, $H = 0.180, p = 0.925$) (Figure 2.11 B), which demonstrated similar locomotor activity between animals of all treatment groups. In addition, no significant difference was observed in the frequency of rearing (one-way ANOVA, $F = 0.378, p = 0.693$) (Figure 2.11 C), which was an indicator of vertical exploratory activity. Although measures of innate anxiety including latency to first inner zone entry (one-way ANOVA, $F = 0.646, p = 0.541$) (Figure 2.11 D), frequency of inner zone entry (one-way ANOVA, $F = 2.423, p = 0.131$) (Figure 2.11 E) and total time spent in inner zone (one-way ANOVA, $F = 1.610, p = 0.240$) (Figure 2.11 F) appeared to be more variable between treatment groups, no significant differences were found for all three parameters.
Figure 2.10 Body weight change in rats during the immunisation period

Body weights of rats were recorded before each injection, three consecutive days post-immunisation and on a weekly basis. Times at which injections were carried out are labelled on the graph. Animals in all treatment groups gained weight over the immunisation period. No visible difference in body weight was observed between animals in any of the treatment groups.

Data represent mean ± SEM.
Figure 2.11 Effects of protein immunisation on rats in the open field test

An open field test was conducted at Week 12 to investigate locomotor and exploratory activity as well as innate anxiety in rats undergoing protein immunisation. Animals were tested in turn for a total of 20 min each. Rats treated with PBS or immunised with Luc protein were combined into one sample group (PBS/Luc) of \( n = 5 \) to match the sample size of recNR1 and NR1extra2 treatment group (\( n = 5 \) each). Parameters were assessed either by software or manual analysis and included total distance moved (A), velocity (B), frequency of rearing (C), latency to first inner zone entry (D), frequency of inner zone entry (E) and time spent in inner zone (F). No significant differences were observed between any of the treatment groups for the measured parameters.

Data represent mean ± SEM.
Figure 2.11
2.3.3. Characterisation of sera from best responding rats

All rats were euthanised with pentobarbitone at the end of the immunisation period (Week 13) for terminal blood collection. Approximately 12 to 18 mL of blood was collected from each animal by cardiac puncture, and 6 to 10 mL of serum was harvested from each blood sample. These final serum samples were used to determine which animals were the best responders to recombinant protein immunisation in each treatment group by Western blot (Figure 2.12). By the end of the immunisation period, all three animals in the Luc treatment group (Rats #3, 4, 5) successfully produced IgG against the full-length Luc protein at similar robust levels. All three Luc samples also reacted to secondary bands comparably. On the other hand, immune responses to protein injection were highly variable in the recNR1 and NR1extra2 treatment groups. While all recNR1-immunised animals produced IgG to various secondary bands in the recNR1 protein preparation, only three out of five samples (Rats #6, 8, 9) expressed IgG against the full-length recNR1 protein. Band intensities were also lower compared to Luc samples. Lastly, sera from four out of five animals in the NR1extra2 treatment group (Rats # 11, 12, 14, 15) exhibited reactivity to the full-length NR1extra2 protein at Week 13. However, these samples also expressed IgG against the unknown protein species at ~28 kDa at higher levels as demonstrated by stronger band intensities.

Serum samples from the aforementioned rats were assessed for cross-reactivity with recombinant protein preparations other than their respective immunogen before pooling. Sera from the two PBS-immunised animals were screened against all three recombinant protein preparations (Figure 2.13 A). No signals were detected when serum from Rat # 1 was used to probe the three protein preparations. Unexpectedly, serum from Rat #2 reacted to few protein bands in each preparation but they did not match the molecular weights of the full-length proteins. The three Luc samples detected multiple bands of the same molecular weight at varying intensities in the recNR1 protein preparation but none matched the molecular weights of the two main recNR1 protein species (Figure 2.13 B). On the other hand, incubating Luc samples with NR1extra2 protein preparation resulted in minimal levels of reactivity. All recNR1 samples reacted with non-specific secondary bands in Luc and NR1extra2 protein preparations (Figure 2.13 C). However, they also successfully detected the full-length NR1extra2 protein at 20 kDa. This was expected because recNR1 protein contains the GluN1 subunit M3-M4 region which makes up the entire recombinant NR1extra2 protein. The four NR1extra2 samples reacted to Luc and recNR1 protein preparations with similar patterns. Multiple secondary bands with matching molecular weights were observed with both protein
preparations, particularly in the lower range. It was not possible to ascertain whether NR1extra2 serum samples were able to bind specifically to the full-length recNR1 protein or its degradation products due to weak band intensities and the presence of non-specific proteins.

Sera from the best responding rats were pooled for each treatment group and used for epitope mapping with synthetic peptides of the mouse GluN1 extracellular domains (Figure 2.14). The use of overlapping 16-mer peptides facilitated the identification of short stretches of amino acid residues that may be targeted by IgG in the pooled sera. Pooled serum samples from PBS- and Luc-immunised animals showed negligible reactivity to the GluN1 peptides when compared to those from recNR1- and NR1extra2-immunised animals. This confirmed the absence of non-specific binding to GluN1 extracellular domains by sera from control animals. Polyclonal reactivity against GluN1 was observed with both recNR1 and NR1extra2 samples, albeit in different regions. Pooled recNR1 serum reacted to multiple sites in both the N-terminus and the M3-M4 region. N-terminal peptides recognised by recNR1 serum included #10 (a.a. 92-107), #37 (a.a. 362-377) and #38 (a.a. 372-387), of which #38 had the highest specificity z-score of 2949. However, most peptides recognised by recNR1 serum were located in the GluN1 glycine-binding region, namely #53 (a.a. 522-537), #54 (a.a. 532-547), #58 (a.a. 654-665), #59 (a.a. 660-675), #60 (a.a. 670-685), #62 (a.a. 690-705), #64 (a.a. 710-725) and #65 (a.a. 720-735). The specificity z-score of peptide 59 for recNR1 serum at 2851 was also very high and comparable to that of peptide 38. In contrast, pooled NR1extra2 serum recognised fewer peptides compared to recNR1, which were restricted within the GluN1 M3-M4 loop, and their resulting specificity z-scores were several folds lower. The peptide which displayed the best reactivity when probed by NR1extra2 serum (#69) only had a specificity z-score of 647. Peptides detected by NR1extra2 serum were #59 (a.a. 660-675), #68 (a.a. 750-765), #69 (a.a. 760-775) and #71 (a.a. 780-795). Epitope mapping demonstrated and further supported the presence of IgG targeted against GluN1 in recNR1 and NR1extra2 sera.
Figure 2.12 Western blot analysis of immune response to recombinant protein immunisation in rats

Following intracardiac blood collection at Week 13, final rat serum samples were isolated and used to assess the immune response of each animal. Western blot was performed with recombinant protein preparations and probed with Luc, recNR1 or NR1extra2 final serum sample from each animal. Sera from the best responding animals of each treatment group (circled) were selected for pooling, further analysis and IgG purification. All animals in the Luc treatment group (Rats #3, 4, 5) responded similarly to immunisation and serum samples displayed reactivity to the full-length Luc protein (arrow). Expression of IgG against secondary bands was also comparable between the three Luc samples. Three out of five samples in the recNR1 treatment group (Rats #6, 8, 9) detected the full-length recNR1 protein at 83 kDa (top arrow) but their reactivity profiles for secondary bands were variable in pattern. Four out of five samples in the NR1extra2 treatment group (Rats # 11, 12, 14, 15) displayed reactivity to the full-length NR1extra2 protein (arrow) but band intensities were visibly lower compared to the unknown protein species at ~28 kDa.
Figure 2.13 Western blot analysis of serum cross-reactivity with recombinant proteins

Individual serum samples from the best responding animals of each treatment group were tested for cross-reactivity to other recombinant protein preparations. Sera from the two PBS-treated animals were used to probe Luc, recNR1 and NR1extra2 protein preparations (A). The sample from Rat #1 did not react to any of the preparations but Rat #2 detected a few non-specific proteins in all three preparations. All three Luc samples reacted to the recNR1 protein preparation in a similar pattern but not at the molecular weights of the two main recNR1 protein species (83 kDa and ~32 kDa) (B). Minimal reactivity was observed when Luc serum samples were used to probe the NR1extra2 protein preparation. The three recNR1 samples showed some non-specific reactivity to both Luc and NR1extra2 protein preparations but also clearly detected the full-length NR1extra2 protein at 20 kDa as expected (C). NR1extra2 serum samples all demonstrated similar non-specific reactivity to Luc and recNR1 protein preparations, binding to multiple secondary bands, especially ones with lower molecular weights (D).

Luc, Luc protein; rec, recNR1 protein; e2, NR1extra2 protein.
## Figure 2.13

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### D. NR1extra2

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Figure 2.14 Epitope mapping of pooled sera against extracellular GluN1 peptides

Rat serum samples were screened for reactivity against overlapping 16-mer peptides spanning the extracellular domains of the mouse GluN1 by ELISA. Pooled sera from the best responding animals for each treatment group were probed against 250 pmol of peptide per well. Specificity was expressed as a z-score. Pooled PBS and Luc serum samples exhibited low, background levels of reactivity to GluN1 peptides. Both recNR1 and NR1extra2 pooled serum samples displayed polyclonal reactivity to multiple sites in the extracellular regions of GluN1. Pooled recNR1 serum reacted to N-terminal peptides (#10, a.a. 92-107; #37, a.a. 362-377; #38, a.a. 372-387) but most reactivity was observed in the GluN1 glycine-binding region (#53, a.a. 522-537; #54, a.a. 532-547; #58, a.a. 654-665; #59, a.a. 660-675; #60, a.a. 670-685; #62, a.a. 690-705; #64, a.a. 710-725; #65, a.a. 720-735). Reactivity was only observed in the M3-M4 region for pooled NR1extra2 serum (#59, a.a. 660-675, #68, a.a. 750-765; #69, a.a. 760-775; #71, a.a. 780-795).
Figure 2.14
2.3.4. Purification and validation of IgG from rat sera

Following serum characterisation, final serum samples from the best responding rats were pooled for each treatment group for IgG purification. Non-IgG proteins were precipitated from solution when caprylic acid was added to serum/sodium acetate mixtures with acidic pH. White protein precipitates were completely pelleted and the IgG-containing supernatant was added to PBS for concentration and desalting. Purified IgG solutions with volumes around 500 to 600 µL and concentrations ranging from 7.661 to 12.861 µg/µL were purified from each 3 mL volume of pooled serum.

The purity of IgG solution for each treatment group was qualitatively assessed by SDS-PAGE and Western blot analyses. Coomassie-stained SDS-PAGE gel revealed prominent bands at ~50 kDa and ~25 kDa, which corresponded with the molecular weights of IgG heavy chain and light chain, respectively (Figure 2.15 A). There were also a few bands in the higher molecular weight range and the protein staining profiles were very similar between all treatment groups. Protein bands subsequently identified by Western blot with anti-rat IgG antibody precisely matched with bands observed in the SDS-PAGE gel (Figure 2.15 B). The high molecular weight protein species were likely to be multimers of IgG heavy chains and light chains because they were also recognised by the anti-rat IgG antibody. The absence of non-specific protein bands in the SDS-PAGE and Western blot analyses indicated that all IgG samples prepared from rat sera were very pure and visibly free of non-IgG protein contaminants such as albumin.

Purified IgG samples were tested against their respective protein immunogen as well as rat hippocampal brain lysate to assess binding capacity following the purification procedure. IgG solutions purified from pooled Luc, recNR1 or NR1extra2 sera all appeared to have retained binding capacity to their immunogens when Western blot band profiles in Figure 2.16 were compared to the final serum samples in Figure 2.12. Purified Luc IgG successfully detected the full-length Luc protein (Figure 2.16 A) while purified recNR1 IgG recognised the two main recNR1 protein species, the full-length protein and the degradation product at ~32 kDa (Figure 2.16 B). Both IgG samples also reacted to a number of secondary bands in the protein preparations. Similar to NR1extra2 rat sera, purified NR1extra2 IgG reacted with the full-length NR1extra2 protein and the non-specific secondary protein band at ~28 kDa (Figure 2.16 C). Although the protein preparations used were different, the intensity of the bands detected by NR1extra2 IgG was visibly weaker compared to both Luc and recNR1 IgG.
To assess reactivity against proteins present in brain tissue, purified IgG samples were also used to probe rat hippocampal lysate in Western blot. Commercial anti-GluN1 antibodies were used as positive controls for comparison and they both detected a band at ~120 kDa, the corresponding molecular weight of GluN1 (Figure 2.16 D). Out of all the IgG samples tested, only recNR1 IgG successfully recognised a band at the same molecular weight. Few secondary bands were detected by all four IgG samples at various molecular weights but none matched with the positive controls.
Figure 2.15 SDS-PAGE and Western blot analyses of purified rat IgG

IgG samples purified from rat sera were resolved by SDS-PAGE for Coomassie blue staining (A) and for Western blot with anti-rat IgG antibody (B). Bands for heavy chain and light chain of IgG were observed at the expected molecular weights of ~50 kDa and ~25 kDa, respectively, in both the gel and the blot (arrows). IgG samples for all treatment groups appeared relatively pure and free of non-IgG protein contaminants.
Figure 2.16 Western blot analysis of IgG specificity against recombinant protein preparations and brain lysate

Purified IgG samples were assessed for reactivity against their respective recombinant protein immunogen and rat hippocampal lysate by Western blot. Comparisons were made with commercial antibodies. Luc IgG was able to detect the full-length Luc protein (arrow), as did the commercial anti-Luc antibody (A). Luc IgG also displayed reactivity to a number of secondary bands. recNR1 IgG and commercial anti-GluN1 M3-M4 antibody had similar reactivity profiles in that they both detected the two main protein products in the recNR1 protein preparation (arrows) as well as a number of secondary bands (B). The commercial anti-GluN1 N-term antibody only reacted with the full-length recNR1 protein and a lesser number of secondary bands compared to the anti-GluN1 M3-M4 antibody. NR1extra2 IgG was able to detect the full-length NR1extra2 protein (arrow) like the commercial anti-GluN1 M3-M4 antibody, but also bound to the secondary band at ~28 kDa (C). Both commercial anti-GluN1 antibodies detected a band at the predicted molecular weight of GluN1 (~120 kDa) (arrow), as well as some secondary bands in rat hippocampal lysate (D). Among the four treatment groups, only recNR1 IgG was able to detect a band at ~120 kDa. All IgG samples reacted to a small number of secondary bands in hippocampal lysate.
2.3.5. Preabsorption of recNR1 IgG against Luc and recNR1 proteins

Preabsorbed recNR1 IgG samples were produced in order to investigate the specificity of recNR1 IgG-mediated effects in later studies. Purified recNR1 IgG sample was incubated with Luc or recNR1 protein preparations. After insoluble recombinant proteins were removed from the mixtures, IgG samples were concentrated down to their original volumes before preabsorption. Quantification of concentration by protein assay was undesirable due to a limitation in the amount of preabsorbed samples available. The binding capacity of preabsorbed recNR1 IgG samples were compared with non-preabsorbed IgG samples by SDS-PAGE analysis with recNR1 protein as an alternative method of quantification, which also provided a validation of our protocol. Preabsorbed recNR1 IgG samples were assumed a concentration of 1.0 µg/µL because they were concentrated down to their original volumes. Obvious differences in band profiles were observed when comparisons were made between preabsorbed and non-preabsorbed recNR1 IgG samples applied to the blot at the same concentration of 2.0 µg/mL (Figure 2.17 A). Non-preabsorbed recNR1 IgG and recNR1 IgG preabsorbed against Luc protein (pre-Luc) shared similarities in their band detection. Both IgG samples reacted with the two major recNR1 protein species with similar intensities but the band profile of recNR1 pre-Luc IgG was comparatively cleaner with fewer secondary bands. This indicated that Luc and recNR1 protein preparations contained common non-specific proteins because antibodies against these proteins were removed following preabsorption with Luc protein preparation. In stark contrast, preabsorption of recNR1 IgG against recNR1 protein (pre-recNR1) appeared rather successful as evidenced by the absence of all protein bands, save for a very weak band at ~32 kDa. The equivalent binding concentrations of recNR1 pre-Luc and pre-recNR1 IgG samples were determined to be 2.294 and 0.009 µg/mL, respectively, using the standard curves plotted from the integrated intensity values of the two major protein species at different concentrations of non-preabsorbed recNR1 IgG (Figure 2.17 B).
Non-preabsorbed recNR1 and preabsorbed recNR1 IgG samples were used to probe against recNR1 protein to assess their binding capacities. recNR1 protein preparation was loaded at 1 µg per lane and resolved on a 12% acrylamide gel. Non-preabsorbed recNR1 IgG was applied at 0.25, 0.5, 1.0, 2.0 or 4.0 µg/mL to generate a standard curve, while the two preabsorbed recNR1 IgG samples were applied at 2.0 µg/mL. The blot shows a progressive increase in band intensity as the non-preabsorbed recNR1 IgG concentration increased (A). Band 1 is the full-length recNR1 protein at 83 kDa and Band 2 is the recNR1 protein degradation product at ~32 kDa. recNR1 IgG preabsorbed against Luc protein (pre-Luc) exhibited a similar pattern of reactivity to non-preabsorbed recNR1 IgG of the same concentration (2 µg/mL), but with fewer secondary bands. Near complete attenuation of binding was demonstrated by recNR1 IgG preabsorbed against recNR1 protein (pre-recNR1). A very weak band remained slightly visible at ~32 kDa. The volume measurements were plotted IgG concentration for Bands 1 and 2 (B). Using the least squares method, the data for the two bands were fitted with second order polynomial regressions to determine the equivalent binding concentration of the preabsorbed recNR1 IgG samples.

Figure 2.17 Western blot analysis of the binding capacity of recNR1 IgG samples
2.4. Discussion

In this chapter, functional antibodies against recombinant proteins purified from bacterial lysates were successfully generated by immunisation in rats. BL21 (DE3) E. coli cells were transformed with pET-Luc, pTriEx-recNR1 or pET-NR1extra2 plasmids to produce recombinant Luc, recNR1 and NR1extra2 proteins, respectively. Proteins purified from bacterial lysates by IMAC were used to immunise Sprague Dawley rats over a period of three months to stimulate IgG production. Most immunised animals successfully developed IgG against their respective immunogen but with variability in immune response. Sera from the best responding animals were harvested and characterised for epitope binding before IgG was purified by the caprylic acid method. Finally, IgG solutions of high purity were obtained and characterised for binding to their immunogens prior to use in primary cell culture experiments in later chapters.

2.4.1. IMAC purification with BL21 (DE3) lysate yielded recombinant protein preparations of high purity.

BL21 (DE3) competent cells were easily transformed with the three expression constructs. Although the cDNAs used for the two GluN1 proteins were of mouse origin, there is more than 99% homology between the mouse, rat and human GluN1 amino acid sequences, and differences are only located in the N-terminal extracellular domain (Karp et al., 1993; Le Bourdellès et al., 1994; Meguro et al., 1992; Yamazaki et al., 1992). The growth rate prior to induction at mid log phase was substantially slower for cells expressing pTriEx-recNR1. The pTriEx-recNR1 cultures took twice as long to reach 0.6 OD\textsubscript{600} compared to pET-Luc and pET-NR1extra2. Basal expression of T7 RNA polymerase by BL21 (DE3) cells at low levels could result in ‘leakiness’ of the T7 promoter and cause small amounts of recombinant protein to be produced before induction with IPTG. Therefore, it was possible that low levels of recNR1 protein in cultures may have been toxic or inhibited the growth of cells in some way. Basal expression of T7 RNA polymerase could be controlled by \textit{lacI}\textsuperscript{Q} repressor or T7 lysozyme co-expression (Dubendorf & Studier, 1991; Moffatt & Studier, 1987). The lac repressor and T7 lysozyme inhibit transcription initiation from the T7 promoter by binding to the lac operator and T7 RNA polymerase, respectively. Basal expression can be further controlled through a hybrid T7/lac promoter, generated by inserting a lacO operator downstream of the T7 promoter (Dubendorf & Studier, 1991). The pET expression system utilised both the lac repressor and the T7/lac promoter which may have allowed pET-Luc and
pET-NR1extra2 cultures to propagate at a faster rate. Impaired growth in pTriEx-recNR1 cultures may have had a direct impact on protein production as demonstrated by the low yield of recNR1 protein per volume of culture.

All three recombinant proteins were successfully purified from insoluble inclusion bodies by IMAC using the Profinia™ Protein Purification system. The full-length protein was confirmed to be the major protein species in each preparation, as shown by SDS-PAGE and Western blot analyses with commercial antibodies where the most prominent band was observed at the expected molecular weight of the protein. NR1extra2 had the highest purity out of the three preparations, as demonstrated by the single visible specific band at 20 kDa. In addition to the full-length proteins, the majority of secondary bands present in the Luc and recNR1 protein preparations were also recognised by the commercial anti-Luc and anti-GluN1 antibodies, indicating that they were degradation products of the two recombinant proteins. Therefore, Luc and recNR1 preparations were considered to be of relatively high purity in the sense that they had little contamination with bacterial proteins.

2.4.2. Variable immune responses to recombinant protein immunisation were observed in rats

Purified protein preparations were used to immunise rats over a three month period and IgG production was qualitatively assessed by Western blot analysis using rat sera from different time points as primary antibodies. All three Luc-immunised rats rapidly developed IgG against the Luc protein preparation, which persisted until the end of the immunisation period. Only three out of five recNR1-immunised rats responded well to injections and produced stable levels of IgG against the full-length protein. On the other hand, NR1extra2 sera had to be screened at a lower dilution compared to Luc and recNR1 serum samples due to low levels of IgG expressed. IgG against the full-length NR1extra2 protein was detectable in sera from four out of five NR1extra2-immunised rats but its expression peaked half way into the immunisation period and gradually declined. Unexpectedly, NR1extra2 serum samples also reacted with a non-specific protein species at 28 kDa, which was not visible in the SDS-PAGE analysis previously conducted. Therefore, it became apparent that the NR1extra2 protein preparation contained a small amount of bacterial protein. Unlike IgG against the full-length NR1extra2 protein, the expression of IgG against the protein species at 28 kDa peaked and remained relatively stable until the end of the immunisation period. The immunogenicity of protein is influenced by various factors including foreignness, size, chemical complexity,
conformation and degradability (Crumpton, 1974). Considering our three recombinant protein preparations, it was not surprising that the fully foreign Luc protein was the most effective in eliciting an immune response. In spite of its high homology with the extracellular domains of the native GluN1 subunit, the immunogenicity of the recNR1 protein was probably enhanced by its chimeric nature and size. In contrast, the NR1extra2 protein shared 100% amino acid homology with the rat GluN1 M3-M4 region and was smaller in size (Karp et al., 1993; Yamazaki et al., 1992), which probably reduced its immunogenicity. Depending on how they are presented, ‘self’ proteins can induce low levels of immune response which may or may not subside over time (Diamond, 2003). This could be an explanation for the IgG expression pattern observed in the NR1extra2-immunised animals. It was possible that the NR1extra2 protein was eventually recognised as a ‘self’ protein halfway into the immunisation period, which caused the focus of the immune response to shift towards the 28 kDa foreign bacterial protein, albeit only present in a minute amount.

2.4.2.1. No differences in general health or open-field behaviours were observed in immunised animals

Immunisation with recombinant proteins did not cause any adverse reactions in the rats as shown by the absence of weight loss. A decision to perform an open field test was made upon the observation that NR1extra2-immunised animals appeared more hyperactive and excitable when handled at around Weeks 8 and 10. This was particularly interesting because NMDA receptor blockade by antagonists has been associated with locomotor hyperactivity in rodents (Danysz et al., 1994; Leriche et al., 2003; Maj et al., 1992). IgG is known to transfer from serum into the CSF basally at low levels and is the third most abundant protein type in the CSF (Keir & Thompson, 1986; Thompson, 2005). Since this was the first time rats had been immunised with the NR1extra2 protein, it became of interest to determine if the treatment could elicit changes in behaviour with the open field test as a first screen for locomotion, exploratory activity and anxiety (Prut & Belzung, 2003). However, no statistical differences were detected in any of the parameters measured. The test was limited by small sample sizes and it was possible that results were confounded by variations in antibody titre, which was not measured in animals. It is imperative for test animals to have similar antibody titres because the concentration of IgG present in the CSF is proportional to the plasma concentration on the assumption that the BBB is intact (Keir & Thompson, 1986). It was also
possible that differences in behaviour had subsided with the decline in NR1extra2-specific IgG expression in animals because the test was conducted at Week 12.

2.4.3. Sera from recNR1 and NR1extra2 but not PBS and Luc-immunised animals exhibited reactivity to GluN1

Individual serum samples from the best responding animals reacted non-specifically with protein preparations other than their respective immunogen when Western blot analysis was conducted. Most positive bands were likely bacterial proteins common to all protein preparations. In addition, recNR1 sera detected the full-length NR1extra2 protein but not vice versa, most likely as a result of low NR1extra2 IgG levels in serum samples. The low expression of NR1extra2 IgG was further reflected in the epitope mapping results by ELISA. Pooled serum samples from recNR1- and NR1extra- immunised animals both exhibited polyclonal reactivity against peptides spanning the mouse GluN1 extracellular domains but the pattern and specificity z-scores differed greatly. Similar observations have been made in previous studies where rats were immunised with GluN1 proteins (Chen, 2010; During et al., 2000). To expectation, pooled recNR1 serum recognised peptides with high reactivity across both extracellular domains, mainly in the GluN1 glycine-binding region but also in the N-terminus. Although pooled NR1extra2 serum successfully detected several peptides within the M3-M4 region, the low specificity z-scores were indicative of low expression of specific IgG. Importantly, pooled sera from each of the two control treatment groups, PBS and Luc, both displayed negligible reactivity against GluN1 peptides.

2.4.4. Purification and validation of IgG from rat sera

Sera from the best responding animals were pooled for IgG purification by the caprylic acid method. This purification method was proven to be very effective in isolating IgG from serum samples as demonstrated by the high IgG purity determined by SDS-PAGE and Western blot analyses. However, the purified solutions were crude IgG preparations which also contained endogenous IgG. This was undoubtedly a disadvantage of the caprylic acid purification method. To purify specific IgG from sera, we would have had to use affinity purification with immobilised antigens (Chumpia et al., 2003). However, this method requires a large amount of pure antigens and recombinant protein preparations are not suitable for use due to the presence of bacterial proteins. Also, given the limited amount of serum samples that were available, it was less risky to perform a crude purification. All IgG
samples retained their binding capacity against their respective recombinant protein immunogen following purification. Also, purified recNR1 IgG was shown to detect GluN1 in rat hippocampal lysate when Western blot analysis was performed.

In addition to regular purified IgG samples, we also prepared preabsorbed recNR1 IgG samples for use in later studies to investigate the specificity of recNR1 IgG-mediated effects. Purified recNR1 IgG samples were successfully preabsorbed against Luc (pre-Luc) and recNR1 (pre-NR1) protein preparations. As expected, Western blot analysis showed that recNR1 pre-Luc IgG retained its binding capacity against recNR1 protein preparation, which was comparable to that of non-preabsorbed IgG. On the other hand, the binding capacity of recNR1 IgG was almost completely attenuated following preabsorption with recNR1 protein preparation. IgG samples prepared in this chapter were utilised in primary culture experiments detailed in subsequent chapters to characterise IgG-mediated effects on synaptic protein expression and function.
Chapter 3.
Anti-GluN1 IgG-mediated effects in primary rat hippocampal neurons
3.1. Introduction

The primary aim of this chapter was to apply control and anti-GluN1 IgG samples prepared in Chapter 2 to primary rat hippocampal neuronal cultures to investigate their effects at the cellular level with a specific focus on protein expression in the excitatory glutamatergic synapse. We aimed to elucidate the mechanisms that underlie the neuroprotective and cognitive enhancing properties of anti-GluN1 IgG that were previously reported by our laboratory in rats and mice (Chen, 2010; Chen, 2015; During et al., 2000). Behavioural phenotypes of animals in these past studies suggest that anti-GluN1 IgG may act as a partial agonist of NMDA receptors (Bado et al., 2011; Flood et al., 1992; Zlomuzica et al., 2007). Possible epitope-dependent effects were explored using the two types of GluN1-targeting antibodies, namely recNR1 IgG and NR1extra2 IgG.

The hippocampus was chosen as the brain region of interest for the work in this thesis because of its pivotal and well established role in learning and memory (Malenka & Nicoll, 1999). Cultures of dissociated hippocampal neurons have been utilised for several decades as an in vitro model to study various research questions relating to neuronal development, activity and function, as well as investigate pharmacological treatments. Dissociated hippocampal cultures were first developed by Banker and Cowan (1977) to investigate factors which determine neuronal morphology and connectivity. Banker and Cowan revealed strong similarities in neurite formation patterns, rate of growth and cell shape between hippocampal neurons in dissociated cultures and those in vivo. Dissociated hippocampal neurons form multiple synaptic contacts with each other in vitro and can be grown at low density, which allows complete visualisation of individual neurons and their dendritic arbours (Bartlett & Banker, 1984). Additional advantages such as direct access to cells with the freedom to control extracellular environments permit investigation of neuronal connectivity, synaptic transmission and plasticity under various conditions.

We hypothesised that anti-GluN1 IgG may behave as a partial agonist and treatment in primary rat hippocampal neurons could result in alterations in synaptic protein expression including that of NMDA and AMPA receptors, in the absence of neurotoxicity. Also, the two types of GluN1-targeting IgG, recNR1 IgG and NR1extra2 IgG may elicit differential effects due to differences in their target epitopes.
3.2. Materials and methods

3.2.1. Primary culture of rat hippocampal neurons

3.2.1.1. Animals

Cultures were prepared from male and female Sprague Dawley rat pups at P0. Animals were obtained from the Vernon Jansen Unit (Faculty of Medical and Health Sciences, University of Auckland). Experimental work involving animals were carried out in accordance with the guidelines for laboratory animal care, with approval from the University of Auckland Animal Ethics Committee (AEC number 1038).

3.2.1.2. Coverslip preparation

In a fume hood, 22 x 22 mm square glass coverslips (Menzel-Glaser) were soaked in concentrated nitric acid (69%) for 18-36 hours in the dark and then washed 10 times with dH2O over a 2 hour period. After draining off excess water, acid-washed coverslips were transferred into 100% ethanol for storage in the dark until use. Single coverslips were held with forceps and sterilised by passing them through the flame of a gas burner. Once the ethanol has fully evaporated, the sterilised coverslips were placed in 6-well tissue culture plates (BD Falcon, 353046), one in each well. To ensure cell adhesion, each coverslip was then coated by incubating with 1.25 mL of 10 µg/mL poly-D-lysine (PDL) (Sigma, p1149) diluted in PBS at 37 °C overnight. The PDL solution was removed the following day and each well was washed twice with PBS, first with 2 mL and then with 1 mL.

3.2.1.3. Dissociated neuronal culture

3.2.1.3.1. Dissection of the rat hippocampus

Dissociated hippocampal cultures were prepared and maintained using methods described by Banker and Goslin (1998) with some modifications. Dissecting tools were sterilised by autoclaving and exposed to UV light for at least 30 min. Hippocampal dissections were carried out in a laminar flow hood with aseptic technique. P0 newborn rat pups were gently wiped with tissue sprayed with 70% ethanol and euthanised by decapitation with scissors. The head was placed into the lid of a 35 mm cell culture dish (BD Falcon, 353001) and incisions through the skin and skull were made along the midline of the head and between the eyes. The skin and skull were peeled away with a pair of blunt curved forceps to expose the
brain, which was scooped out and placed into a 35 mm cell culture dish containing 4.3 mL of ice-cold sterile Hank’s balanced salt solution (HBSS) (0.95% (w/v) Hanks’ Balanced Salts (Sigma, H2387), 10 mM HEPES, pH 7.2 with NaOH). Hippocampi from each brain were carefully dissected under a Zoom Stereomicroscope (Olympus, SZ61) using fine Jeweller’s No. 5 forceps (World Precision Instruments, 555229F). The cerebral hemispheres were gently separated while anchoring the brain at the cerebellum to reveal the hippocampi sitting in the medial temporal lobes. Any meninges attached to the hippocampi were carefully removed. Cuts were made first to the anterior and posterior ends, followed by the outer convex edge of the hippocampus to free it from the cortex (Figure 3.1). Carefully, using the fine forceps, hippocampi were transferred immediately after dissection into a separate 35 mm cell culture dish containing ice-cold sterile HBSS.

![Figure 3.1 Hippocampal dissection](image)

The inner concave edge of the rat hippocampus does not require cutting as it faces the lateral ventricle. Therefore, the only cuts needed to free the hippocampus are to its anterior ① and posterior ② ends, as well as along the outer convex edge ③.

### 3.2.1.3.2. Cell dissociation and plating

All cell culture procedures were performed in a Class II Biological Safety Cabinet (HERAsafe, Thermo Scientific) under sterile conditions with aseptic technique. Isolated hippocampi were transferred from ice-cold HBSS into 5 mL of pre-warmed HBSS in a 15 mL tube containing freshly added papain at 20 U/mL (Worthington, LK003178). Hippocampi were incubated at 37 °C for 15 min and gently mixed every 5 min by inversion. The papain solution was removed and replaced with 5 mL of pre-warmed Minimum Essential Medium (MEM) (Gibco, 11090-081) containing 10% (v/v) fetal bovine serum (Thermo Scientific, HYCSH30406.02) for 1-2 min to inactivate enzyme activity. The enzyme inactivation solution was fully removed and 2 mL of pre-warmed complete NBM culture medium
(Neurobasal® Medium (Gibco, 21103-049) containing B-27® Supplement (Gibco, 17504-044) and freshly added GlutaMAX™ Supplement (Gibco, 35050-061)) was added to the tube. Cells were then dissociated by gentle trituration (~10 times) with a fire-polished glass Pasteur pipette that has a bore size of ~0.8 mm. With the tube slightly tilted, large tissue chunks were moved to one side to maximise transfer of the dissociated cell suspension into a suitable volume of pre-warmed complete NBM for plating at the desired density. Cells were plated at an approximate density of one hippocampus per 6-well plate with 2 mL of culture medium per well. Primary hippocampal cultures were maintained in a humidified incubator (HERAcell 150, Thermo Scientific) at 37 °C in an atmosphere of 5% CO2/95% air. Cells were refed by replacing ~25% or 0.5 mL of culture medium with fresh complete NBM on 1 day in vitro (DIV) and weekly thereafter.

3.2.2. Treatment of neuronal cultures with rat IgG

Purified IgG solutions prepared in Section 2.2.5 were diluted to 1 µg/µL with PBS before they were applied to cultured neurons on DIV 8, a scheduled day for refeeding. Each well was refed by replacing ~25% or 0.5 mL of culture medium with fresh complete NBM containing 1% (v/v) penicillin-streptomycin (Gibco, 15140) because purified IgG solutions were not sterile. Following refeed, IgG solutions were directly pipetted into each well to obtain a final concentration of 1.75, 3.5 or 7.0 µg/mL. Plates were gently mixed once by swirling to disperse the IgG. Treatments were typically left on for 7 days before cells were fixed (Figure 3.2).

![Figure 3.2 Timeline of a typical primary culture experiment involving IgG treatment](image-url)
3.2.3. Immunocytochemistry

Culture medium was removed from each well and coverslips were rinsed twice with 2 mL of PBS. Cells were fixed by incubating each coverslip in 1 mL of 4% (w/v) paraformaldehyde (PFA) or Shandon™ Glyo-Fixx™ (Thermo Scientific, 6764262) for 20 min at RT. Following fixation, coverslips were washed twice with 2 mL of PBS for 5 min each time with gentle shaking. Cells were permeabilised with 1 mL of PBS containing 0.25% (v/v) Triton X-100 for 5 min and then washed twice with 2 mL of PBS for 5 min each time for gentle shaking. For immunofluorescent staining, non-specific binding sites were first blocked by incubating each coverslip in the plate with 600 µL of PBS containing 3% (v/v) normal goat serum (NGS) (Gibco, 16210-064) for 1 hour at RT with gentle shaking. Improvised humidified chambers were made in order to reduce the volume of antibody or staining reagent used (Figure 3.3).

![Figure 3.3 Construction of a simple humidified chamber](image)

A simple chamber can be constructed by placing a square piece of paper towel hydrated with distilled water and a layer of parafilm into a 15 cm Petri dish. Coverslips were handled with forceps and placed on the parafilm with the cells facing upwards and solutions were gently pipetted onto the coverslips.

Incubation steps hereon were performed in humidified chambers while wash steps with PBS were performed in the original culture plates. Coverslips were transferred to humidified chambers and 200 µL of primary antibody cocktail (Table 3.1) diluted in PBS containing 3% (v/v) NGS was applied for 2 hours at RT or overnight at 4 °C. Coverslips were moved back to their original wells and washed three times with 2 mL of PBS for 10 min each time with gentle shaking. Parafilm sheets were replaced before coverslips were once again transferred into humidified chambers and 200 µL of fluorophore-conjugated secondary antibody cocktail (Table 3.2) diluted in PBS containing 3% (v/v) NGS was applied for 1 hour at RT in the dark. Subsequent steps were all performed in the dark to prevent photobleaching of the fluorophores. Coverslips were washed three times with 2 mL of PBS for 10 min each time.
with gentle shaking in the plates. Excess PBS solution was blotted away with tissue before coverslips were mounted cell-side down onto a glass microscope slide (Thermo Scientific, LBSP4981) with 10 µL of AF1 antifade mountant solution (Citifluor). Mounted coverslips were left overnight to allow the antifade to fully permeate before sealing with clear nail polish and stored in the dark at 4 °C.

Table 3.1 List of primary antibodies used for immunostaining neuronal cultures

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<tr>
<td>Anti-GluN1</td>
<td>Mouse</td>
<td>BD Pharmingen</td>
<td>556308</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-Homer</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-15321</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Anti-MAP2</td>
<td>Rabbit</td>
<td>Chemicon</td>
<td>ab5622</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Anti-MAP2A, 2B</td>
<td>Mouse</td>
<td>Chemicon</td>
<td>mab378</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Anti-PSD-95</td>
<td>Mouse</td>
<td>Neuromab</td>
<td>75-028</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Anti-VGLUT1</td>
<td>Guinea pig</td>
<td>Millipore</td>
<td>AB5905</td>
<td>1:2,000</td>
</tr>
</tbody>
</table>
### Table 3.2 List of secondary antibodies used for immunostaining neuronal cultures

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Host species</th>
<th>Company</th>
<th>Catalogue no.</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 488 Anti-mouse IgG</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A21202</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 488 Anti-rabbit IgG</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A21206</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 594 Anti-guinea pig IgG</td>
<td>Donkey</td>
<td>Jackson ImmunoResearch</td>
<td>706-585-148</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 594 Anti-rabbit IgG</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A21207</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 594 Anti-mouse IgG</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A21203</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 647 Anti-rabbit IgG</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A31573</td>
<td>1:500</td>
</tr>
<tr>
<td>Alexa Fluor 647 Anti-mouse IgG</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A31571</td>
<td>1:500</td>
</tr>
</tbody>
</table>

*Note* Stock solutions of Anti-guinea pig IgG secondary antibodies were diluted 1:1 with water and glycerol.

### 3.2.4. Assessment of potential toxicity

To assess potential toxicity following treatment, primary neuronal cultures were prepared according to Section 3.2.1 and challenged with different concentrations of IgG. On DIV 8, cells were treated with PBS IgG, Luc IgG, recNR1 IgG or NR1extra2 IgG at 1.75, 3.5 or 7.0 µg/mL as described in Section 3.2.2. A naïve group was included as an additional control. A total of three individual cultures were used for the experiment.
3.2.4.1. MAP2 immunocytochemistry

To assay for neurotoxicity, cells were fixed with 4% (w/v) PFA on DIV 15. Immunocytochemistry was then conducted on cultures as described in Section 3.2.3 using a mouse primary antibody for the neuronal marker microtubule-associated protein 2 (MAP2) (Chemicon, mab378) (Carrier et al., 2006). Donkey Alexa Fluor 594 anti-mouse IgG antibody (Invitrogen, A21203) was used as the secondary antibody.

3.2.4.2. DAPI staining

Nuclear staining with 4',6-Diamidino-2-phenylindole (DAPI) (Sigma, D-9542) was performed on cultured cells after immunostaining against MAP2 (Section 3.2.4.1), before they were mounted. After secondary antibody incubation and washes in PBS, the coverslips were transferred back into humidified chambers containing fresh parafilm sheets. DAPI was diluted to 50 ng/mL with PBS and 200 µL was applied to each coverslip for 20 min at RT. Coverslips were then transferred back to the plates, washed twice with 2 mL of PBS for 10 min each time with gentle shaking, and mounted onto slides as described in Section 3.2.3.

3.2.4.3. Image acquisition

Images were captured on an Olympus AX70 microscope with appropriate filters and a MBF CX9000 camera at 10x magnification using PictureFrame Software Version 2.2 (Optronics). Five fields from each coverslip were randomly selected for quantification, usually from the four corners and the centre. Edges of coverslips and regions with prominent pools of fibroblasts were avoided.

3.2.4.4. Image analysis

MAP2 immunostaining is frequently used as a measure of neurotoxicity by quantifying the number of MAP2-positive cells in random fields of view following treatments (Jeohn et al., 1998). The number of surviving neurons was quantified by manually counting MAP2-positive cells using the built-in Cell Counter plugin in ImageJ Software Version 1.48p (NIH Image). MAP2-immunopositive area was also measured using ImageJ as another indicator of neuronal viability (Vandame et al., 2007; Vandame et al., 2013). In brief, MAP2 immunofluorescence images were converted from RGB to 8-bit greyscale before a set threshold was applied to highlight MAP2-immunopositive dendrites. Measurements of the area were then made with the resultant binary images by selecting and adding the areas to the
‘region of interest (ROI) Manager’ in ImageJ (Figure 3.4). Qualitative assessment of neuronal morphology was also conducted. The number of DAPI-positive cells was quantified to assess overall cell survival, including non-neuronal cells. This was performed firstly by converting the DAPI fluorescence images to 8-bit grayscale and applying a set threshold. The ‘Watershed’ function was then used to separate neighbouring nuclei which were in contact and the number of spots was counted using the ‘Analyse Particles’ function with the ROI Manager (Figure 3.5). The same MAP2 and DAPI threshold values were used for all images. Neuronal purity was also calculated by dividing the number of MAP2-positive cells by the number of DAPI-positive cells and expressed as a percentage.

3.2.4.5. Statistical analysis

Data for the five fields of view from each coverslip were combined into a single total value. To compensate for possible variability in cell density, results from each culture (except neuronal purity) were expressed as a percentage of their respective culture naïve control.

Statistical analysis was performed using GraphPad Prism Software Version 6. All data were presented as mean ± SEM. The Kolmogorov-Smirnov test for normality was used to determine if data sets were parametric. Bartlett’s test and Levene's test were used to test for equality of variances in parametric and non-parametric data, respectively. If parametric methods could be used, statistical differences between treatment groups were determined by one-way ANOVA with comparison to the naïve group by Dunnett’s multiple comparisons test. If assumptions of the parametric method were not satisfied, the non-parametric Kruskal-Wallis test was used with comparison to the naïve group by Dunn’s multiple comparisons test. A difference was considered statistically significant when the p-value was less than 0.05.
Figure 3.4 Measurement of MAP2-immunopositive area in ImageJ Software

Figure 3.5 Quantification of DAPI-positive cells in ImageJ Software
3.2.5. Quantification of synaptic protein expression following IgG treatment

To assess and quantify changes in synaptic protein expression following IgG treatment, primary neuronal cultures were prepared according to Section 3.2.1 and IgG solutions were applied on DIV 8 as described in Section 3.2.2. Control groups included naïve cells and neurons treated with PBS IgG or Luc IgG. Preabsorbed recNR1 IgG solutions prepared in Section 2.2.7 were used in one experiment. Previous studies from our laboratory have estimated the concentration of IgG in the CSF of immunised rats to be approximately 3.5 µg/mL (unpublished data). Therefore, we decided to apply IgG treatments to cultures at 1.75, 3.5 and 7.0 µg/mL. All cultures were fixed on DIV 15 with 4% (w/v) PFA except those used for the VGLUT1/GluN1 and VGLUT1/GluA1 experiments, which were fixed with Glyo-Fixx. Following fixation, immunocytochemistry was performed as described in Section 3.2.3. A total of at least three individual cultures were used for each immunostaining experiment. Synaptic proteins of interest, treatment groups, IgG concentrations, primary and secondary antibodies used for each experiment are detailed in Table 3.3.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment groups</th>
<th>1° antibodies</th>
<th>2° antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSD-95</td>
<td>Naïve, PBS IgG, Luc IgG, recNR1 IgG, NR1extra2 IgG IgG at 1.75, 3.5, and 7.0 µg/mL</td>
<td>Anti-MAP2</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-PSD-95</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td>PSD-95 with preabsorbed IgG</td>
<td>Luc IgG, recNR1 IgG, recNR1 pre-Luc IgG, recNR1 pre-recNR1 IgG IgG at 7.0 µg/mL</td>
<td>Anti-MAP2</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-PSD-95</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td>VGLUT1/Homer (Excitatory synapses)</td>
<td>Naïve, PBS IgG, Luc IgG, recNR1 IgG, NR1extra2 IgG IgG at 3.5 and 7.0 µg/mL</td>
<td>Anti-MAP2A, 2B</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-VGLUT1</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-Homer</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>VGLUT1/GluN1 (Synaptic NMDAR)</td>
<td>Naïve, PBS IgG, Luc IgG, recNR1 IgG, NR1extra2 IgG IgG at 3.5 and 7.0 µg/mL</td>
<td>Anti-GluN1</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-VGLUT1</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-MAP2</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>VGLUT1/GluA1 (Synaptic AMPAR)</td>
<td>Naïve, PBS IgG, Luc IgG, recNR1 IgG, NR1extra2 IgG IgG at 3.5 and 7.0 µg/mL</td>
<td>Anti-GluA1</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-VGLUT1</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-MAP2A, 2B</td>
<td>Alexa Fluor 647</td>
</tr>
</tbody>
</table>
3.2.5.1. Image acquisition

Images of cultured neurons were acquired using the Zeiss Axioplan 2 microscope in the Biomedical Imaging Research Unit (Faculty of Medical and Health Sciences, University of Auckland). Neurons on coverslips were randomly selected based on their morphology and only healthy pyramidal neurons that did not have excessive overlaps with other cells were imaged to ensure experimental consistency. The selection criteria included having a triangular shaped soma, visually intact cell membranes, as well as having thick and extended dendrites (Spruston, 2008). The following filters were used for visualising the emission of different fluorophores: Fluorescein isothiocyanate (FITC) (longpass filter) or green fluorescent protein (GFP) (bandpass filter) for Alexa Fluor 488; Rhodamine (bandpass filter) for Alexa Fluor 594; Cy5 (bandpass filter) for Alexa Fluor 647. Z-series stacks (z-stacks) of 0.2 µm interval were digitally captured at 63x magnification with an oil immersion objective lens (1.4 numerical aperture) using a Princeton Micromax cooled charge-coupled device (CCD) camera (RTE/CCD-1317-K/2 model) controlled by MetaMorph® Software Version 7.8.3.0. Given that the depth of field was approximately 0.4 µm, sampling z-stacks at an interval of 0.2 µm satisfied the Nyquist Criterion (Bolte & F.P., 2006). This ensured an accurate three-dimensional representation of dendrites and synapses. Exposure times were optimised to minimise fluorophore excitation to prevent specimen photobleaching, but still achieve adequate signal acquisition without compromising image quality due to saturation. Images were collected at 12-bit grayscale and a 2 x 2 binning was used in order to reduce exposure time. If necessary, neutral density (ND) filters were applied to attenuate light intensity and reduce photobleaching. Absence of spectral fluorescence bleed-through was confirmed for each channel prior to image acquisition (see Appendix). Identical imaging conditions were used for cells from all treatment groups imaged for the same experiment. Imaging conditions used for each immunostaining experiment are detailed in Table 3.4.
Table 3.4 Imaging conditions used for immunostaining experiments

Details of target proteins, fluorophores, filter blocks, exposure times, and the number of ND filters used for each immunostaining experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Target protein</th>
<th>Fluorophore</th>
<th>Filter block</th>
<th>Exposure time (ms)</th>
<th>ND filter(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSD-95 and PSD-95 with preabsorbed IgG</td>
<td>MAP2</td>
<td>Alexa Fluor 488</td>
<td>FITC</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PSD-95</td>
<td>Alexa Fluor 594</td>
<td>Rhodamine</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>VGLUT1/Homer</td>
<td>MAP2</td>
<td>Alexa Fluor 488</td>
<td>FITC</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>VGLUT1</td>
<td>Alexa Fluor 594</td>
<td>Rhodamine</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Homer</td>
<td>Alexa Fluor 647</td>
<td>Cy5</td>
<td>500</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>GluN1</td>
<td>Alexa Fluor 488</td>
<td>GFP</td>
<td>600</td>
<td>0</td>
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<tr>
<td>VGLUT1/GluN1</td>
<td>VGLUT1</td>
<td>Alexa Fluor 594</td>
<td>Rhodamine</td>
<td>150</td>
<td>2</td>
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<tr>
<td></td>
<td>MAP2</td>
<td>Alexa Fluor 647</td>
<td>Cy5</td>
<td>200</td>
<td>1</td>
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<tr>
<td>VGLUT1/GluA1</td>
<td>VGLUT1</td>
<td>Alexa Fluor 594</td>
<td>Rhodamine</td>
<td>150</td>
<td>2</td>
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<tr>
<td></td>
<td>MAP2</td>
<td>Alexa Fluor 647</td>
<td>Cy5</td>
<td>50</td>
<td>1</td>
</tr>
</tbody>
</table>
3.2.5.2. Image analysis

All images were analysed using ImageJ Software Version 1.48p (NIH Image). The following parameters were assessed for each protein of interest: 1) puncta fluorescence intensity (total); 2) puncta fluorescence intensity (colocalised); 3) puncta density (total); 4) puncta density (colocalised), where ‘total’ refers to all puncta along dendritic regions analysed and ‘colocalised’ refers to puncta which colocalised with its double-labelling protein partner.

The image analysis method employed is graphically described in Figure 3.6. First, a substack was extracted from each original z-stack image using the ‘Create Substack’ function in order to eliminate out-of-focus slices. The same slice range was used to generate substacks from all z-stacks acquired from the same cell. Each substack was then converted to a maximum projection image using the ‘Z project’ function. The use of maximum projection images was chosen over 3D reconstruction or analysing slices within a substack individually because it improves contrast-and signal-to-noise ratios and consumes less time (Brown & Riederer, 1992; Cheyne & Montgomery, 2008; Gruden et al., 2002). Although it is possible for two separate puncta in different slices to overlap if they share the same x- and y-coordinates, this is not a frequent occurrence and would be unlikely to affect the data if sample sizes are sufficiently large.

Maximum projection images from the same cell were combined into a single RGB image using the ‘Merge Channels’ function with appropriate colours. Images where immunostaining was of poor quality or had high background were excluded from further analysis. The ‘Paintbrush Tool’ was set to the colour white with a brush width of 30 pixels and was used to highlight dendrites on the merged RGB image of the cell to create a dendritic mask ROI. Dendritic branching points and regions of overlap were avoided. A digital graphic tablet (Bamboo Fun Tablet, Wacom) was used to maximise the accuracy of dendritic lengths traced. The highlighted RGB image was converted to 8-bit grayscale and threshold was applied to convert the dendritic mask ROI into a binary image. The dendritic mask ROI was selected and added to the ‘ROI Manager’ in ImageJ for later use. The binary image was then transformed into a one-pixel width image using the ‘Skeletonize’ function, allowing dendritic length to be measured in pixels.

Next, display values of a maximum projection image for a protein of interest were adjusted before the image was converted from 12-bit to 8-bit grayscale. Background intensity was removed using the ‘Subtract Background’ function and a suitable Rolling Ball Radius (RBR).
RBR background subtraction also reduced diffuse dendritic fluorescence and made punctate immunostaining more definitive. A set threshold was applied to create a binary image. Five random cells were selected for each treatment group in a particular experiment and their maximum projection images were used to determine the RBR and threshold values. This was optimised separately for proteins of interest in each set of experiment. Various combinations of RBR and threshold values were trialled to make sure highlighted puncta were of accurate sizes and had intensities that were more than twice the background (see Figure 3-7). The average RBR and threshold values obtained from the five random cells were applied to all cells within the same experiment. Values used for individual experiments are detailed in the Appendix. The ‘Watershed’ function was used to separate multiple adjacent puncta that have amalgamated into a single punctum, if any, in the binary image.

The dendritic mask ROI was selected in the ROI Manager and the ‘Analyse Particles’ function was performed to add puncta within the dendritic mask to the ROI Manager. A particle size threshold (see Appendix) was implemented during this step to prevent smaller noise pixels from being added. ROIs of puncta added to the ROI Manager were overlaid onto the MAP2 image of the cell. Puncta ROIs not localised to dendrites of the cell of interest were deleted from the ROI Manager. The original maximum projection image for the protein of interest was loaded for analysis. Puncta ROIs were selected in the ROI Manager to measure the total (surface and internal) puncta along dendrites and their mean gray values (intensity). The mean gray values for each image were averaged to obtain an average puncta fluorescence intensity value. Average puncta fluorescence intensity values were normalised and expressed as a ratio between each treatment group and a respective control. The ratio provided a semi-quantitative and comparative measurement of the amount of protein expression for each treatment group. Puncta density was calculated by dividing the number of puncta by the dendritic length and expressed as number of puncta per 10 µm. The entire analysis process was repeated for the double-labelling protein partner.

Parameters of colocalised puncta for each protein pair were also assessed. The two maximum projection images were combined using the ‘Merge Channels’ function and the puncta ROIs for both proteins were overlaid on top. Each ROI was carefully examined for colocalisation, indicated by an overlap in shape or colour e.g. colocalisation of red and green puncta results in a yellow colour. Non-colocalised puncta ROIs were deleted from the ROI Manager. Similar to the analysis for total puncta parameters, puncta for each protein were selected in
the ROI Manager in turn and measured for number of puncta and intensity. Calculations for average fluorescence intensity and density were then performed for colocalised puncta.
Figure 3.6 Image analysis in ImageJ Software

Key steps involved in the analysis of immunofluorescent punctate staining of synaptically localised proteins using ImageJ Software. A primary hippocampal neuron immunostained for VGLUT1 (red), GluA1 (green), and MAP2 (blue) was used as an example. Substacks were extracted from the original z-stacks (1) before projection images (maximum intensity) were created (2). Projection images were then merged into a single RGB image (3) which was used to highlight a dendritic mask ROI before converting it to 8-bit grayscale (4). Threshold was applied (5) and the dendritic mask ROI was added to the ROI manager (6). The dendritic mask ROI was skeletonised to allow the measurement of dendritic length (7). Display values were adjusted before maximum projection images were converted to 8-bit grayscale (8) and background was subtracted using a suitable RBR (9). Threshold was applied (10) and Watershed was used on the binary image to separate neighbouring puncta which may have fused (11). Puncta within the dendritic mask ROI were selected and added to the ROI Manager (12), which enabled the measurement of total puncta parameters (13). Non-colocalised puncta ROIs viewed over a new merged RGB image were deleted from the ROI manager (14) to allow colocalised puncta parameters to be measured (15).
Figure 3.6 (Part 1)

1) Create substack

2) Z project (maximum intensity)

3) Merge channels
**Figure 3.6 (Part 2)**

4) Highlight dendritic mask ROI and convert image to 8-bit grayscale

5) Apply threshold

6) Select and add dendritic mask ROI to ROI manager

7) Skeletonize and measure dendritic length
Figure 3.6 (Part 3)

8) Adjust displayed values and convert image to 8-bit grayscale

9) Subtract background with an appropriate rolling ball radius value

10) Apply threshold

11) Apply watershed
12) Select and add puncta within dendritic mask ROI to ROI manager

13) Measure total puncta parameters

14) Delete non-colocalising puncta ROIs

15) Measure colocalised puncta parameters
Figure 3.7 Determination of background subtraction RBR and threshold values

Examples images where different RBR and threshold values were applied to the same maximum projection image. Diffuse punctate immunostaining required lower RBR value to remove fluorescence in the dendrites. Higher threshold values yielded smaller but distinct puncta while lower threshold values produced larger puncta with higher rates of amalgamation. The most suitable RBR and threshold values should fully and accurately highlight puncta with fluorescence intensities more than twice the background signal.
3.2.5.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 6. All data were presented as mean ± SEM. IgG treatments of the same concentration were tested for differences. The Kolmogorov-Smirnov test for normality was used to determine if data sets followed a Gaussian distribution. To test for equality of variances, Bartlett’s test was used on parametric data while Levene's test was used on non-parametric data. If assumptions for the parametric method were met, statistical differences between treatment groups were determined by one-way ANOVA with Tukey’s multiple comparisons test. Otherwise, the non-parametric Kruskal-Wallis test was used with Dunn’s multiple comparisons test. A difference was considered statistically significant when the $p$-value was less than 0.05.

3.2.6. Stability of IgG in cell culture

To assess the stability of rat IgG in culture medium, a single plate of primary neuronal culture was prepared according to Section 3.2.1 until the point of plating in 6-well plates. Cells were plated into three wells while culture medium without cells was added into the remaining three wells. All wells were refed on DIV 1 and recNR1 IgG was applied at 7.0 µg/mL on DIV 8 as described in Section 3.2.2. Day 0 samples were collected immediately after recNR1 IgG was added by sampling 55 µL of culture medium from each well. Samples from wells containing cells were pooled and the same was done for wells without cells. Further samples were collected at 1, 2, 3, 5 and 7 days post-treatment. ELISA was performed as described in Section 2.2.4.2 using peptide 59, one of two peptides with the highest reactivity when probed by pooled recNR1 rat serum sample (Section 2.3.3). Instead of rat serum, 50 µL of undiluted culture medium was applied to each well after peptide coating. Culture medium from DIV 8 containing 7.0 µg/mL of Luc IgG was used as the blank. After background absorbance was subtracted, absorbance measurements were expressed as a percentage of their respective Day 0 sample value.
3.2.7. Changes in synaptic protein expression over time following IgG treatment

To assess and quantify changes in synaptic protein expression over time following IgG treatment, primary neuronal cultures were prepared according to Section 3.2.1 and treated with IgG on DIV 8 as described in Section 3.2.2. Luc IgG (control) and recNR1 IgG were used at 7.0 µg/mL for time course experiments. Cells were fixed at three different time points, at 2 days (DIV 10), 4 days (DIV 12) or 7 days (DIV 15) post-IgG treatment. VGLUT1/Homer experiment cultures were fixed with 4% (w/v) PFA while VGLUT1/GluN1 experiment cultures were fixed with Glyo-Fixx. Immunocytochemistry was performed following fixation according to Section 3.2.3. Primary and secondary antibodies used are detailed in Table 3.1 and Table 3.2, respectively. Images were acquired and analysed as described in Sections 3.2.5.1 and 3.2.5.2, respectively. Imaging conditions used for time course experiments were the same as those described for VGLUT1/Homer and VGLUT1/GluN1 experiments in Table 3.4. A total of three individual cultures were used for each time course experiment. Synaptic proteins of interest, treatment groups, IgG concentrations, primary and secondary antibodies used for each experiment are detailed in Table 3.5.

Table 3.5 Summary of immunostaining time course experiments on IgG-treated neurons

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment groups</th>
<th>1° antibodies</th>
<th>2° antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>VGLUT1/Homer (Excitatory synapses)</td>
<td>Luc IgG, recNR1 IgG</td>
<td>Anti-MAP2A, 2B</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td>Time course</td>
<td>IgG at 7.0 µg/mL</td>
<td>Anti-VGLUT1</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td></td>
<td>2, 4, and 7 days post-treatment</td>
<td>Anti-Homer</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>VGLUT1/GluN1 (Synaptic NMDAR)</td>
<td>Luc IgG, recNR1 IgG</td>
<td>Anti-GluN1</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td>Time course</td>
<td>IgG at 7.0 µg/mL</td>
<td>Anti-VGLUT1</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td></td>
<td>2, 4, and 7 days post-treatment</td>
<td>Anti-MAP2</td>
<td>Alexa Fluor 647</td>
</tr>
</tbody>
</table>
3.2.7.1. Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 6. All data were presented as mean ± SEM. The Kolmogorov-Smirnov test for normality was used to determine if data at each time point were parametric. The F-test was used to test for homogeneity of variance. The two-tailed, unpaired t-test was performed to determine statistical differences between the two treatment groups at each time point if data sets were parametric. The Mann-Whitney test was used for non-parametric data. A difference was considered statistically significant when the $p$-value was less than 0.05.
3.3. Results

3.3.1. No alterations in morphology or toxicity were observed in neurons following IgG treatment up to 7.0 µg/mL.

In addition to the potential therapeutic effects of anti-GluN1 antibodies observed in previous studies by our laboratory (Chen, 2010; Chen, 2015; During et al., 2000), antibodies against glutamate receptors are also associated with various autoimmune disorders including Rasmussen’s encephalitis (Rogers et al., 1994), anti-NMDA receptor encephalitis (Dalmau et al., 2008; Manto et al., 2010; Sansing et al., 2007), anti-AMPA receptor encephalitis (Lai et al., 2009) and systemic lupus erythematosus (DeGiorgio et al., 2001; Kowal et al., 2006) where pathological seizures and neurotoxicity have been observed. Therefore, potential toxicity caused by IgG treatment was first investigated by conducting immunocytochemistry against the neuronal marker MAP2 and DAPI staining on primary neuronal cultures treated with IgG up to 7.0 µg/mL for a week. MAP2 immunoreactivity was distributed in the somatic and dendritic compartments (Figure 3.8). Immunostaining patterns and intensities were visually comparable between IgG treatment groups, regardless of IgG type and concentration, and appeared similar to the naïve group (Figure 3.8 A). There were also no discernible differences in the general shape, cell size, extent of dendritic branching and length in neurons between any of the treatment groups. Large numbers of DAPI-stained cells which were not immunopositive for MAP2 were also present on coverslips from all cultures. This indicated that our dissociated culture method yielded a mix population of cells from the rat hippocampus. The amount of DAPI staining appeared similar between treatment groups and there was no indication of nuclear fragmentation.

Images of MAP2 immunoreactivity and DAPI staining were analysed to assess neuronal and total cell survival. There were no significant differences in the number of surviving MAP2-positive cells (Kruskal-Wallis test, \(H = 6.039, p = 0.914\)) or total somatic and dendritic MAP2-immunopositive area (one-way ANOVA, \(F = 0.787, p = 0.662\)) for any of the IgG treatments at any concentration when comparisons were made with the naïve group (Figure 3.9 A, B). These results demonstrated that IgG treatments up to the concentration of 7.0 µg/mL were not neurotoxic and did not cause aberrant morphological changes in hippocampal neurons. The number of surviving DAPI-stained cells (one-way ANOVA, \(F = 0.321, p = 0.983\)) was not significantly different between IgG treatments and the naïve group (Figure 3.9 C). In addition, the neuronal purity of cultures ranged between 15-20% but were
not statistically different (Kruskal-Wallis test, $H = 9.780$, $p = 0.635$). In summary, IgG treatments up to 7.0 µg/mL did not affect cell viability in experiments with our primary hippocampal neuronal cultures, which incidentally comprised a high proportion of non-neuronal cells.
Figure 3.8 MAP2 immunostaining and DAPI staining in primary neuronal cultures following IgG treatment

Representative images of hippocampal neurons labelled for MAP2 (Alexa Fluor 594, red) and all cell nuclei with DAPI (blue) in a single field of view following IgG treatment. A naïve group (A) was included as an additional control for variability in cell density. Cells were treated with PBS IgG (B, C, D), Luc IgG (E, F, G), recNR1 IgG (H, I, J) or NR1extra2 IgG (K, L, M) at one of three concentrations, 1.75 µg/mL, 3.5 µg/mL or 7.0 µg/mL on DIV 8 for a week, until DIV 15. No visible changes in the morphology of MAP2-positive neurons or any nuclear fragmentation in DAPI staining were observed.

Scale bar, 250 µm.
Figure 3.9 Effect of IgG treatment on neuronal and total cell survival in primary hippocampal cultures

Coverslips of cells were imaged from three independent cultures (Naïve, n = 6; PBS IgG: 1.75 µg/mL, n = 5; 3.5 µg/mL, n = 6; 7.0 µg/mL, n = 5; Luc IgG: 1.75 µg/mL, n = 5; 3.5 µg/mL, n = 5; 7.0 µg/mL, n = 6; recNR1 IgG: 1.75 µg/mL, n = 5; 3.5 µg/mL, n = 6; 7.0 µg/mL, n = 5; NR1extra2 IgG: 1.75 µg/mL, n = 5; 3.5 µg/mL, n = 5; 7.0 µg/mL, n = 5). Images of MAP2 immunostaining and DAPI staining were analysed to quantify the number of surviving MAP2-positive cells (A), MAP2-immunopositive area (B), number of surviving DAPI-stained cells (C) and neuronal purity of cultures (D). Coverslips of cells received no treatment (naïve) or an IgG treatment at 1.75 µg/mL, 3.5 µg/mL or 7.0 µg/mL. All results except neuronal purity were expressed as a percentage of their respective culture naïve control. No statistical differences were observed in any of the measured parameters when comparisons were made with the naïve group.

Data represent mean ± SEM.
Figure 3.9

A

Surviving MAP2-positive cells (%) vs. IgG concentration (µg/mL)

B

MAP2 immunopositive area (%) vs. IgG concentration (µg/mL)

C

Surviving DAPI-stained cells (%) vs. IgG concentration (µg/mL)

D

Neuronal purity (%) vs. IgG concentration (µg/mL)
3.3.2. recNR1 IgG treatment increased PSD-95 puncta density in hippocampal neurons in a concentration-dependent manner

Given its close association with the NMDA receptor, immunocytochemistry to detect PSD-95, a prominent postsynaptic protein of the excitatory synapse (Lim et al., 2003; Sheng, 2001), was conducted on primary neuronal cultures as a first screen to detect changes in synaptic protein expression that may be caused by IgG treatment. Neurons were labelled for MAP2 to visualise their dendrites and PSD-95 (Figure 3.10). PSD-95 immunostaining has a definitive punctate pattern of localisation along MAP2-positive dendrites. The pattern and intensity of PSD-95 immunostaining appeared comparable between all treatment groups from qualitative analysis.

Fluorescence intensity and puncta density were determined from the images to quantify PSD-95 expression following IgG treatment. Fluorescence intensity values were normalised to the naïve control group of each culture for the experiment. No significant differences in PSD-95 fluorescence intensity were found following IgG treatments at any of the concentrations (1.75 µg/mL, one-way ANOVA, $F = 0.705, p = 0.590$; 3.5 µg/mL, one-way ANOVA, $F = 1.219, p = 0.306$; 7.0 µg/mL, Kruskal-Wallis test, $H = 4.765, p = 0.312$) (Figure 3.11 A, C, E). The two lower concentrations of IgG treatment did not induce any significant changes in PSD-95 puncta density (1.75 µg/mL, one-way ANOVA, $F = 0.324, p = 0.862$; 3.5 µg/mL, one-way ANOVA, $F = 1.500, p = 0.206$) (Figure 3.11 B, D). Although not statistically significant, the number of PSD-95 puncta per length of dendrite was slightly higher for neurons treated with 3.5 µg/mL recNR1 IgG (6.63 ± 0.46) compared to the naïve control and other IgG treatments at the same concentration (Naïve, 5.82 ± 0.36; PBS IgG, 5.80 ± 0.43; Luc IgG, 5.42 ± 0.41; NR1extra2 IgG, 5.43 ± 0.34). On the other hand, recNR1 IgG treatment at 7.0 µg/mL significantly increased PSD-95 puncta density (7.40 ± 0.37) compared to all control groups (one-way ANOVA, $F = 5.651, p < 0.001$; Tukey’s post-hoc: Naïve, 5.82 ± 0.36, $p = 0.037$; PBS IgG, 5.41 ± 0.39, $p = 0.004$; Luc IgG, 5.63 ± 0.46, $p = 0.011$) and NR1extra2 IgG (Tukey’s post-hoc, 5.02 ± 0.35, $p < 0.001$) (Figure 3.11 F).

To confirm that the increase in PSD-95 puncta density was elicited by IgG specific to the recNR1 protein, the experiment was repeated on hippocampal neurons treated with preabsorbed recNR1 IgG (prepared in Section 2.2.7) and comparisons were made with Naïve, PBS IgG, Luc IgG and non-preabsorbed recNR1 IgG. Preabsorption of recNR1 IgG was carried with Luc (pre-Luc) or recNR1 (pre-recNR1) protein preparations. All IgG treatments

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were applied at 7.0 µg/mL. Once again, differences in PSD-95 expression between treatment groups were not easily distinguished by qualitative comparison of images (Figure 3.12). This time, PSD-95 fluorescence intensity was shown to be significantly higher in recNR1 IgG-treated neurons (1.08 ± 0.02) compared to the naïve (Kruskal-Wallis test, $H = 18.53$, $p = 0.002$; Dunn’s post-hoc, $1.00 ± 0.02, p = 0.048$) and recNR1 pre-recNR1 IgG groups (Dunn’s post-hoc, $1.00 ± 0.02, p = 0.010$), but not other control groups (Figure 3.13 A). There were clear differences in PSD-95 puncta density between treatment groups (Kruskal-Wallis test, $H = 30.88$, $p < 0.0001$). As expected, cells treated with recNR1 IgG had significantly higher PSD-95 puncta density (7.11 ± 0.35) compared to all control groups (Dunn’s post-hoc: Naïve, 5.30 ± 0.27, $p = 0.004$; PBS IgG, 5.34 ± 0.32, $p = 0.015$; Luc IgG, 5.48 ± 0.32, $p = 0.035$) as well as recNR1 pre-recNR1 IgG (Dunn’s post-hoc, 5.30 ± 0.35, $p = 0.006$) (Figure 3.13 B). Furthermore, recNR1 pre-Luc IgG also significantly increased PSD-95 puncta density to a similar extent (7.06 ± 0.35) when comparisons were made with the same treatment groups (Dunn’s post-hoc: Naïve, $p = 0.005$; PBS IgG, $p = 0.017$; Luc IgG, $p = 0.038$; recNR1 pre-recNR1 IgG, $p = 0.006$). Therefore, the increase in PSD-95 puncta density was ablated when neurons were treated with recNR1 IgG preabsorbed against recNR1 protein.

Taken together, the results from these two experiments demonstrated that recNR1 IgG specifically increased the number of PSD-95 puncta in hippocampal neurons in a concentration-dependent manner, but the average PSD-95 expression levels of individual puncta were unchanged. In contrast, no changes in PSD-95 expression were detected following treatment with NR1extra2 IgG at any of the concentrations.
**Figure 3.10 (Part 1)**

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Figure 3.10 (Part 2)

Figure 3.10 PSD-95 immunostaining in primary hippocampal neurons following IgG treatment

Representative dendritic images of hippocampal neurons double-labelled for MAP2 (Alexa Fluor 488, green) and PSD-95 (Alexa Fluor 594, red) following IgG treatment. Individual channels and merged images are shown. One group received no treatment (naïve) as an additional control. Cells were treated with PBS IgG, Luc IgG, recNR1 IgG or NR1extra2 IgG at 1.75 µg/mL, 3.5 µg/mL or 7.0 µg/mL.

Scale bar, 10 µm.
Figure 3.11 Effect of IgG treatment on PSD-95 expression in primary hippocampal neurons

Cells were imaged from three individual cultures (Naïve, n = 27; PBS IgG: 1.75 µg/mL, n = 25; 3.5 µg/mL, n = 25; 7.0 µg/mL, n = 27; Luc IgG: 1.75 µg/mL, n = 27; 3.5 µg/mL, n = 26; 7.0 µg/mL, n = 29; recNR1 IgG: 1.75 µg/mL, n = 28; 3.5 µg/mL, n = 26; 7.0 µg/mL, n = 29; NR1extra2 IgG: 1.75 µg/mL, n = 26; 3.5 µg/mL, n = 25; 7.0 µg/mL, n = 27). Images of hippocampal neurons which received no treatment (naïve) or an IgG treatment at 1.75 µg/mL, 3.5 µg/mL or 7.0 µg/mL were analysed to assess PSD-95 expression. Fluorescence intensity values were normalised to the naïve control group. Quantification of PSD-95 fluorescence intensity showed no significant differences between any of the treatment groups at 1.75 µg/mL (A), 3.5 µg/mL (C) or 7.0 µg/mL (E). No significant changes in PSD-95 puncta density were observed following IgG treatments at 1.75 µg/mL (B) or 3.5 µg/mL (D). At 7.0 µg/mL, recNR1 IgG was found to significantly increase PSD-95 puncta density in hippocampal neurons while the other treatment groups remained comparable (F).

Data represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 3.11

A

![Graph A](image)

IgG treatment (1.75 μg/mL)

B

![Graph B](image)

IgG treatment (1.75 μg/mL)

C

![Graph C](image)

IgG treatment (3.5 μg/mL)

D

![Graph D](image)

IgG treatment (3.5 μg/mL)

E

![Graph E](image)

IgG treatment (7.0 μg/mL)

F

![Graph F](image)

IgG treatment (7.0 μg/mL)
Figure 3.12 PSD-95 immunostaining in primary hippocampal neurons following treatment with preabsorbed recNR1 IgG

Representative dendritic images of hippocampal neurons double-labelled for MAP2 (Alexa Fluor 488, green) and PSD-95 (Alexa Fluor 594, red) following treatment with preabsorbed recNR1 IgG solutions and controls. Individual channels and merged images are shown. One group received no treatment (naïve) as an additional control. Cells were treated with PBS IgG, Luc IgG, recNR1 IgG, recNR1 pre-Luc IgG or recNR1 pre-recNR1 IgG at 7.0 µg/mL.

Scale bar, 10 µm.
Figure 3.13 Comparison of changes in PSD-95 expression following treatment with preabsorbed and non-preabsorbed recNR1 IgG

Cells were imaged from three individual cultures (Naïve, n = 26; PBS IgG, n = 25; Luc IgG, n = 25; recNR1 IgG, n = 27; recNR1 pre-Luc IgG, n = 26; recNR1 pre-recNR1 IgG, n = 25). Images of hippocampal neurons which received no treatment (naïve) or an IgG treatment at 7.0 µg/mL were analysed to assess PSD-95 expression. Fluorescence intensity values were normalised to the naïve control group. PSD-95 fluorescence intensity was significantly increased in recNR1 IgG-treated cells compared to naïve and recNR1 pre-recNR1 IgG-treated cells (A). Both recNR1 IgG and recNR1 pre-Luc IgG treatments resulted in similar significant increases in PSD-95 puncta density in comparison to all other treatment groups (B).

Data represent mean ± SEM. *p < 0.05, **p < 0.01.
3.3.3. recNR1 IgG treatment increased excitatory glutamatergic synapse density in hippocampal neurons

To further characterise the effects of IgG treatment in primary neuronal cultures, double-labelling with presynaptic marker VGLUT1 and postsynaptic protein Homer was performed to examine changes in excitatory glutamatergic synapses. Neurons were labelled for MAP2 to visualise dendrites, VGLUT1 and Homer (Figure 3.14). VGLUT1 and Homer immunostainings were clearly punctate in nature. Qualitatively, the total immunostaining pattern and intensity of both VGLUT1 and Homer appeared similar between all treatment groups.

Fluorescence intensity values from each image were normalised to the naïve group of its respective culture. Analysis showed that total VGLUT1 fluorescence intensity was comparable between all treatment groups at 3.5 µg/mL (one-way ANOVA, $F = 2.407, p = 0.052$) but significantly increased in cells treated with 7.0 µg/mL recNR1 IgG (1.19 ± 0.04) in comparison to the naïve control (Kruskal-Wallis test, $H = 12.53, p = 0.014$; Dunn's post-hoc, 1.00 ± 0.02, $p = 0.005$) (Figure 3.15 A, B). There were no statistical differences in synaptic VGLUT1 fluorescence intensity with any IgG treatment at 3.5 µg/mL (one-way ANOVA, $F = 1.942, p = 0.107$) (Figure 3.15 C) but a significant increase was observed in neurons treated with 7.0 µg/mL recNR1 IgG (1.12 ± 0.04) compared to naïve cells (Kruskal Wallis test, $H = 12.850, p = 0.012$; Dunn's post-hoc, 1.00 ± 0.02, $p = 0.005$) (Figure 3.15 D).

On the other hand, IgG treatments at both concentrations did not result in any change in total (3.5 µg/mL, one-way ANOVA, $F = 0.882, p = 0.476$; 7.0 µg/mL, Kruskal-Wallis test, $H = 5.237, p = 0.264$) or synaptic (3.5 µg/mL, one-way ANOVA, $F = 1.187, p = 0.319$; 7.0 µg/mL, Kruskal-Wallis test, $H = 7.034, p = 0.134$) Homer fluorescence intensities (Figure 3.15 E-H).

The density of total and colocalised VGLUT1 and Homer puncta along dendrites were derived from the images. No significant differences in the number of total VGLUT1 puncta were observed following IgG treatment at 3.5 µg/mL (one-way ANOVA, $F = 1.280, p = 0.281$) or 7.0 µg/mL (Kruskal-Wallis test, $H = 6.562, p = 0.161$) (Figure 3.15 I, J). Total Homer puncta density was significantly higher for recNR1 IgG-treated neurons at 3.5 µg/mL (8.39 ± 0.46) when compared to the naïve group (one-way ANOVA, $F = 5.358, p < 0.001$; Tukey's post-hoc, 6.64 ± 0.32, $p = 0.006$), PBS IgG (Tukey's post-hoc, 6.51 ± 0.34, $p = 0.002$) and NR1extra2 IgG (Tukey's post-hoc, 6.43 ± 0.26, $p = 0.002$) (Figure 3.15 K).
Strangely, no statistical differences were found with 7.0 µg/mL IgG treatments (Kruskal-Wallis, $H = 9.465, p = 0.051$) (Figure 3.15 L). Finally, the excitatory synapse density was determined by quantifying the number of VGLUT1/Homer colocalised puncta. Although not statistically different (Kruskal-Wallis test, $H = 7.342, p = 0.119$), it was notable that the excitatory synapse density was comparatively higher for 3.5 µg/mL recNR1 IgG (4.50 ± 0.33) than other groups at the same concentration (Naïve, 3.63 ± 0.23; PBS IgG, 3.66 ± 0.22; Luc IgG, 3.66 ± 0.29; NR1extra2 IgG, 3.54 ± 0.24) (Figure 3.15 M). On the other hand, recNR1 IgG treatment at 7.0 µg/mL increased excitatory synapse numbers in neurons compared to naïve (one-way ANOVA, $F = 3.880, p = 0.005$; Tukey's post-hoc, 3.63 ± 0.23, $p = 0.007$), Luc IgG (Tukey's post-hoc, 3.70 ± 0.25, $p = 0.015$) and NR1extra2 IgG (Tukey's post-hoc, 3.80 ± 0.21, $p = 0.028$) treatment groups (Figure 3.15 N).

These results indicated that recNR1 IgG treatment induced some concentration-dependent changes in VGLUT1 and Homer expressions in hippocampal neurons which included total and synaptic VGLUT1 expression levels, total Homer puncta density and the number of excitatory synapses. Conversely, VGLUT1 and Homer expression patterns and levels in cells treated with NR1extra2 IgG remained comparable to that of the controls.
Figure 3.14 (Part 1)
Representative dendritic images of hippocampal neurons labelled for MAP2 (Alexa Fluor 488, green), VGLUT1 (Alexa Fluor 594, red) and Homer (Alexa Fluor 647, blue) following IgG treatment. Boxed regions in the left panels are enlarged on the right and shown as individual channels and merged images. VGLUT1/Homer colocalised puncta are purple in colour (arrows). One group received no treatment (naïve) as an additional control. Cells were treated with PBS IgG, Luc IgG, recNR1 IgG, or NR1extra2 IgG at 3.5 µg/mL or 7.0 µg/mL.

Scale bars, 10 µm (left) and 5 µm (right).
Figure 3.15 Effect of IgG treatment on excitatory glutamatergic synapses in primary hippocampal neurons

Cells were imaged from three individual cultures (Naïve, n = 30; PBS IgG: 3.5 µg/mL, n = 29; 7.0 µg/mL, n = 31; Luc IgG: 3.5 µg/mL, n = 31; 7.0 µg/mL, n = 29; recNR1 IgG: 3.5 µg/mL, n = 30; 7.0 µg/mL, n = 30; NR1extra2 IgG: 3.5 µg/mL, n = 30; 7.0 µg/mL, n = 31). Images of hippocampal neurons which received no treatment (naïve) or an IgG treatment at 3.5 µg/mL or 7.0 µg/mL were analysed to assess excitatory synaptic protein expression. Fluorescence intensity values were normalised to the naïve control group. Total VGLUT1 fluorescence intensity was similar between treatment groups at 3.5 µg/mL (A) but significantly increased for recNR1 IgG 7.0 µg/mL when compared to the naïve group (B). There were also no changes in synaptic VGLUT1 fluorescence intensity at 3.5 µg/mL (C) but a significant increase was observed after treatment with 7.0 µg/mL recNR1 IgG in comparison with the naïve group (D). No significant differences in fluorescence intensity were observed for total Homer following IgG treatments at 3.5 µg/mL (E) and 7.0 µg/mL (F), or for synaptic Homer at 3.5 µg/mL (G) and 7.0 µg/mL (H). No significant differences were detected in total VGLUT1 puncta density following IgG treatments at both 3.5 µg/mL (I) and 7.0 µg/mL (J). Total Homer puncta density was found to be significantly increased for recNR1 IgG-treated cells compared to the naïve, PBS IgG and NR1extra2 IgG groups at 3.5 µg/mL (K) but not 7.0 µg/mL (L). The number of VGLUT1/Homer colocalised puncta were analysed for excitatory synapse density and no significant differences were found after IgG treatment at 3.5 µg/mL (M). Treatment with recNR1 IgG at 7.0 µg/mL significantly increased the number of excitatory synapses in comparison to the naïve, Luc IgG and NR1extra2 IgG groups (N).

Data represent mean ± SEM. *p < 0.05, **p < 0.01.
Figure 3.15 (Part 1)

A

B

Total VGLUT1 fluorescence intensity

IgG treatment (3.5 μg/mL)

IgG treatment (7.0 μg/mL)

C

D

Synaptic VGLUT1 fluorescence intensity

IgG treatment (3.5 μg/mL)

IgG treatment (7.0 μg/mL)

E

F

Total Homer fluorescence intensity

IgG treatment (3.5 μg/mL)

IgG treatment (7.0 μg/mL)

G

H

Synaptic Homer fluorescence intensity

IgG treatment (3.5 μg/mL)

IgG treatment (7.0 μg/mL)
Figure 3.15 (Part 2)

I

Total VGLUT1 puncta density (number/10 μm)

IgG treatment (3.5 μg/mL)

J

Total VGLUT1 puncta density (number/10 μm)

IgG treatment (7.0 μg/mL)

K

Total Homer puncta density (number/10 μm)

IgG treatment (3.5 μg/mL)

L

Total Homer puncta density (number/10 μm)

IgG treatment (7.0 μg/mL)

M

Excitatory synapse density (number/10 μm)

IgG treatment (3.5 μg/mL)

N

Excitatory synapse density (number/10 μm)

IgG treatment (7.0 μg/mL)
3.3.4. recNR1 IgG treatment increased synaptic NMDAR puncta density in hippocampal neurons

The results in the previous section have confirmed that anti-GluN1 IgG treatment led to alterations in excitatory synapses. The expression of specific glutamate receptor subtypes in the synapse were examined to expand on this particular finding. To investigate changes in NMDA receptor expression following IgG treatment, double-labelling with presynaptic VGLUT1 and the GluN1 subunit was conducted on primary neuronal cultures. Cultures were fixed using Glyo-Fixx instead of 4% PFA to enhance the quality of GluN1 immunostaining. Neurons were labelled for MAP2 to visualise dendrites, VGLUT1 and GluN1 (Figure 3.16). No obvious differences in total VGLUT1 or GluN1 immunostaining patterns and intensities were discernible by qualitative visual comparison. However, the intensity of VGLUT1 immunostaining in cells fixed with Glyo-Fixx was noticeably stronger than those fixed with 4% PFA in the previous VGLUT1/Homer double-labelling experiment (Section 3.3.3). A shorter exposure time and an additional neutral density filter were therefore used for imaging VGLUT1 immunostaining in this experiment. In addition to punctate GluN1 immunostaining, diffuse dendritic immunostaining of intracellular receptor pools was also observed because the cells had been permeabilised.

Images of neurons were analysed to determine the fluorescence intensities of VGLUT1 and GluN1 puncta, which were normalised to the naïve control from the same culture. Contrary to findings in the VGLUT1/Homer experiment (Section 3.3.3), there were no significant differences in total VGLUT1 fluorescence intensity when IgG treatments were applied to neurons at 3.5 µg/mL (one-way ANOVA, $F = 0.521, p = 0.720$) or 7.0 µg/mL (one-way ANOVA, $F = 1.245, p = 0.294$) (Figure 3.17 A, B). Similarly, no significant differences were found in the fluorescence intensity of VGLUT1 puncta which colocalised with GluN1 puncta following IgG treatment at 3.5 µg/mL (Kruskal-Wallis test, $H = 3.078, p = 0.545$) and 7.0 µg/mL (Kruskal-Wallis test, $H = 3.582, p = 0.466$) (Figure 3.17 C, D). The fluorescence intensity of total GluN1 puncta was comparable between IgG treatment groups at 3.5 µg/mL (one-way ANOVA, $F = 1.500, p = 0.205$) and 7.0 µg/mL (one-way ANOVA, $F = 1.877, p = 0.117$) (Figure 3.17 E, F). There were also no significant differences in synaptic GluN1 fluorescence intensity with IgG treatments at 3.5 µg/mL (Kruskal-Wallis test, $H = 4.815, p = 0.307$) or 7.0 µg/mL (Kruskal-Wallis test, $H = 6.982, p = 0.137$) (Figure 3.17 G, H).
Puncta density was measured for total and colocalised puncta of VGLUT1 and GluN1. Treatment with recNR1 IgG at 3.5 µg/mL significantly increased total VGLUT1 puncta density in neurons (9.27 ± 0.28) when compared to the naïve group (Kruskal-Wallis test, $H = 11.49$; $p = 0.022$; Dunn's post-hoc, 8.24 ± 0.24, $p = 0.046$) (Figure 3.17 I). A significant increase was also observed when recNR1 IgG was applied at 7.0 µg/mL (9.28 ± 0.35) but only in comparison to Luc IgG-treated neurons (one-way ANOVA, $F = 3.019$, $p = 0.020$; Tukey’s post-hoc, 8.00 ± 0.27, $p = 0.012$) (Figure 3.17 J). These results were in contrast to the observations made in the VGLUT1/Homer experiment (Section 3.3.3) where no changes were detected at either recNR1 IgG concentrations. These discrepancies in VGLUT1 expression pattern may have resulted from the use of Glyo-Fixx as the fixative since it had greatly enhanced the VGLUT1 immunostaining intensity in comparison to PFA fixation. On the other hand, total GluN1 puncta density was not statistically different between any of the treatment groups at 3.5 µg/mL (Kruskal-Wallis test, $H = 7.875$, $p = 0.096$) or 7.0 µg/mL (Kruskal-Wallis test, $H = 7.445$, $p = 0.114$) (Figure 3.17 K, L). Finally, the density of synaptic GluN1 puncta was significantly higher in neurons treated with 3.5 µg/mL recNR1 IgG (6.37 ± 0.22) in comparison to all three control groups (one-way ANOVA, $F = 3.733$, $p = 0.006$; Tukey’s post-hoc: Naïve, 5.52 ± 0.24, $p = 0.047$; PBS IgG, 5.35 ± 0.19, $p = 0.009$; Luc IgG, 5.39 ± 0.22, $p = 0.013$) (Figure 3.17 M). Furthermore, a similar observation was made with recNR1 IgG at 7.0 µg/mL where synaptic GluN1 puncta density was significantly increased (6.41 ± 0.28) compared to the naïve (one-way ANOVA, $F = 3.489$, $p = 0.009$; Tukey’s post-hoc, 5.52 ± 0.24, $p = 0.048$), Luc IgG (Tukey’s post-hoc, 5.44 ± 0.21, $p = 0.024$) and NR1extra2 IgG treatment groups (Tukey’s post-hoc, 5.36 ± 0.23, $p = 0.012$) (Figure 3.17 N).

The most notable finding from this experiment was that both concentrations of recNR1 IgG treatment had resulted in an increase in synaptic GluN1 puncta density or the number of NMDAR-containing synapses. Although alterations in VGLUT1 expression following recNR1 IgG treatment varied between the VGLUT1/Homer and VGLUT1/GluN1 double-labelling experiments, only increases but not decreases in VGLUT1 fluorescence intensity or density had been observed thus far. No changes in VGLUT1 or GluN1 expression were detected in NR1extra2 IgG-treated neurons.
Representative dendritic images of hippocampal neurons labelled for MAP2 (Alexa Fluor 647, blue), VGLUT1 (Alexa Fluor 594, red) and GluN1 (Alexa Fluor 488, green) following IgG treatment. Boxed regions in the left panels are enlarged on the right and shown as individual channels and merged images. VGLUT1/GluN1 colocalised puncta are yellow in colour (arrows). One group received no treatment (naïve) as an additional control. Cells were treated with PBS IgG, Luc IgG, recNR1 IgG, or NR1extra2 IgG at 3.5 µg/mL or 7.0 µg/mL.

Scale bars, 10 µm (left) and 5 µm (right).
Figure 3.17 Effect of IgG treatment on synaptic NMDAR expression in primary hippocampal neurons

Cells were imaged from four individual cultures and the same number of hippocampal neurons were imaged for each treatment group (n = 32). Images of hippocampal neurons which received no treatment (naïve) or an IgG treatment at 3.5 µg/mL or 7.0 µg/mL were analysed to assess synaptic NMDA receptor expression. Fluorescence intensity values were normalised to the naïve control group. Quantification revealed no statistical differences in total VGLUT1 fluorescence intensity following IgG treatments at 3.5 µg/mL (A) or 7.0 µg/mL (B). The fluorescence intensity of GluN1-colocalising VGLUT1 puncta in neurons were comparable for all treatment groups at both 3.5 µg/mL (C) and 7.0 µg/mL IgG (D). None of the IgG treatments resulted in changes in total GluN1 fluorescence intensity when applied at 3.5 µg/mL (E) and 7.0 µg/mL (F). There were also no differences in synaptic GluN1 fluorescence intensity at 3.5 µg/mL (G) or 7.0 µg/mL (H). Total VGLUT1 puncta density was found to be significantly higher for recNR1 IgG-treated neurons compared to naïve cells at 3.5 µg/mL (I) and Luc IgG-treated cells at 7.0 µg/mL (J). No differences in total GluN1 puncta density were detected following IgG treatments at 3.5 µg/mL (K) or 7.0 µg/mL (L). Synaptic GluN1 puncta density was significantly increased in neurons treated with recNR1 IgG at 3.5 µg/mL compared to all control groups (naïve, PBS IgG and Luc IgG) (M). Treatment with recNR1 IgG at 7.0 µg/mL also significantly increased synaptic GluN1 puncta in comparison to naïve, Luc IgG and NR1extra2 IgG (N).

Data represent mean ± SEM. *p < 0.05, **p < 0.01.
Figure 3.17 (Part 1)

A

Total VGLUT1 fluorescence intensity

IgG treatment (3.5 µg/mL)

B

Total VGLUT1 fluorescence intensity

IgG treatment (7.0 µg/mL)

C

GluN1-colocalising VGLUT1 fluorescence intensity

IgG treatment (3.5 µg/mL)

D

GluN1-colocalising VGLUT1 fluorescence intensity

IgG treatment (7.0 µg/mL)

E

Total GluN1 fluorescence intensity

IgG treatment (3.5 µg/mL)

F

Total GluN1 fluorescence intensity

IgG treatment (7.0 µg/mL)

G

Synaptic GluN1 fluorescence intensity

IgG treatment (3.5 µg/mL)

H

Synaptic GluN1 fluorescence intensity

IgG treatment (7.0 µg/mL)
Figure 3.17 (Part 2)
3.3.5. No changes in synaptic AMPAR expression were observed following IgG treatment in hippocampal neurons

In addition to NMDA receptors, changes in the expression of AMPA receptors, another glutamate receptor subtype, following IgG treatment were also investigated by double-labelling VGLUT1 and the GluA1 subunit. Glyo-Fixx was the chosen fixative for this experiment to enhance GluA1 immunostaining. Neurons were labelled for MAP2, VGLUT1 and GluA1 (Figure 3.18). Strong VGLUT1 immunostaining was again observed in neurons fixed with Glyo-Fixx like the VGLUT1/GluN1 experiment in Section 3.3.4. Similar to GluN1 immunostaining, GluA1 immunostaining was punctate but also diffusely distributed in the dendrites. Otherwise, there were no notable differences between treatment groups from qualitative assessment of the images alone.

Like previous experiments, puncta fluorescence intensities were normalised to the naïve control. Analysis of puncta fluorescence intensity for VGLUT1 and GluA1 failed to detect any differences between treatment groups at both IgG concentrations tested. Total VGLUT1 fluorescence intensity was similar between all treatment groups at 3.5 µg/mL (one-way ANOVA, \( F = 1.590, p = 0.180 \)) and 7.0 µg/mL (Kruskal-Wallis test, \( H = 1.771, p = 0.778 \)) (Figure 3.19 A, B). Also, no differences were observed in the fluorescence intensity of VGLUT1 puncta colocalised with GluA1 at both concentrations (3.5 µg/mL, one-way ANOVA, \( F = 1.419, p = 0.230 \); 7.0 µg/mL, one-way ANOVA, \( F = 0.628, p = 0.644 \)) (Figure 3.19 C, D). As for GluA1 immunostaining, there were no differences in total or synaptic puncta fluorescence intensities between treatment groups at 3.5 µg/mL (total, Kruskal-Wallis test, \( H = 5.148, p = 0.273 \); synaptic, Kruskal-Wallis test, \( H = 4.631, p = 0.327 \)) or 7.0 µg/mL (total, one-way ANOVA, \( F = 0.691, p = 0.599 \); synaptic, one-way ANOVA, \( F = 0.688, p = 0.602 \)) (Figure 3.19 E-H).

Puncta density of VGLUT1 and GluA1 were also determined for all treatment groups. No statistical differences were found in total VGLUT1 density following IgG treatments at 3.5 µg/mL (one-way ANOVA, \( F = 0.514, p = 0.726 \)) or 7.0 µg/mL (one-way ANOVA, \( F = 0.7229, p = 0.578 \)) (Figure 3.19 I, J). This observation was in contrast to the VGLUT1/GluN1 experiment in Section 3.3.4 where recNR1 IgG treatment at both concentrations resulted in significantly higher total VGLUT1 density in comparison to at least one of the control groups. Total GluA1 puncta density was comparable between neurons of all treatment groups at both concentrations (3.5 µg/mL, Kruskal-Wallis test, \( H = 2.196, p = 0.700 \); 7.0 µg/mL,
Kruskal-Wallis test, $H = 2.501, p = 0.645$) (Figure 3.19 K, L). There were also no significant differences in the synaptic GluA1 puncta density of neurons treated with IgG at 3.5 µg/mL (one-way ANOVA, $F = 0.799, p = 0.528$) or 7.0 µg/mL (Kruskal-Wallis test, $H = 3.841, p = 0.428$) (Figure 3.19 M, N).

In summary, none of the IgG treatments resulted in any change in GluA1-containing AMPA receptor expression in hippocampal neurons at the concentrations used. It should also be noted that increases in VGLUT1 fluorescence intensity and density reported previously in Sections 3.3.3 and 3.3.4, respectively, were not observed in this experiment.
Figure 3.18 (Part 1)
Figure 3.18 VGLUT1 and GluA1 immunostaining in primary hippocampal neurons following IgG treatment

Representative dendritic images of hippocampal neurons labelled for MAP2 (Alexa Fluor 647, blue), VGLUT1 (Alexa Fluor 594, red) and GluA1 (Alexa Fluor 488, green) following IgG treatment. Boxed regions in the left panels are enlarged on the right and shown as individual channels and merged images. VGLUT1/GluA1 colocalised puncta are yellow in colour (arrows). One group received no treatment (naïve) as an additional control. Cells were treated with PBS IgG, Luc IgG, recNR1 IgG, or NR1extra2 IgG at 3.5 µg/mL or 7.0 µg/mL.

Scale bars, 10 µm (left) and 5 µm (right).
Figure 3.19 Effect of IgG treatment on synaptic AMPAR expression in primary hippocampal neurons

Cells were imaged from four individual cultures and the same number of hippocampal neurons were imaged for each treatment group (n = 32). Images of hippocampal neurons which received no treatment (naïve) or an IgG treatment at 3.5 µg/mL or 7.0 µg/mL were analysed to assess synaptic AMPA receptor expression. Fluorescence intensity values were normalised to the naïve control group. Image analysis showed that IgG treatments at 3.5 µg/mL or 7.0 µg/mL did not result in any changes in the parameters measured. No statistical differences were detected in total VGLUT1 (A, B) and GluA1-colocalised VGLUT1 (C, D) puncta fluorescence intensities as well as total GluN1 (E, F) and synaptic GluN1 (G, H) puncta fluorescence intensities between treatment groups. There were also no statistical differences in total VGLUT1 (I, J), total GluA1 (K, L) and synaptic GluA1 (M, N) puncta densities between treatment groups.

Data represent mean ± SEM.
Figure 3.19 (Part 2)

**I**

Total VGluT1 puncta density (number/10 μm)

- Naive
- PBS
- Luc
- rectR1
- NR1extra2

IgG treatment (3.5 μg/mL)

**J**

Total VGluT1 puncta density (number/10 μm)

- Naive
- PBS
- Luc
- rectR1
- NR1extra2

IgG treatment (7.0 μg/mL)

**K**

Total GluA1 puncta density (number/10 μm)

- Naive
- PBS
- Luc
- rectR1
- NR1extra2

IgG treatment (3.5 μg/mL)

**L**

Total GluA1 puncta density (number/10 μm)

- Naive
- PBS
- Luc
- rectR1
- NR1extra2

IgG treatment (7.0 μg/mL)

**M**

Synaptic GluA1 puncta density (number/10 μm)

- Naive
- PBS
- Luc
- rectR1
- NR1extra2

IgG treatment (3.5 μg/mL)

**N**

Synaptic GluA1 puncta density (number/10 μm)

- Naive
- PBS
- Luc
- rectR1
- NR1extra2

IgG treatment (7.0 μg/mL)
3.3.6. The approximate amount of IgG remained stable in culture for at least 7 days following application

Due to the lack of effects observed in NR1extra2 IgG-treated hippocampal neurons, the stability of rat IgG was determined in culture medium in the primary neuronal culture environment. To investigate the stability of IgG in culture medium during the treatment period, ELISA was performed using samples of recNR1 IgG-containing medium with the assumption that the results obtained were also representative of IgG against other antigens. The recNR1 IgG treatment was chosen for this experiment because binding could be easily quantified with one of the synthetic GluN1 peptides used previously in epitope mapping. Peptide 59 was selected for this purpose because of its high reactivity with pooled recNR1 serum, which was reported in Section 2.3.3. Raw absorbance values were similar between samples from wells plated with and without primary hippocampal neurons (not shown). In addition, absorbance values were shown to be relatively stable during the 7-day treatment period when expressed as a percentage of the Day 0 sample collected on DIV 8 (Figure 3.20). Percentage absorbance increased by 6.56% and 8.33% after 7 days on DIV 15 in samples with and without cells, respectively. There were no signs of reduction in IgG level during the treatment period, which eliminated the possibility that IgG-mediated effects may have been obscured by degradation.
Figure 3.20 Stability of recNR1 IgG in primary culture medium

ELISA was performed by probing recNR1 IgG-containing medium samples against GluN1 peptide 59. Absorbance values at 492 nm for medium samples from wells with and without cells were expressed as a percentage of their respective Day 0 values. Percentage absorbance was not drastically different between the two types of samples and remained relatively stable throughout the 7-day treatment period.
3.3.7. Alterations in excitatory glutamatergic synapses were only detectable after at least 7 days of recNR1 IgG treatment

Time course experiments were conducted to determine the onset of recNR1 IgG-induced effects on neurons identified previously in the VGLUT1/Homer and VGLUT1/GluN1 double-labelling experiments (Sections 3.3.3 and 3.3.4). Primary neuronal cultures were treated with Luc IgG (control) or recNR1 IgG at 7.0 µg/mL on DIV 8 as before but were fixed after 2 (DIV 10), 4 (DIV 12) or 7 (DIV 15) days of treatment. It was clear that neurons in culture continued to develop during the treatment period as shown by gradual changes in neuronal morphology and dendritic arborisation of MAP2-positive neurons over time (Figure 3.21). The size of MAP2-positive neurons increased between DIV 10 and DIV 15, along with the length of dendrites and amount of branching.

3.3.7.1. VGLUT1/Homer time course experiment

To examine changes in excitatory synaptic protein expression over time, neurons were once again labelled for MAP2, VGLUT1 and Homer (Figure 3.22). Images of the three time points revealed a progressive increase in the number of VGLUT1 and Homer puncta present on hippocampal neurons regardless of the IgG treatment. However, there were no obvious differences in immunostaining patterns or intensities in cells fixed at the same time point when they were qualitatively assessed.

Image analysis was subsequently carried out to see if these observations were true. Total VGLUT1 fluorescence intensity increased in neurons between 2 and 7 days of treatment for Luc IgG (1.00 ± 0.05 to 1.25 ± 0.05) and recNR1 IgG (0.97 ± 0.04 to 1.39 ± 0.06) (Figure 3.23 A). However, no significant differences were found at any of the time points (unpaired t-tests; 2 days, t = 0.570, p = 0.572; 4 days, t = 0.470, p = 0.641; 7 days, t = 1.862, p = 0.069). Synaptic VGLUT1 fluorescence intensity also increased between 2 and 7 days of treatment for Luc IgG (1.00 ± 0.05 to 1.25 ± 0.05) and recNR1 IgG (0.96 ± 0.04 to 1.39 ± 0.06) but no differences were observed at any time point (unpaired t-tests: 2 days, t = 0.623, p = 0.536; 4 days, t = 0.477, p = 0.636; 7 days: t = 1.819, p = 0.075) (Figure 3.23 B). Total Homer fluorescence intensity became significantly higher for recNR1 IgG at 7 days (1.16 ± 0.03) compared to Luc IgG (unpaired t-test, 1.07 ± 0.03, t = 2.205, p = 0.033) but not at earlier time points (unpaired t-tests: 2 days, t = 1.131, p = 0.264; 4 days, t = 0.531, p = 0.598) (Figure 3.23 C). Similarly, synaptic Homer fluorescence intensity was significantly increased for
recNR1 IgG at 7 days (1.15 ± 0.03) compared to Luc IgG (unpaired t-test, 1.05 ± 0.03, \( t = 2.273, p = 0.028 \)) but not at earlier time points (unpaired t-tests: 2 days, \( t = 0.991, p = 0.327 \); 4 days, \( t = 0.589, p = 0.559 \)) (Figure 3.23 D). These changes in Homer fluorescence intensity at 7 days were not observed in the previous VGLUT1/Homer experiment (Section 3.3.3).

Total VGLUT1 puncta density approximately doubled in numbers between 2- and 7-days post-treatment for both Luc IgG (3.56 ± 0.37 to 7.06 ± 0.33) and recNR1 IgG (3.75 ± 0.39 to 7.60 ± 0.21) but no significant differences were detected at any of the time points (unpaired t-tests: 2 days, \( t = 0.363, p = 0.719 \); 4 days, \( t = 1.546, p = 0.129 \); 7 days, \( t = 1.372, p = 0.177 \)) (Figure 3.23 E). Total Homer puncta density increased to a lesser extent, by approximately 60\%, for Luc IgG (4.01 ± 0.32 to 6.46 ± 0.29) and recNR1 IgG (4.29 ± 0.24 to 6.85 ± 0.28) between 2- and 7-days post-treatment but there were no significant differences (unpaired t-tests: 2 days, \( t = 0.704, p = 0.485 \); 4 days, \( t = 0.428, p = 0.670 \); 7 days, \( t = 0.986, p = 0.329 \)) (Figure 3.23 F). In agreement with the first VGLUT1/Homer double-labelling experiment in Section 3.3.3, treatment with recNR1 IgG at 7.0 µg/mL again increased the number of excitatory synapses in cultured neurons. Excitatory synapse density in recNR1 IgG-treated neurons (5.42 ± 0.22) was significantly higher compared to Luc IgG after 7 days of treatment (unpaired t-test, 4.47 ± 0.20, \( t = 3.151, p = 0.003 \)) (Figure 3.23 G). Excitatory synapse density approximately doubled between 2- and 7-days post-treatment for Luc IgG (2.33 ± 0.26 to 4.47 ± 0.20) and recNR1 IgG (2.47 ± 0.23 to 5.42 ± 0.22) but no statistical differences were found at the two earlier time points (unpaired t-tests: 2 days, \( t = 0.408, p = 0.685 \); 4 days, \( t = 1.339, p = 0.187 \)).
Figure 3.21 MAP2 immunostaining in primary neuronal cultures at different time points following IgG treatment

Representative low power images of hippocampal neurons labelled for MAP2 (green) at 2 days (DIV 10) (A, B), 4 days (DIV 12) (C, D) and 7 days (DIV 15) (E, F) post-IgG treatment. Cells were treated with Luc IgG or recNR1 IgG at 7.0 µg/mL on DIV 8. Neurons fixed at earlier time points were smaller than neurons on DIV 15. A progressive increase in dendritic length and branching was observed in MAP2-positive neurons between DIV 10 and DIV 15.

Scale bar, 200 µm.
Figure 3.22 (Part 1)
Figure 3.22 VGLUT1 and Homer immunostaining in primary hippocampal neurons at different time points following IgG treatment

Representative dendritic images of hippocampal neurons labelled for MAP2 (Alexa Fluor 488, green), VGLUT1 (Alexa Fluor 594, red) and Homer (Alexa Fluor 647, blue) following IgG treatment. Boxed regions in the left panels are enlarged on the right and shown as individual channels and merged images. VGLUT1/Homer colocalised puncta are purple in colour (arrows). Cells were treated with Luc IgG or recNR1 IgG at 7.0 µg/mL on DIV 8 for 2, 4 or 7 days. There was a progressive increase in the number of VGLUT1 and Homer puncta over time.

Scale bars, 10 µm (left) and 5 µm (right).
Figure 3.23 Time course of changes in excitatory glutamatergic synapses in primary hippocampal neurons following IgG treatment

Cells were imaged from three individual cultures and the same number of hippocampal neurons were imaged for each treatment group, at each time point (n = 24). Images of hippocampal neurons which received Luc IgG or recNR1 IgG at 7.0 µg/mL were analysed to assess changes in excitatory synaptic protein expression at 2, 4, and 7 days post-treatment. Fluorescence intensity values for the time course experiment were normalised to Luc IgG after 2 days of treatment. Image analysis showed that most of the measured parameters increased over time during the treatment period but significant differences between Luc IgG and recNR1 IgG-treated were only found in a few parameters. No differences were observed between Luc IgG and recNR1 IgG in total VGLUT1 (A) and synaptic VGLUT1 (B) fluorescence intensities at any of the time points. Total Homer (C) and synaptic Homer (D) fluorescence intensities were both significantly increased in recNR1 IgG-treated neurons at 7 days post-treatment compared to Luc IgG. Total VGLUT1 (E) and total Homer (F) puncta densities increased between 2- and 7-days post-treatment but no significant differences were detected. The number of VGLUT1/Homer colocalised puncta also increased over time and the excitatory synapse density was found to be significantly higher in neurons treated with recNR1 IgG for 7 days compared to Luc IgG (G).

Data represent mean ± SEM. *p < 0.05, **p < 0.01.
3.3.7.2. VGLUT1/GluN1 time course experiment

In the second time course experiment conducted, neurons were labelled for MAP2, VGLUT1 and GluN1 to investigate changes in synaptic NMDA receptor expression over time (Figure 3.24). Once again, images showed a progressive increase in the number of VGLUT1 puncta in hippocampal neurons over time for both IgG treatments. On the other hand, differences in GluN1 immunostaining were difficult to distinguish by eye because it was more diffuse.

Analysis of images showed that total VGLUT1 fluorescence intensity was significantly increased for recNR1 IgG at 4 days (1.16 ± 0.04) when compared to Luc IgG (unpaired t-test, 1.05 ± 0.04, t = 2.056, p = 0.046) (Figure 3.25 A). A significant increase was also observed for recNR1 IgG at 7 days (1.27 ± 0.04) in comparison to Luc IgG (unpaired t-test, 1.12 ± 0.03, t = 2.800, p = 0.007) but not at 2-days post-treatment (unpaired t-test, t = 0.047, p = 0.963). A similar increase in fluorescence intensity was observed for GluN1-colocalising VGLUT1 puncta after 7 days of recNR1 IgG treatment (1.27 ± 0.04) compared to Luc IgG (unpaired t-tests: 2 days, t = 0.135, p = 0.893; 4 days, t = 1.643, p = 0.107) (Figure 3.25 B). Total GluN1 fluorescence intensity increased between 2- and 7-days post-treatment for Luc IgG (1.00 ± 0.03 to 1.22 ± 0.04) and recNR1 IgG (1.01 ± 0.02 to 1.22 ± 0.04) but no significant differences were detected (unpaired t-tests: 2 days, t = 0.020, p = 0.840; 4 days, t = 0.579, p = 0.566; 7 days, t = 0.039, p = 0.969) (Figure 3.25 C). Similarly, fluorescence intensity of synaptic GluN1 increased between 2- and 7-days post-treatment (Luc IgG, 1.00 ± 0.03 to 1.24 ± 0.05; recNR1 IgG, 1.03 ± 0.03 to 1.25 ± 0.04) but there were also no significant differences at any time point (unpaired t-tests: 2 days, t = 0.645, p = 0.522; 4 days, t = 0.397, p = 0.693; 7 days, t = 0.186, p = 0.854) (Figure 3.25 D). The number of VGLUT1 puncta more than doubled between 2- and 7-days post-treatment for Luc IgG (3.31 ± 0.35 to 7.70 ± 0.27) and recNR1 IgG (3.39 ± 0.26 to 8.96 ± 0.38). A significant increase was observed following 7 days of recNR1 IgG treatment (8.96 ± 0.38) compared to Luc IgG (unpaired t-test, 7.70 ± 0.27, t = 2.696, p = 0.010) but not at earlier time points (unpaired t-tests: 2 days, t = 0.177, p = 0.860; 4 days, t = 0.264, p = 0.793) (Figure 3.25 E). This finding concurred with the results described in Section 3.3.4. Total GluN1 puncta density increased slightly between 2 and 7 days of treatment for both treatment groups (Luc IgG, 7.81 ± 0.38 to 8.30 ± 0.47; recNR1 IgG, 7.76 ± 0.31 to 8.97 ± 0.44) but no statistical differences were detected (unpaired t-tests: 2 days, t = 0.099, p = 0.922; 4 days, t = 0.623, p = 0.537; 7 days, t = 1.048, p = 0.300) (Figure 3.25 F). The number of synapses containing NMDA receptors approximately doubled
in neurons between 2- and 7-days post-treatment (Luc IgG, 2.14 ± 0.20 to 4.00 ± 0.18; recNR1 IgG, 2.20 ± 0.15 to 4.78 ± 0.22) (Figure 3.25 G). Consistent with the previous VGLUT1/GluN1 experiment in Section 3.3.4, synaptic GluN1 puncta density was significantly higher following 7 days of recNR1 IgG treatment (4.78 ± 0.22) in comparison to Luc IgG (unpaired t-test, 4.00 ± 0.18, t = 2.742, p = 0.009). No significant differences in synaptic GluN1 puncta were detected at the two earlier time points (unpaired t-tests: 2 days, t = 0.247, p = 0.806; 4 days, t = 0.964, p = 0.340).

In summary, we were able to replicate the main findings reported in Sections 3.3.3 and 3.3.4, where recNR1 IgG increased excitatory synapse numbers and synaptic NMDA receptor density after 7 days of treatment. No differences were observed between recNR1 IgG and Luc IgG at 2- and 4-days post-treatment for most parameters measured, save for total VGLUT1 fluorescence intensity after 4 days in the VGLUT1/GluN1 time course experiment. Discrepancies in some of the observations made between the first double-labeling experiments and these time course experiments may be accounted by slight adjustments in image analysis settings to accommodate detection of immunostaining at earlier time points where fluorescence intensities were weaker.
Figure 3.24 (Part 1)
Figure 3.24 VGLUT1 and GluN1 immunostaining in primary hippocampal neurons at different time points following IgG treatment

Representative dendritic images of hippocampal neurons labelled for MAP2 (Alexa Fluor 647, blue), VGLUT1 (Alexa Fluor 594, red) and GluN1 (Alexa Fluor 488, green) following IgG treatment. Boxed regions in the left panels are enlarged on the right and shown as individual channels and merged images. VGLUT1/GluN1 colocalised puncta are yellow in colour (arrows). Cells were treated with Luc IgG or recNR1 IgG at 7.0 µg/mL on DIV 8 for 2, 4 or 7 days. There was a progressive increase in the number of VGLUT1 puncta over time but changes in GluN1 puncta were less distinguishable.

Scale bars, 10 µm (left) and 5 µm (right).
Figure 3.25 Time course of changes in synaptic NMDAR expression in primary hippocampal neurons following IgG treatment

Cells were imaged from three individual cultures and the same number of hippocampal neurons were imaged for each treatment group, at each time point (n = 24). Images of hippocampal neurons which received Luc IgG or recNR1 IgG at 7.0 µg/mL were analysed to assess changes in synaptic NMDA receptor expression at 2, 4, and 7 days post-treatment. Fluorescence intensity values for the time course experiment were normalised to Luc IgG after 2 days of treatment. Image analysis of VGLUT1 and GluN1 immunostaining at different time points revealed most parameters increased over time during the 7-day treatment period. Total VGLUT1 fluorescence intensity was significantly higher in neurons at 4 and 7 days after recNR1 IgG treatment compared to Luc IgG (A). The fluorescence intensity of GluN1-colocalising VGLUT1 puncta in recNR1 IgG-treated neurons was only significantly higher than Luc IgG after 7 days (B). No statistical differences were observed for total GluN1 (C) or synaptic GluN1 (D) fluorescence intensities for recNR1 IgG at any of the time points. There was an obvious increase in total VGLUT1 puncta density over time for both treatment groups and was significantly higher for recNR1 IgG at 7 days (E). Total GluN1 puncta density remained relatively unchanged over the one week period for both IgG treatments and no differences were detected (F). The number of synaptic GluN1 puncta increased over time in neurons and was significantly higher in the recNR1 IgG treatment group compared to Luc IgG after 7 days (G).

Data represent mean ± SEM. *p < 0.05, **p < 0.01.
Figure 3.25

A

B

C

D

E

F

G

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3.4. Discussion

In this chapter, primary rat hippocampal neuronal cultures were treated with IgG prepared in Chapter 2 in order to determine the underlying cellular mechanisms by which anti-GluN1 IgG provides neuroprotection and exerts memory-improving effects in animals (Chen, 2010; Chen, 2015; During et al., 2000). The inclusion of two different types of anti-GluN1 IgG, recNR1 IgG and NR1extra2 IgG facilitated the investigation of potential epitope-dependent effects as a side-aim. Immunostaining of dissociated hippocampal neurons allowed us to assess possible modifications in excitatory synapses and glutamate receptor expression in vitro. We found no signs of toxicity or aberrant alterations in neuronal morphology following IgG treatment at physiological concentrations. Treatment with recNR1 IgG produced a concentration-dependent increase in PSD-95 puncta density, which was abolished by preabsorption of the IgG with recNR1 protein preparation, suggesting the change in density was not mediated by non-specific antibodies. In addition, recNR1 IgG also increased the number of excitatory synapses and synaptic NMDA receptor expression in a concentration-dependent manner. Subsequent time course experiments demonstrated that significant differences in expressions only became apparent after 7 days of IgG treatment. We observed no alterations in the expression of GluA1-containing AMPA receptor in primary hippocampal neuron with any of the treatment groups in this chapter.

3.4.1. IgG treatments did not exhibit neurotoxic properties in primary culture

The first functional excitatory synapses are identified in cultured hippocampal neurons at DIV 7 (Grabrucker et al., 2009; Lin et al., 2002) so we commenced IgG treatment soon after on DIV 8. Under basal conditions, the concentration of circulating IgG in serum is around 5 mg/mL in rats (Salauze et al., 1994) and approximately 0.1 to 0.2% transfers into the CNS (Bard et al., 2000; Thompson, 2005). Therefore, the range of concentrations we tested were within physiological levels. Immunostaining against the neuronal marker MAP2 provides useful measures of neuronal viability due to its critical roles in neuronal growth and development as well as degeneration as a cytoskeletal protein (Johnson & Jope, 1992). Autoantibodies against glutamate receptors in the CNS including NMDA and AMPA receptors have been associated with synaptic dysfunction and neurodegeneration in various autoimmune disorders (DeGiorgio et al., 2001; Lai et al., 2009; Manto et al., 2010; Rogers et al., 1994). Neurotoxicity has also resulted from blockade of NMDA receptors by antagonists both in vitro and in vivo (Horváth et al., 1997; Hwang et al., 1999; Olney et al., 1989; Olney...
et al., 1991). Moreover, NMDAR-mediated synaptic potentiation has been shown to alter dendritic morphology by modulating the phosphorylation of MAP2 (Sánchez et al., 1997) and receptor blockade results in complex arborisation (Lüthi et al., 2001). Therefore, it was imperative that we determine the potential toxicity of our IgG treatments before proceeding with immunocytochemical characterisation in hippocampal neurons because of possible confounding effects. Neurons in our primary cultures were easily identified by labelling with MAP2 and, as expected, MAP2 expression was concentrated in their soma and dendrites (De Camilli et al., 1984; Kosik & Finch, 1987). The number of MAP2-positive neurons and MAP2-immunopositive area were similar between the naïve group and IgG treatments at all concentrations used, showing the absence of neurotoxicity. This finding is in agreement with a previous study from our laboratory where no indications of neurodegeneration were reported in rats immunised with recombinant Luc and recNR1 proteins (Chen, 2015). This could either suggest our anti-GluN1 IgG do not possess full antagonistic activity like conventional NMDA receptor antagonists, or do not elicit neurotoxicity at the concentrations tested. Some NMDA receptor antagonists like memantine have been shown to not increase constitutive neuronal apoptosis at neuroprotective doses in immature rats (Manning et al., 2011).

Past studies have also shown no signs of neuroinflammation, specifically reactive astrogliosis and microglial activation, in rats following protein immunisation (Chen, 2015; Dicker, 2010). Through DAPI staining, we demonstrated that our primary cultures contained not only neurons but also other non-neuronal cells, which resulted in a neuronal purity of 15 to 20%. There were no differences in the total number of DAPI-stained cells between treatment groups, suggesting lack of general cytotoxicity. Although most non-neuronal cells in culture were likely astrocytes, further investigation is warranted to confirm their phenotypes. High purity neuronal cultures minimises interference from other cell types in assays. However, mixed cultures are probably more suited for use as a model for this study because they provide a closer, albeit not perfect, representation of in vivo conditions, especially given that glial cells form tripartite synapses and establish dynamic interactions with neurons (Halassa et al., 2007; Haydon, 2001). Astrocytes make direct contact with approximately half of the synapses in the mature rat hippocampus (Ventura & Harris, 1999) and neurons co-cultured with astrocytes produce more functional synapses (Ullian et al., 2001), suggesting a contribution to synaptic development. They have also been shown to actively regulate glutamate levels in the synaptic cleft by glutamate release and uptake (Montana et al., 2004;
Rothstein et al., 1994). In addition, there is growing evidence that glial cells such as astrocytes and microglia express glutamate receptors including the NMDA receptor, which are involved in neuronal-glial transmission (Gallo & Ghiani, 2000; Lalo et al., 2006; Murugan et al., 2013; Parpura et al., 1994). Therefore, it is possible that anti-GluN1 IgG could modulate glia-neuron transmission and future studies should aim to address this potential interaction.

3.4.2. recNR1 IgG increases the number of potential PSD-95 containing postsynaptic sites

We first chose to investigate changes in PSD-95 expression following treatment with anti-GluN1 IgG in cultured hippocampal neurons due to its close association with NMDA receptors. PSD-95 is concentrated at glutamatergic synapses and plays a critical role in the molecular organisation of NMDA receptor at postsynaptic sites as it binds to the C-terminal domain of GluN2 subunits and some GluN1 splice forms through its second PDZ domain (Kornau et al., 1995). It increases synaptic NMDA receptor clustering and channel opening rates (Kim et al., 2005; Lin et al., 2004) but also prevents GluN2B-mediated receptor internalisation (Roche et al., 2001). In addition, PSD-95 regulates surface AMPA receptor expression in cultured hippocampal neurons (Bats et al., 2007). It also has roles in excitatory synapse maturation in vitro (El-Husseini et al., 2002). In this study, we reported a concentration-dependent increase in PSD-95 puncta density in hippocampal neurons following recNR1 IgG treatment. PSD-95 puncta density increased by approximately 30% in neurons treated with 7.0 µg/mL recNR1 IgG in comparison to control groups. No changes were detected with the NR1extra2 IgG group at any of the concentration. It is unclear whether this is an epitope-dependent effect since the proportion of specific IgG to total IgG in the NR1extra2 IgG sample was lower than that of recNR1 IgG. There was a possibility that the alteration in PSD-95 expression was caused by non-specific IgG present in our crudely purified recNR1 IgG solution. This was addressed by repeating the experiment using preabsorbed IgG. Specificity of the recNR1 IgG-mediated effect was confirmed when PSD-95 puncta density increased to a similar extent following treatment with recNR1 pre-Luc IgG, which retained recNR1 protein binding capacity, but was unchanged with recNR1 pre-recNR1 IgG, which has minimal recNR1 protein binding capacity (see Chapter 2).

Overall, our results suggest that there may potentially be more postsynaptic sites available for functional synapse formation in recNR1 IgG-treated neurons. This finding is in contrast to a
previous study from our laboratory where no changes in PSD-95 expression level were detected in Western blots performed on total hippocampal lysates from recNR1-immunised rats or mice passively transferred with recNR1 IgG, both with superior learning and memory functions (Chen, 2015). Although Western blot analysis is useful for characterising protein expression, the semi-quantitative method may not have the sensitivity to detect small changes in expression levels (Kramer et al., 2012; Wang et al., 2006). However, in the case of anti-NMDA receptor encephalitis, there were also no changes in PSD-95 expression or localisation in cultured hippocampal neurons treated with patient IgG, despite a severe reduction in NMDA receptor expression and function (Dalmau et al., 2008; Hughes et al., 2010). This provides support that recNR1 IgG behaves differently to the anti-GluN1 antibodies found in anti-NMDA receptor encephalitis. Furthermore, blockade of NMDA receptor activity by antagonists such as APV and memantine has not been linked to any change in PSD-95 expression both in vitro and in vivo (Manning et al., 2011; Rao & Craig, 1997), suggesting that recNR1 IgG may not exhibit full antagonistic activity. However, it is uncertain whether or not recNR1 IgG possesses agonist-like properties. Short-term activation of NMDA receptors has been shown to reduce the fluorescence intensity of PSD-95 puncta (Colledge et al., 2003) but this is in contrast to the findings in the present study.

3.4.3. recNR1 IgG increases the number of excitatory glutamatergic synapses

Because of the increase in PSD-95 density, we next decided to provide more definitive evidence that recNR1 IgG treatment can lead to changes in excitatory synapses in hippocampal neurons. We used VGLUT1 as the presynaptic marker for glutamatergic synapses (Fujiyama et al., 2001; Takamori et al., 2000) and Homer, another protein of the PSD which also colocalises with NMDA receptors (Shiraishi et al., 2003), to determine if changes in expression similar to PSD-95 could be detected. In this study, we reported a concentration-dependent increase in the number of excitatory synapses in recNR1 IgG-treated hippocampal neurons compared to controls, which only became significant after 7 days of treatment. No changes were observed with the NR1extra2 IgG treatment group. We also observed an increase in total Homer puncta density following recNR1 IgG treatment but only at the lower concentration of 3.5 µg/mL and not at 7.0 µg/mL. Furthermore, recNR1 IgG increased total and synaptic Homer fluorescence intensities in comparison to Luc IgG in the time course experiment after 7 days of treatment. Although they are both proteins of the PSD, the dissimilarities in PSD-95 and Homer puncta density changes following recNR1 IgG
treatment suggest a protein-specific effect. PSD-95 is one of the first proteins to be targeted to developing postsynaptic sites along with NMDA receptors (Rao et al., 1998), which might affect its susceptibility to changes caused by NMDA receptor activity. Changes in VGLUT1 expression differed between all double-labelling experiments conducted, including those with GluN1 and GluA1, and both time course experiments. However, a common observation was that recNR1 IgG treatment only resulted in increases in VGLUT1 puncta fluorescence intensity or density, which might suggest a very slight, but overall upregulation in VGLUT1 expression. Some studies have shown no changes in the expression of presynaptic markers such as synaptophysin (Rao & Craig, 1997) and synapsin (Kato et al., 2007) by immunocytochemistry following APV-mediated NMDA receptor blockade in cultured hippocampal neurons. Interestingly, electrophysiological studies have produced mixed results with regards to presynaptic changes. Changes in the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) or mEPSCs can be attributed to alterations in presynaptic release probability of glutamate, number of synaptic release sites or synaptic density (Turrigiano & Nelson, 2004). Activation of NMDA receptors by spontaneous synaptic activity as a result of APV withdrawal in cultured neurons has been shown to increase the frequency of mEPSCs (Liao et al., 2001). In contrast, blockade of NMDA receptors with APV in cultured neurons did not lead to changes in mEPSC frequency (Kato et al., 2007). However, NMDA receptor blockade by CPP in vivo (Luthi et al 2001) and memantine in hippocampal slice cultures (He et al 2014) increases mEPSC and sEPSC frequencies, respectively. Nevertheless, these studies illustrate changes in presynaptic function as a potential consequence of NMDA receptor modulation.

In complete contrast to our findings, Hughes et al. (2010) detected no change in synapse numbers in hippocampal neurons treated with anti-NMDA receptor encephalitis patient IgG. This provides further support for the differential effects between the two types of anti-GluN1 IgG. This may be attributed to the differences in the target epitope since N368/G369 in the GluN1 N-terminus are crucial residues for determining immunoreactivity in anti-NMDA receptor encephalitis (Gleichman et al., 2012). The N368/G369 residues correspond to peptide #37 used in our epitope mapping procedure, which recNR1 IgG exhibit some, but minimal reactivity to in comparison to other positive peptides (see Chapter 2). Most studies have directly inferred excitatory synapse expression from synaptic glutamate receptor expressions, which makes comparisons difficult. However, the present findings may be in agreement with some studies which utilised NMDA receptor antagonists. A few
electrophysiological studies in hippocampal slice cultures have suggested that blocking NMDA receptor activity could lead to increased glutamatergic transmission or the number of functional excitatory synapses (He & Bausch, 2014; Lüthi et al., 2001). NMDA receptor blockade has been shown to increase synaptic clustering of Homer, albeit with concurrent blockade of cAMP phosphodiesterase activity (Shiraishi et al., 2003).

3.4.4. recNR1 increases the number of synaptic NMDAR but not AMPAR puncta.

Finally, we followed up our observation of the change in excitatory synapse density by examining glutamate receptor expression in hippocampal neurons after IgG treatment. VGLUT1 was again used as the presynaptic marker while NMDA receptors were detected by staining for GluN1, its obligatory subunit (Monyer et al., 1992; Traynelis et al., 2010), and AMPA receptors by immunostaining for the GluA1 subunit, which accounts for approximately 80% of synaptic and 95% of somatic extrasynaptic AMPA receptors in the hippocampus (Lu et al., 2009; Reimers et al., 2011). We reported an increase in synaptic NMDA receptor puncta density following recNR1 IgG treatment at both 3.5 µg/mL and 7.0 µg/mL in comparison to control groups, with no changes in GluN1 puncta fluorescence intensities. The difference in synaptic NMDA receptor puncta density was only significant in comparison to Luc IgG after 7 days of treatment. On the other hand, no alterations in synaptic GluA1-containing AMPA receptor expression were found in recNR1 IgG-treated hippocampal neurons. Once again, NR1extra2 IgG treatment did not result in any changes. Altogether, our results show that hippocampal neurons treated with recNR1 IgG presented a higher number of NMDAR-containing synapses but no change in synapses with GluA1-containing AMPA receptors. Because the functional properties of AMPA receptors are dependent on their subunit composition (Boulter et al., 1990; Meng et al., 2003; Swanson et al., 1997), it may be of interest to investigate changes in the expression of GluA2 and GluA3 subunits in future studies.

Contrary to the findings in the present study, our laboratory did not observe any changes in the expression of NMDA receptor subunits GluN1, GluN2A and GluN2B, or AMPA receptor subunit GluA1 in the hippocampus of recNR1-immunised rats and mice passively transferred with recNR1 IgG when immunohistochemistry and Western blot analysis were conducted in a previous study (Chen, 2015). This could be explained by the inability for immunohistochemical densitometry and Western blot with total tissue lysate to make clear distinction between total and synaptic expressions. This is supported by the absence of
changes in total GluN1 puncta fluorescence intensity or density in this study. Our findings are also in stark contrast to results reported by Dalmau et al. (2008) and Hughes et al. (2010) in neurons treated with anti-NMDA receptor encephalitis patient CSF and IgG where they observed a profound reduction in surface NMDA receptor density and synaptic localisation caused by antibody-mediated receptor internalisation. However, Hughes et al. also did not observe any changes in the expression and localisation of GluA1, as well as GluA2, following treatment. Findings from other studies provide some support for antagonistic properties of recNR1 IgG. Activity blockade by NMDA receptor antagonist APV in cultured hippocampal neurons has been shown to increase synaptic clusters of GluN1 but not GluA1 (Rao & Craig, 1997). Upregulation of NMDA receptor expression in hippocampal neurons following blockade has also been hinted by increases in GluN1 mRNA levels with MK-801 treatment in vitro (Lasoñ et al., 1997; Sinner et al., 2015) and increases in [3H]MK-801 binding after PCP treatment in vivo (du Bois et al., 2009; Gao & Tamminga, 1996). However, there are also contradicting data where one study showed increase in [3H]MK-801 receptor binding only occurs with short-term blockade while long-term PCP treatment reduces binding (Newell et al., 2007).

The finding that recNR1 IgG-treated neurons have proportionally more NMDAR-containing than AMPAR-containing synapses signifies a possibility that there could be an increase in morphological silent synapses. Postsynaptic ‘silent’ synapses contain very little or no AMPA receptors but express NMDA receptors, which are functionally silent at normal resting membrane potentials due to the voltage-dependent block by magnesium ions (Isaac et al., 1995; Liao et al., 1995; Montgomery et al., 2001). Synaptic activation of NMDA receptors by APV withdrawal (Liao et al., 2001) or glycine (Lu et al., 2001) chemically induces LTP in cultured hippocampal neurons, which is accompanied by rapid delivery and clustering of native AMPA receptors at the synaptic membrane. Insertion of AMPA receptors following NMDA receptor stimulation is driven by an increase in intracellular calcium and activation of CAMKII (Barria et al., 1997; Hayashi et al., 2000; Shi et al., 1999). Incorporation of AMPA receptor at previously silent synapses could be one of the underlying mechanisms of LTP plasticity (Isaac et al., 1995; Liao et al., 1995), which is a putative cellular basis of learning and memory (Alkon & Nelson, 1990; Berger, 1984; Bliss & Collingridge, 1993; Skelton et al., 1987). Therefore, it is possible that silent synapses played a role in the determining the behavioural phenotypes of recNR1 IgG-treated animals previously observed in our laboratory (Chen, 2010; Chen, 2015). A double-labelling experiment for AMPA and NMDA receptors
could provide further confirmation of the presence of silent synapses (Liao et al., 1999). Also, electrophysiological studies are warranted to determine the functionality of these glutamate receptors in recNR1 IgG-treated neurons.

3.4.5. Limitations and future studies

Although IgG treatments were concentration-matched, the proportion of specific IgG to total IgG in each preparation is unknown due to the crude purification method used. We were able to demonstrate the stability of rat IgG in culture medium during the 7-day treatment period. Therefore, it was likely that the low expression of specific NR1extra2 IgG (see Chapter 2) made it difficult to ascertain whether it had the capacity to mediate changes in primary hippocampal neurons. Testing should be performed with a high-titre IgG preparation before proper conclusions can be drawn. Studies which investigated the effects of anti-NMDA receptor encephalitis patient CSF and IgG on cultured neurons applied treatments at much higher concentrations and employed very different treatment schedules. For example, Dalmau et al. (2008) applied patient CSF to neurons for 7 days by replacing 20 µL of the 300 µL total medium volume with fresh 20 µL CSF daily. On the other hand, Hughes et al. (2010) treated neurons with 1 µg to 1 mg/mL of IgG for 1, 3 or 7 days in their experiments. Differences in treatment application may have, in part, contributed to the discrepancies in our observations. Therefore, it may also be of interest for future studies to investigate the effects of IgG treatment at concentrations beyond physiological levels to see if responses can be augmented or if they could change entirely. However, there is a risk of protein aggregation which could lead to nonspecific binding (Pease et al., 2008; Sukumar et al., 2004). Also, IgG treatments could potentially be extended for a longer period of time. Lastly, comparisons should be made between recNR1 IgG and a known partial agonist of the NMDA receptor, such as D-cycloserine (Bado et al., 2011; Gabriele & Packard, 2007), to see if they confer similar pharmacological effects.
Chapter 4.
Electrophysiological and further immunocytochemical characterisation of anti-GluN1 IgG-treated hippocampal neurons
4.1. Introduction

The primary aim of this chapter was to determine if alterations in glutamate receptor expression in the excitatory synapse following recNR1 IgG treatment observed in Chapter 3 translated to functional changes in synaptic transmission. This was achieved through electrophysiological characterisation of IgG-treated primary hippocampal neurons by whole-cell patch-clamp recordings. NR1extra2 IgG was excluded from further investigation due to lack of effect on glutamate receptor expression. To continue our exploration of possible epitope-dependent effects mediated by anti-GluN1 IgG, a third antibody which targeted the allosteric region of the GluN1 subunit N-terminus that we named NR1.NTD IgG was included in the experiments described in this chapter. In addition to electrophysiological recordings, immunocytochemical characterisation of protein expression in the excitatory glutamatergic synapse was also carried out on NR1.NTD IgG-treated neurons.

The recombinant NR1.NTD protein used for immunisation procedures to generate NR1.NTD IgG contained the N368/G369 amino acid residues essential for immunoreactivity in anti-NMDA receptor encephalitis (Gleichman et al., 2012). NR1.NTD IgG was generated to contrast the first two anti-GluN1 antibodies, recNR1 IgG and NR1extra2 IgG, by targeting an extracellular N-terminal segment outside of the glycine-binding site of the NMDA receptor.

We employed electrophysiological techniques to investigate changes in synaptic transmission following IgG treatment. Basal excitatory synaptic activity was first examined by single whole-cell patch clamping and recording mEPSCs in hippocampal neurons. Paired cell recordings were also performed to assess synaptic AMPA and NMDA receptor functions. This technique involves simultaneous recordings from a pair of synaptically connected neurons and can be performed in both slice and dissociated cultures (Arons et al., 2012; Montgomery & Madison, 2002; Montgomery et al., 2001; Montgomery et al., 2005). The stimulation of a single presynaptic neuron permits the analysis of monosynaptic connections between cells.

We hypothesised that changes in synaptic protein expression as a result of anti-GluN1 IgG treatment will be reflected in modifications of excitatory synaptic transmission mediated by AMPA and NMDA receptors in primary rat hippocampal neurons. In addition, the N-terminus-targeting NR1.NTD IgG may induce different effects compared to recNR1 IgG.
4.2. Materials and methods

4.2.1. Recombinant NR1.NTD protein production

Methods for recombinant NR1.NTD protein production were the same as those described in Section 2.2.1. NR1.NTD protein was produced by Ms Rebecca N. Marnane.

4.2.1.1. Plasmid

Plasmid construct expressing an allosteric GluN1 N-terminal domain segment, pTriEx-NR1.NTD (Figure 4.1), was cloned and verified by Dr Alexander Mouravlev. The construct contains an ampicillin resistance gene for bacterial selection and has a HIS-tag fused to the cDNA of the 42 kDa recombinant mouse NR1.NTD protein. The NR1.NTD protein is a truncated form of the GluN1 N-terminal domain and borders the proposed ligand binding site (Figure 4.2). A plasmid map was generated with DNA sequence data using Vector NTI software (InforMax).

Figure 4.1 pTriEx-NR1.NTD plasmid construct

Plasmid map of pTriEx-NR1.NTD depicting the location of various restriction enzyme cleavage sites, as well as the size and position of the cDNA sequence for the NR1.NTD protein. The position of the HIS-tag is in the C-terminus of the cDNA sequence.
Figure 4.2 Schematic diagram of the GluN1 subunit and the boundaries of recombinant GluN1 (recNR1 and NR1.NTD) proteins

The diagram depicts the amino acid boundaries of recombinant NR1.NTD protein in comparison to recNR1 protein in the extracellular domain. The recNR1 protein spans both GluN1 extracellular domains, encompassing amino acids 1-562 and 654-812, while the NR1.NTD protein comprises of amino acids 21-372 in the N-terminal domain and borders the proposed ligand binding site.

4.2.2. Analysis of recombinant NR1.NTD protein preparation

4.2.2.1. SDS-PAGE and Coomassie blue staining

The purified NR1.NTD protein sample was analysed by SDS-PAGE and Coomassie blue staining, which were performed as described in Sections 2.2.2.1 and 2.2.2.2, respectively. NR1.NTD protein was loaded at 1 µg per lane.

4.2.2.2. Western blotting

Purity of the NR1.NTD protein preparation was assessed by Western blot as described in Section 2.2.2.4, respectively. Mouse primary antibodies against the GluN1 N-terminus (Neuromab, 75-272) (1:5,000) and GluN1 M3-M4 (BD Pharmingen, 556308) (1:10,000)
were used. Goat HRP-conjugated anti-mouse IgG (Santa Cruz, sc-2005) (1:5,000) was used as the secondary antibody.

4.2.3. Recombinant NR1.NTD protein immunisation in rats

All procedures regarding NR1.NTD protein immunisation in rats including injections, monitoring IgG production and blood collection were performed by Ms Rebecca N. Marnane according to Section 2.2.3.

4.2.4. Assessment of NR1.NTD IgG cross-reactivity.

Pooled serum sample from the best responding NR1.NTD-immunised rats at the end of the immunisation period was tested for cross-reactivity to Luc, recNR1 and NR1extra2 protein preparations. SDS-PAGE and Western blots were performed according to Sections 2.2.2.1 and 2.2.2.4. NR1.NTD pooled serum sample was used as the primary antibody (1:10,000) and Goat HRP-conjugated anti-rat IgG antibody (Santa Cruz, sc-2006) was used as the secondary antibody (1:5,000).

4.2.5. Purification of NR1.NTD IgG from rat serum

Pooled NR1.NTD serum sample from the best responding rats was used for IgG purification after it was confirmed to react to N-terminal GluN1 peptides #5, 10, 20, 33, 34 and 71 through epitope mapping conducted by Ms Rebecca N. Marnane. IgG purification was performed according to Section 2.2.5 by Ms Rebecca N. Marnane.

4.2.6. Quality analysis of purified NR1.NTD IgG

4.2.6.1. Assessment of NR1.NTD IgG purity

Purity of NR1.NTD IgG solution was assessed by Coomassie staining and Western blot according to Section 2.2.6.1.
4.2.6.2. Examination of NR1.NTD IgG reactivity against NR1.NTD protein

Purified NR1.NTD IgG solution was screened for reactivity against recombinant NR1.NTD protein preparation and comparisons were made with commercial antibodies by Western blot according to Section 2.2.6.2.

4.2.7. Electrophysiology

4.2.7.1. Primary culture of rat hippocampal neurons

Primary rat hippocampal neuronal cultures were prepared and maintained as described in Section 3.2.1 with some adjustments. Circular 13 mm glass coverslips (Menzel-Glaser) were used for electrophysiology experiments. Following sterilisation by flaming, 4 round coverslips were placed in each well of a 6-well tissue culture plates (BD Falcon, 353046). Each well was then coated by incubating with 1 mL of 10 µg/mL PDL (Sigma, p1149) diluted in PBS at 37 °C overnight. Cells were plated at an approximate density of two hippocampi per 6-well plate with 2 mL of culture medium per well.

4.2.7.2. Treatment of neuronal cultures with rat IgG

Luc, recNR1 and NR1.NTD IgG treatments were applied to primary neuronal cultures at 7.0 µg/mL on DIV 8 as described in Section 3.2.2. Cells were treated for varying lengths of time and electrophysiological recordings were performed from DIV 13 to DIV 16 to maximise use of cultures.

4.2.7.3. Recording setup

All recordings were conducted at RT in artificial cerebrospinal fluid (ACSF) (119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO_4·7H_2O, 2.5 mM CaCl_2, 1 mM NaH_2PO_4, 26 mM NaHCO_3, 11 mM Glucose) bubbled with carbogen (95% O_2/5% CO_2) and perfused at a rate of 1 mL/min using a gravity-fed bath perfusion system. Micropipette electrodes between 6-9 MΩ were pulled from filamented standard wall borosilicate tubing (Sutter Instrument Company, F150-86-7.5) using a horizontal micropipette puller (Sutter, P-97).
4.2.7.3.1. mEPSC recordings

To record spontaneous mEPSC recordings, dissociated hippocampal neurons grown on coverslips were transferred to a recording chamber (Warner Instrument, RC-26GLP) on a Zeiss Axioskop 2 FS Plus microscope mounted on an air table for vibration isolation. The setup was enclosed in a Faraday cage (TMC, 81-333-06) and all equipment was electrically grounded to ensure minimisation of electrical noise. Hippocampal neurons were visualised by differential interference contrast (DIC) microscopy with a 40x water dipping objective lens and a CCD video camera (Hamamatsu, C7500-51). Membrane current and potential recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices) and its Commander software and digitised at 10kHz with Digidata 1440 (Molecular Devices) to convert analogue to digital signals. Recordings were sampled at 10kHz with a low-pass Bessel filter at 1kHz. Data acquisition was performed using pClamp Software Version 10.2 (Molecular Devices).

4.2.7.3.2. Paired cell recordings

To record paired recordings, cultured neurons on coverslips were transferred to a recording chamber (Warner Instrument, RC-26GLP) on an Olympus BX51WI mounted on an air table in a Faraday cage. Neurons were visualised by DIC with a 40x water dipping objective lens and a high resolution 70 series Newvicon (NC70) video camera (Dage-MTI). Recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices) and its Commander software and digitised at 10kHz with Digidata 1322A (Molecular Devices). Recordings were sampled at 10kHz with a low-pass Bessel filter at 1kHz. Data acquisition was performed using pClamp Software Version 9.2 (Molecular Devices).

4.2.7.4. mEPSC recordings

Spontaneous mEPSC recordings were conducted in ACSF containing 1µM tetrodotoxin (TTX) (Alomone Lab, #T-550) and 100µM picrotoxin (Sigma, P1675) to prevent action potential-evoked excitatory postsynaptic currents (EPSCs) and all inhibitory postsynaptic currents (IPSCs), respectively. Whole-cell recordings were made in single cells using potassium gluconate internal solution (120mM K Gluconate, 40mM HEPES, 5mM MgCl$_2$, 2mM NaATP, 0.3mM NaGTP, pH 7.2 with KOH, 298mOsm). Data collection was initiated 5min after cell break-in to allow equilibration and stabilisation of membrane properties. A 10min gap-free recording was made for each cell, held under voltage clamp at -65mV.
4.2.7.5. mEPSC recording analysis

Series resistance (Rs) of cells were measured at the beginning and the end of recordings. Recordings were discarded and excluded from data analysis if changes in Rs were greater than 20%. Analysis was performed in Clampfit Software Version 10.2 (Molecular Devices). An offline low-pass Bessel filter at 1 kHz was applied to mEPSC recordings before analysis. Individual mEPSCs were detected using the ‘Event Detection’ and ‘Template Search’ function in Clampfit using a negative-going template generated by averaging 20 randomly selected mEPSC events from a Luc control trace. The baseline was set according to the created template and the match threshold was set at 4. The inter-event interval and event frequency for each recording were calculated by the ‘Event Statistics’ function. The amplitude and 90-10% decay time of individual mEPSC events within a recording were measured using cursors and the ‘Statistics’ function.

4.2.7.6. Paired cell recordings

Paired whole-cell patch clamping was achieved using potassium gluconate internal solution (120 mM K Gluconate, 40 mM HEPES, 5 mM MgCl₂, 2 mM NaATP, 0.3 mM NaGTP, pH 7.2 with KOH, 298 mOsm) in the presynaptic recording pipette and caesium gluconate internal solution (120 mM Cs Gluconate, 40 mM HEPES, 5 mM MgCl₂, 2 mM NaATP, 0.3 mM NaGTP, 5 mM QX314, pH 7.2, 298 mOsm) in the postsynaptic recording pipette. Evoked AMPAR-mediated EPSC recordings were conducted in normal ACSF. The presynaptic neuron was held under current clamp at approximately -65 mV and stimulated at 0.1 Hz with a 300-450 pA current step for 20 ms to induce an action potential. Simultaneously, the postsynaptic neuron was held under voltage clamp at -65 mV to measure monosynaptic AMPAR-mediated EPSCs. A current response was defined as monosynaptic if its onset latency was within 5 ms after the peak of the presynaptic action potential. To determine whether a peak monosynaptic response was an EPSC or IPSC, the postsynaptic neuron was held at -30 mV for a few sweeps to assess for reversal from an inward current to an outward current. Postsynaptic responses were determined to be excitatory if reversal failed to occur at -30 mV (Pavlidis & Madison, 1999). Once the response was determined to be excitatory, the pair of neurons was stimulated and AMPAR-mediated EPSCs were recorded for 10 min. To record evoked NMDAR-mediated EPSCs in the same pair of neuron, ACSF containing 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Sigma, C239) was applied to the bath to block AMPA receptors. When blockade of AMPAR-mediated EPSC has been
confirmed, the postsynaptic neuron was slowly depolarised to +55 mV. After the holding current of the postsynaptic neuron has stabilised, synaptic NMDAR-mediated EPSCs were recorded for 10 min.

**4.2.7.7. Paired cell recording analysis**

Rs of cells were measured at the beginning and the end of recordings. Recordings were discarded and excluded from data analysis if changes in Rs were greater than 20%. Analysis was performed in Clampfit Software Version 10.2 (Molecular Devices). The baseline was adjusted to 0 pA by subtracting the average current for the first 50 ms of baseline recording. For each AMPAR-mediated EPSC recording, the peak amplitude of the monosynaptic current was measured using cursors and the ‘Statistics’ function in Clampfit. The failure rate of evoked excitatory synaptic transmission was also calculated by dividing the number of sweeps where monosynaptic current was absent by the total number of sweeps recorded for each pair of neurons. Offline low-pass Bessel filter at 1 kHz was applied to NMDAR-mediated EPSC recordings before peak amplitude was measured using cursors and the ‘Statistics’ function. The peak amplitude ratio was calculated if both AMPAR- and NMDAR-mediated EPSCs were successfully recorded from a pair of neurons.

**4.2.7.8. Statistical analysis**

Statistical analysis was performed using GraphPad Prism Software Version 6. All data were presented as mean ± SEM. The Kolmogorov-Smirnov test for normality was used to determine if data sets were parametric. Bartlett’s test and Levene’s test were used to test for equality of variances in parametric and non-parametric data, respectively. Statistical differences between treatment groups were determined by one-way ANOVA with Tukey’s multiple comparisons test if assumptions of the parametric method were satisfied. Otherwise, the non-parametric Kruskal-Wallis test was used with Dunn’s multiple comparisons test.

**4.2.8. Quantification of synaptic protein expression following NR1.NTD IgG treatment**

To assess and quantify changes in synaptic protein expression following treatment with NR1.NTD IgG, primary neuronal culture were prepared according to Section 3.2.1. Luc IgG (control) and NR1.NTD IgG were applied to cultures at 7.0 µg/mL on DIV 8 as described in
Section 3.2.2. Cultures were fixed on DIV 15 with 4% (w/v) PFA for VGLUT1/Homer or Glyo-Fixx for VGLUT1/GluN1 and VGLUT1/GluA1 experiments. Immunocytochemistry was performed as described in Section 3.2.3. Primary and secondary antibodies used were the same as those detailed in Table 3.1 and Table 3.2, respectively. Images were acquired and analysed as described in Sections 3.2.5.1 and 3.2.5.2, respectively. Imaging conditions used were the same as those described for VGLUT1/Homer, VGLUT1/GluN1 and VGLUT1/GluA1 experiments in Table 3.4. A total of three individual cultures were used for each immunostaining experiment. Synaptic proteins of interest, treatment groups, IgG concentrations, primary and secondary antibodies used for each experiment are detailed in Table 4.1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment groups</th>
<th>1° antibodies</th>
<th>2° antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>VGLUT1/Homer (Excitatory synapses) with NR1.NTD IgG</td>
<td>Luc IgG and NR1.NTD IgG IgG at 7.0 µg/mL</td>
<td>Anti-MAP2A, 2B, Anti-VGLUT1, Anti-Homer</td>
<td>Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647</td>
</tr>
<tr>
<td>VGLUT1/GluN1 (Synaptic NMDAR) with NR1.NTD IgG</td>
<td>Luc IgG and NR1.NTD IgG IgG at 7.0 µg/mL</td>
<td>Anti-GluN1, Anti-VGLUT1, Anti-MAP2</td>
<td>Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647</td>
</tr>
<tr>
<td>VGLUT1/GluA1 (Synaptic AMPAR) with NR1.NTD IgG</td>
<td>Luc IgG and NR1.NTD IgG IgG at 7.0 µg/mL</td>
<td>Anti-GluA1, Anti-VGLUT1, Anti-MAP2A, 2B</td>
<td>Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647</td>
</tr>
</tbody>
</table>
4.2.8.1. Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 6. All data were presented as mean ± SEM. The Kolmogorov-Smirnov test for normality was used to determine if data sets were normally distributed. The F-test was used to test for homogeneity of variance. The two-tailed, unpaired t-test was performed to determine statistical differences between Luc IgG and NR1.NTD IgG treatment groups if data sets were parametric. Otherwise, the Mann-Whitney test was used for non-parametric data. A difference was considered statistically significant when the $p$-value was less than 0.05.
4.3. Results

4.3.1. NR1.NTD IgG was purified from rats immunised with recombinant NR1.NTD protein

Recombinant NR1.NTD protein was used for immunisation in rats to generate antibodies against the N-terminus of GluN1 subunit. The final protein preparation was analysed by SDS-PAGE and Coomassie blue staining (Figure 4.3 A) as well as Western blot with commercial antibodies (Figure 4.3 B) to validate the protein species present in the preparation and to assess purity. SDS-PAGE analysis showed the purified protein preparation was relatively free of secondary bands and the main protein species was at the expected molecular weight of the full-length NR1.NTD protein (42 kDa). Reactivity of the commercial anti-GluN1 N-term antibody against this protein species confirmed its identity as the full-length protein. Anti-GluN1 N-term antibody also reacted strongly to a prominent protein species at ~84 kDa, which suggested possible dimerisation of the full-length NR1.NTD protein. The presence of some degradation products in the protein preparation was indicated by the detection of several secondary bands of lower molecular weights. As expected, signals were absent in the secondary antibody-only control lane and the anti-GluN1 M3-M4 antibody failed to detect the N-terminal protein.

Purified NR1.NTD protein preparation was used to immunise Sprague Dawley rats for 12 weeks, same as the immunisation schedule used for other recombinant proteins previously in Chapter 2. Pre-immune serum samples were free of IgG against NR1.NTD protein and IgG expression in the best responding animals generally plateaued between Weeks 8 and 10 (data not shown). Serum samples from the best responding NR1.NTD-immunised rats at the end of the immunisation period were pooled and assessed for cross-reactivity against other recombinant protein preparations (Figure 4.3 C). Pooled NR1.NTD serum was able to recognise the full-length NR1.NTD protein as well as various secondary bands in the protein preparation. It also displayed minimal reactivity to Luc and NR1extra2 protein preparations with single bands at ~30 kDa. Several bands were detected by pooled NR1.NTD serum in the recNR1 protein preparation, which were probably common bacterial proteins as the bands were shared by recNR1 and NR1.NTD protein preparations. However, pooled NR1.NTD serum also appeared to have successfully detected the full-length recNR1 protein at 83 kDa.
Following characterisation, pooled NR1.NTD serum was used for IgG purification. The purity of NR1.NTD IgG solution purified by the caprylic acid method was qualitatively assessed by SDS-PAGE (Figure 4.3 D) and Western blot analyses (Figure 4.3 E). Prominent bands which corresponded to the molecular weight of IgG heavy chain (~50 kDa) and light chain (~25 kDa) were observed in both Coomassie blue-stained gel and Western blot with anti-rat IgG antibody. Anti-rat IgG antibody reacted with most visible secondary bands, which showed that NR1.NTD IgG prepared from rat sera was relatively pure and free of non-IgG protein contaminants. Western blot demonstrated that NR1.NTD IgG retained its binding capacity for the full-length NR1.NTD protein even after purification (Figure 4.3 F). There were similarities in the reactivity profiles of NR1.NTD IgG and the commercial anti-GluN1 N-term antibody but some non-specific secondary protein bands were also detected by the former.

Purified NR1.NTD IgG samples were utilised in subsequent electrophysiological and immunocytochemical experiments in primary rat hippocampal neuronal cultures.
Figure 4.3 Validation of purified recombinant NR1.NTD protein and NR1.NTD IgG

Purified recombinant NR1.NTD protein and NR1.NTD IgG were resolved on 12% acrylamide gels for analysis. SDS-PAGE analysis with Coomassie blue staining (A) and Western blot analysis of purified NR1.NTD protein preparation (B) both showed the full-length NR1.NTD protein at the expected molecular weight of 42 kDa (with the commercial anti-GluN1 N-term antibody only) (arrows). Few secondary bands were also observed, including a prominent protein species at ~84 kDa. No signals were detected by the anti-GluN1 M3-M4 antibody or the secondary antibody-only control. Pooled serum from the best responding NR1.NTD-immunised rats detected the full-length NR1.NTD protein (arrow) but also exhibited some cross-reactivity to Luc, recNR1 (rec) and NR1extra2 (e2) protein preparations as shown by Western blot analysis (C). SDS-PAGE analysis with Coomassie blue staining (D) and Western blot of purified NR1.NTD IgG using anti-rat IgG antibody (E) both showed the bands for heavy chain and light chain of IgG at the expected molecular weights of ~50 kDa and ~25 kDa, respectively (arrows). The IgG preparation appeared relatively pure and free of contamination. Purified NR1.NTD IgG was tested for reactivity against recombinant NR1.NTD protein by Western blot and comparisons were made with commercial anti-GluN1 antibodies (F). NR1.NTD IgG and the anti-GluN1 N-term antibody both detected the full-length NR1.NTD protein at 42 kDa (arrow). The pattern and intensity of secondary bands varied between NR1.NTD IgG and the anti-GluN1 N-term antibody but they both detected the other prominent protein species at ~84 kDa.
Figure 4.3
4.3.2. recNR1 IgG did not alter mEPSC activity but event frequency was decreased in NR1.NTD IgG-treated neurons

To investigate whether the increase in excitatory synapse density mediated by recNR1 IgG treatment was accompanied by functional modulation of glutamatergic synapses, basal excitatory synaptic transmission was assessed first by recording mEPSC activity of primary hippocampal neurons following IgG treatment (Figure 4.4 A). Basal neuronal activity of cells treated with NR1.NTD IgG was also examined and compared with Luc IgG and recNR1 IgG. Average mEPSC event traces from the three IgG treatment groups appeared similar in shape and size (Figure 4.4 B). This was confirmed by quantitative analysis of mEPSC events where no significant differences were detected between treatment groups in mEPSC amplitude (one-way ANOVA, \( F = 0.017, p = 0.984 \)) or 90-10% decay time (one-way ANOVA, \( F = 0.237, p = 0.790 \)) (Figure 4.4 E, F). The mEPSC amplitude distribution histograms (10 pA bins) were also comparable between treatment groups with no visible skew (Figure 4.4 C). Although cumulative probability curves of mEPSC amplitude had similar trajectories, the maximum amplitude for NR1.NTD IgG (-140.58 pA) was smaller in size compared to both Luc IgG (-212.24 pA) and recNR1 IgG (-204.02 pA) (Figure 4.4 D). On the other hand, NR1.NTD IgG treatment significantly increased mEPSC inter-event intervals (1126 ± 166 ms) in comparison to recNR1 IgG (Kruskal-Wallis test, \( H = 7.965, p = 0.019 \); Dunn’s post-hoc, 640 ± 92 ms, \( p = 0.026 \)) but not Luc IgG (Dunn’s post-hoc, 775 ± 150 ms, \( p = 0.082 \)) (Figure 4.4 G). Furthermore, the frequency of mEPSC events was significantly lower in neurons treated with NR1.NTD IgG (1.26 ± 0.16 Hz) compared to those treated with Luc IgG (one-way ANOVA, \( F = 4.426, p = 0.016 \); Tukey's post-hoc, 2.04 ± 0.27 Hz, \( p = 0.041 \)) or recNR1 IgG (Tukey's post-hoc, 2.10 ± 0.22 Hz, \( p = 0.027 \)) (Figure 4.4 H).

The results obtained from analysis of mEPSC recordings indicated no difference in basal excitatory synaptic transmission in recNR1 IgG-treated hippocampal neurons compared to Luc IgG. However, the lower maximum mEPSC amplitude, increased inter-event interval and reduction in event frequency in hippocampal neurons treated with NR1.NTD IgG suggested possible presynaptic and postsynaptic impairments.
Figure 4.4 Effect of IgG treatment on spontaneous mEPSC activity of primary hippocampal neurons

Recordings were made from IgG-treated hippocampal neurons across three individual cultures with $n = 20$ per treatment group in the presence of TTX and picrotoxin to isolate spontaneous mEPSCs. Cells were treated with Luc IgG, recNR1 IgG or NR1.NTD IgG at 7.0 µg/mL. Example mEPSC traces showing 30 s of continuous current recording (A) and average mEPSC event traces (B) for each treatment group are shown. Distribution histograms showing the number of events in 10 pA bins (C) and cumulative probability curves of mEPSC amplitudes (D) appeared similar between treatment groups but the maximum mEPSC amplitude for the NR1.NTD IgG was smaller in size compared to Luc IgG and recNR1 IgG (arrows). No significant differences were observed in mEPSC amplitude (E) or 90-10% decay time (F) between any of the treatment groups. NR1.NTD IgG-treated neurons had significantly longer mEPSC inter-event intervals compared to recNR1 IgG (G), and significantly lower mEPSC event frequency compared to both Luc IgG and recNR1 IgG (H).

Data represent mean ± SEM. *$p < 0.05$
Figure 4.4

A

Luc
recNR1
NR1.NTD

B

Luc
recNR1
NR1.NTD

C

Number of events

mEPSC amplitude (pA)

D

Cumulative probability

mEPSC amplitude (pA)

E

mEPSC amplitude (pA)

IgG treatment (7.0 μg/mL)

F

mEPSC decay time (90–10%) (ms)

IgG treatment (7.0 μg/mL)

G

mEPSC inter-event interval (ms)

IgG treatment (7.0 μg/mL)

H

mEPSC event frequency (Hz)

IgG treatment (7.0 μg/mL)
4.3.3. recNR1 IgG treatment increased the amplitude of NMDAR-mediated synaptic response in hippocampal neurons

The mEPSCs recorded in the previous section were predominantly mediated by AMPA receptors because they were recorded at -65 mV. Therefore, the function of glutamate receptor subtypes, AMPA and NMDA receptors, were further examined by performing paired recordings of evoked synaptic responses. Recordings were made between two connected hippocampal neurons in culture, where action potential was triggered in the presynaptic neuron in current clamp and the response of the postsynaptic neuron was recorded in voltage clamp (Figure 4.5 A). NMDAR-mediated EPSC recordings were successfully obtained in approximately half the number of pairs of cells recorded for AMPAR-mediated EPSCs. In general, AMPAR-mediated responses were almost always polysynaptic and NMDAR-mediated responses often fluctuated after peaking, which prevented accurate quantification of changes in synaptic current kinetics (Figure 4.5 B, C). Quantitative analysis of AMPAR-mediated EPSCs revealed no significant differences in peak amplitude between treatment groups (Kruskal-Wallis test, \( H = 0.195, p = 0.907 \)) (Figure 4.6 A). Cumulative probability curves of AMPAR-mediated EPSC peak amplitude and maximum peak amplitudes were also similar between treatment groups (Luc IgG, -1543.60 pA; recNR1 IgG, -1374.45 pA; NR1.NTD IgG, -1253.17 pA) (Figure 4.6 B). On the other hand, the peak amplitude of NMDAR-mediated EPSCs was significantly increased following recNR1 IgG treatment in neurons (44.03 ± 7.90 pA) when comparisons were made with Luc IgG (Kruskal-Wallis test, \( H = 9.142, p = 0.010 \); Dunn's post-hoc, 23.71 ± 3.74 pA, \( p = 0.014 \)) and NR1.NTD IgG (Dunn's post-hoc, 26.38 ± 4.21 pA, \( p = 0.046 \)) (Figure 4.6 C). The increase was also demonstrated by a clear rightward shift in the NMDAR-mediated EPSC cumulative probability curve for recNR1 IgG and resulted in a higher maximum peak amplitude (170.81 pA) compared to Luc IgG (105.34 pA) and NR1.NTD IgG (106.39 pA) (Figure 4.6 D). There were no significant differences in the failure rate of AMPAR-mediated EPSC (Kruskal-Wallis test, \( H = 0.753, p = 0.686 \)) or the ratio of NMDAR- and AMPAR-mediated EPSC peak amplitudes (Kruskal-Wallis test, \( H = 1.785, p = 0.410 \)) between any of the treatment groups (Figure 4.6 E, F).

Taken together, paired cell recordings revealed no alterations in AMPA or NMDA receptor function in hippocampal neurons following NR1.NTD IgG treatment. On the other hand, recNR1 IgG-treated neurons exhibited enhanced NMDAR-mediated currents but no changes in AMPA receptor function in comparison to both Luc IgG and NR1.NTD IgG.
Figure 4.5 Evoked AMPAR- and NMDAR-mediated EPSCs in primary hippocampal neurons following IgG treatment

Schematic diagram of a paired recording between a presynaptic (red) and a postsynaptic (blue) hippocampal neuron and example current traces (A). Action potential firing was induced by a 20 ms current step in the presynaptic cell, which directly evoked a monosynaptic AMPAR-mediated response (arrow) in the postsynaptic cell within a 5 ms window. NMDAR-mediated responses were recorded in a similar manner but at a depolarising membrane potential and in the presence of CNQX to block AMPA receptors. Representative traces of AMPAR- (B) and NMDAR-mediated EPSCs (C) for each treatment group are shown. Monosynaptic AMPA receptor-mediated responses are indicated by arrows.
Figure 4.6 Effect of IgG treatment on evoked AMPAR- and NMDAR-mediated EPSCs in primary hippocampal neurons

Recordings were made from pairs of hippocampal neurons across six individual cultures for AMPAR-mediated EPSC (Luc IgG, n = 25; recNR1 IgG, n = 22, NR1.NTD IgG, n = 20) and NMDAR-mediated EPSC (Luc IgG, n = 13; recNR1 IgG, n = 12; NR1.NTD IgG, n = 15). Cells were treated with Luc IgG, recNR1 IgG or NR1.NTD IgG at 7.0 µg/mL. No significant differences were observed between treatment groups in AMPAR-mediated EPSC peak amplitude (A). The cumulative probability curves of AMPAR-mediated EPSC peak amplitude (B) had similar trajectories between treatment groups. Treatment with recNR1 IgG significantly increased NMDAR-mediated EPSC peak amplitude compared to both Luc IgG and NR1.NTD IgG (C) and a rightward shift was observed in its cumulative probability curve (D). No significant differences were detected in the AMPAR-mediated EPSC failure rate (E) or the NMDAR- and AMPAR-mediated EPSC peak amplitude ratio (F). Maximum peak amplitudes in the cumulative probability graphs are indicated by arrows.

Data represent mean ± SEM. *p < 0.05.
Figure 4.6

**A**

AMPA EPSC peak amplitude (pA)

- Luc
- recNR1
- NR1-NTD

IgG treatment (7.0 μg/mL)

**B**

Cumulative probability

- Luc
- recNR1
- NR1-NTD

AMPAR EPSC peak amplitude (pA)

**C**

NMDA EPSC peak amplitude (pA)

- Luc
- recNR1
- NR1-NTD

IgG treatment (7.0 μg/mL)

**D**

Cumulative probability

- Luc
- recNR1
- NR1-NTD

NMDA EPSC peak amplitude (pA)

**E**

AMPA EPSC failure rate (%)

- Luc
- recNR1
- NR1-NTD

IgG treatment (7.0 μg/mL)

**F**

NMDA/AMPA EPSC peak amplitude ratio

- Luc
- recNR1
- NR1-NTD

IgG treatment (7.0 μg/mL)
4.3.4. Immunostaining analysis of hippocampal neurons treated with NR1.NTD IgG

Electrophysiological data from NR1.NTD IgG-treated hippocampal neurons suggested possible alterations in pre- and postsynaptic function. Therefore, immunocytochemistry was performed to see if functional changes were reflected in protein expression in excitatory synapses following NR1.NTD IgG treatment. Double-labelling experiments were the same as those performed with recNR1 IgG and NR1extra2 IgG in Chapter 3 except NR1.NTD IgG was only applied at 7.0 µg/mL and comparisons were made with a single control group, Luc IgG. Fluorescence intensity values from each image were normalised to the Luc IgG control from the same culture for all three analyses.

4.3.4.1. No changes in excitatory glutamatergic synapse expression were observed following NR1.NTD IgG treatment

To assess changes in excitatory synaptic protein expression, hippocampal neurons were labelled for MAP2, VGLUT1 and Homer following NR1.NTD IgG treatment (Figure 4.7). The immunostaining patterns and intensities of both VGLUT1 and Homer appeared similar between Luc IgG and NR1.NTD IgG treatment groups. This was confirmed by analysis of images to determine fluorescence intensities and densities of VGLUT1 and Homer puncta. No statistical differences were found in total (unpaired t-test, $t = 0.331, p = 0.742$) or synaptic (unpaired t-test, $t = 0.169, p = 0.866$) VGLUT1 puncta fluorescence intensities following NR1.NTD IgG treatment (Figure 4.8 A, B). Similarly, there were also no significant differences in total (unpaired t-test, $t = 0.473, p = 0.638$) or synaptic (unpaired t-test, $t = 0.552, p = 0.583$) Homer puncta fluorescence intensities (Figure 4.8 C, D) between NR1.NTD IgG and the Luc IgG control. Moreover, there were no significant differences in total VGLUT1 (unpaired t-test, $t = 0.260, p = 0.796$) and total Homer (unpaired t-test, $t = 0.410, p = 0.683$) puncta densities or the number of excitatory synapses (unpaired t-test, $t = 0.074, p = 0.941$) (Figure 4.8 E, F, G).

4.3.4.2. No changes in NMDAR expression were observed following NR1.NTD IgG treatment

To assess changes in NMDA receptor expression, hippocampal neurons were labelled for MAP2, VGLUT1 and GluN1 following NR1.NTD IgG treatment (Figure 4.9). VGLUT1 and GluN1 immunostaining patterns were visibly similar between Luc IgG and NR1.NTD IgG
treatment groups. Image analysis confirmed this observation. Total (Mann-Whitney test, $U = 426, p = 0.730$) and GluN1-colocalised (unpaired t-test, $t = 0.096, p = 0.924$) VGLUT1 puncta fluorescence intensities were not significantly different between NR1.NTD IgG- and Luc IgG-treated neurons (Figure 4.10 A, B). In addition, no statistical differences were detected in total (unpaired t-test, $t = 0.598, p = 0.552$) or synaptic (unpaired t-test, $t = 0.081, p = 0.936$) GluN1 fluorescence intensities (Figure 4.10 C, D). There were also no significant differences between NR1.NTD IgG and Luc IgG in total VGLUT1 (unpaired t-test, $t = 0.803, p = 0.425$), total GluN1 (Mann-Whitney test, $U = 401, p = 0.476$) and synaptic GluN1 (unpaired t-test, $t = 0.350, p = 0.728$) puncta densities (Figure 4.10 E, F, G).

4.3.4.3. NR1.NTD IgG treatment increased synaptic AMPAR fluorescence intensity, total and synaptic AMPAR puncta density

To assess changes in AMPA receptor expression, hippocampal neurons were labelled for MAP2, VGLUT1 and GluA1 following NR1.NTD IgG treatment (Figure 4.11). Although VGLUT1 and GluA1 immunostaining patterns appeared comparable between NR1.NTD IgG and Luc IgG, this was proven otherwise when the images were analysed for fluorescence intensities and puncta densities. There were no statistical differences in total (unpaired t-test, $t = 1.519, p = 0.134$) or GluA1-colocalised (unpaired t-test, $t = 1.395, p = 0.168$) VGLUT1 puncta fluorescence intensities (Figure 4.12 A, B). While no statistical difference was detected in total GluA1 fluorescence intensity (Mann-Whitney test, $U = 331, p = 0.080$), synaptic GluA1 fluorescence intensity was significantly higher in NR1.NTD IgG-treated neurons ($1.12 \pm 0.04$) compared to Luc IgG-treated neurons (unpaired t-test, $t = 2.186, 1.00 \pm 0.04, p = 0.033$) (Figure 4.12 C, D). There was no significant difference in total VGLUT1 puncta density (unpaired t-test, $t = 1.352, p = 0.182$) (Figure 4.12 E). However, treatment with NR1.NTD IgG resulted in significant increases in both total ($11.85 \pm 0.59$) and synaptic GluA1 ($6.41 \pm 0.41$) puncta densities when comparisons were made with Luc IgG (total, unpaired t-test, $t = 2.518, 9.82 \pm 0.55, p = 0.015$; synaptic, unpaired t-test, $t = 2.479, 5.14 \pm 0.32, p = 0.016$) (Figure 4.12 F, G).

Altogether, these results showed that although there were no changes in the number of excitatory synapses or GluN1-containing synapses, NR1.NTD IgG treatment increased total and synaptic GluA1 puncta density as well as synaptic GluA1 fluorescence intensity in hippocampal neurons. This demonstrated an overall increase in AMPA receptor expression.
Figure 4.7 VGLUT1 and Homer immunostaining in primary hippocampal neurons following NR1.NTD IgG treatment

Representative dendritic images of hippocampal neurons labelled for MAP2 (Alexa Fluor 488, green), VGLUT1 (Alexa Fluor 594, red) and Homer (Alexa Fluor 647, blue) following IgG treatment. Boxed regions in the left panels are enlarged on the right and shown as individual channels and merged images. VGLUT1/Homer colocalised puncta are purple in colour (arrows). Cells were treated with Luc IgG or NR1.NTD IgG at 7.0 µg/mL.

Scale bars, 10 µm (left) and 5 µm (right).
Figure 4.8 Effect of NR1.NTD IgG treatment on excitatory glutamatergic synapses in primary hippocampal neurons

Cells were imaged from three individual cultures and the same number of hippocampal neurons were imaged for each treatment group \((n = 30)\). Images of hippocampal neurons treated with Luc IgG or NR1.NTD IgG at 7.0 \(\mu\)g/mL were analysed to assess excitatory synaptic protein expression. Fluorescence intensity values were normalised to the Luc IgG control group. Image analysis showed that treatment with NR1.NTD IgG did not result in any changes in the parameters measured when comparisons were made with the Luc IgG control. No statistical differences were detected in total VGLUT1 (A) and synaptic VGLUT1 (B) puncta fluorescence intensities as well as total Homer (C) and synaptic Homer (D) puncta fluorescence intensities. There were also no statistical differences in total VGLUT1 puncta (E), total Homer puncta (F) and excitatory synapse (VGLUT1/Homer colocalised puncta) (G) densities in hippocampal neurons.

Data represent mean ± SEM.
Figure 4.8

A. Total VGLUT1 fluorescence intensity

B. Synaptic VGLUT1 fluorescence intensity

C. Total Homer fluorescence intensity

D. Synaptic Homer fluorescence intensity

E. Total VGLUT1 puncta density (number/10 μm)

F. Total Homer puncta density (number/10 μm)

G. Excitatory synapse density (number/10 μm)

IgG treatment (7.0 μg/mL)
Figure 4.9 VGLUT1 and GluN1 immunostaining in primary hippocampal neurons following NR1.NTD IgG treatment

Representative dendritic images of hippocampal neurons labelled for MAP2 (Alexa Fluor 647, blue), VGLUT1 (Alexa Fluor 594, red) and GluN1 (Alexa Fluor 488, green) following IgG treatment. Boxed regions in the left panels are enlarged on the right and shown as individual channels and merged images. VGLUT1/GluN1 colocalised puncta are yellow in colour (arrows). Cells were treated with Luc IgG or NR1.NTD IgG at 7.0 µg/mL.

Scale bars, 10 µm (left) and 5 µm (right).
Figure 4.10 Effect of NR1.NTD IgG treatment on synaptic NMDAR expression in primary hippocampal neurons

Cells were imaged from three individual cultures and the same number of hippocampal neurons were imaged for each treatment group (n = 30). Images of hippocampal neurons treated with Luc IgG or NR1.NTD IgG at 7.0 µg/mL were analysed to assess synaptic NMDA receptor expression. Fluorescence intensity values were normalised to the Luc IgG control group. Image analysis showed that treatment with NR1.NTD IgG did not result in any changes in the parameters measured when comparisons were made with the Luc IgG control. No statistical differences were detected in total VGLUT1 (A) and GluN1-colocalised VGLUT1 (B) puncta fluorescence intensities as well as total GluN1 (C) and synaptic GluN1 (D) puncta fluorescence intensities. There were also no statistical differences in total VGLUT1 (E), total GluN1 (F) and synaptic GluN1 (G) puncta densities in hippocampal neurons.

Data represent mean ± SEM.
Figure 4.10

A

Total VGluT1 fluorescence intensity

IgG treatment (7.0 μg/mL)

B

Glut1-colocalising VGluT1 fluorescence intensity

IgG treatment (7.0 μg/mL)

C

Total GluN1 fluorescence intensity

IgG treatment (7.0 μg/mL)

D

Synaptic GluN1 fluorescence intensity

IgG treatment (7.0 μg/mL)

E

Total VGluT1 puncta density (number/10 μm)

IgG treatment (7.0 μg/mL)

F

Total GluN1 puncta density (number/10 μm)

IgG treatment (7.0 μg/mL)

G

Synaptic GluN1 puncta density (number/10 μm)

IgG treatment (7.0 μg/mL)
Figure 4.11 VGLUT1 and GluA1 immunostaining in primary hippocampal neurons following NR1.NTD IgG treatment

Representative dendritic images of hippocampal neurons labelled for MAP2 (Alexa Fluor 647, blue), VGLUT1 (Alexa Fluor 594, red) and GluA1 (Alexa Fluor 488, green) following IgG treatment. Boxed regions in the left panels are enlarged on the right and shown as individual channels and merged images. VGLUT1/GluA1 colocalised puncta are yellow in colour (arrows). Cells were treated with Luc IgG or NR1.NTD IgG at 7.0 µg/mL.

Scale bars, 10 µm (left) and 5 µm (right).
Figure 4.12 Effect of NR1.NTD IgG treatment on synaptic AMPAR expression in primary hippocampal neurons

Cells were imaged from three individual cultures and the same number of hippocampal neurons were imaged for each treatment group ($n = 30$). Images of hippocampal neurons treated with Luc IgG or NR1.NTD IgG at 7.0 µg/mL were analysed to assess synaptic AMPA receptor expression. Fluorescence intensity values were normalised to the Luc IgG control group. Image analysis showed no statistical differences in total VGLUT1 (A) and GluA1-colocalised VGLUT1 (B) puncta fluorescence intensities. Total GluA1 fluorescence intensity was not statistically different between the treatment two groups (C) but NR1.NTD IgG significantly increased synaptic GluA1 fluorescence intensity (D). There was no change in total VGLUT1 puncta density (E) but NR1.NTD IgG treatment significantly increased total GluA1 (F) and synaptic GluA1 (G) puncta densities in hippocampal neurons.

Data represent mean ± SEM. *p < 0.05.
Figure 4.12

A  Total VGLUT1 fluorescence intensity

B  GluA1-colocalising VGLUT1 fluorescence intensity

C  Total GluA1 fluorescence intensity

D  Synaptic GluA1 fluorescence intensity

E  Total VGLUT1 puncta density (number/10 μm)

F  Total GluA1 puncta density (number/10 μm)

G  Synaptic GluA1 puncta density (number/10 μm)

IgG treatment (7.0 μg/mL)
4.4. Discussion

In this chapter, characterisation of primary rat hippocampal neurons treated with IgG prepared in Chapter 2 was extended by means of electrophysiology in order to gain further understanding of the mechanisms underlying potential therapeutic effects of anti-GluN1 IgG (Chen, 2010; Chen, 2015; During et al., 2000). An allosteric N-terminal-targeting NR1.NTD IgG was added to our repertoire of anti-GluN1 antibodies to assist with the identification of possible epitope-dependent effects. Electrophysiological recordings of hippocampal neurons allowed functional analysis of excitatory synaptic transmission through AMPA and NMDA receptors in vitro following IgG treatment. We found no differences in basal excitatory synaptic activity, as measured by mEPSCs, following recNR1 IgG treatment. However, NR1.NTD IgG-treated neurons exhibited higher inter-event interval and lower mEPSC frequency, indicating possible dysfunction in synaptic transmission. Interestingly, we observed heightened NMDAR-mediated synaptic response but no change in AMPAR-mediated EPSCs in neurons treated with recNR1 IgG, which paralleled our previous immunocytochemical findings in Chapter 3. In contrast, analysis of evoked current responses from paired cell recordings did not show any changes in AMPA or NMDA receptor function following NR1.NTD IgG treatment. Immunostaining of NR1.NTD IgG-treated hippocampal neurons revealed no changes in excitatory glutamatergic or NMDAR-containing synapse numbers. However, the treatment led to an increase in the synaptic expression of GluA1-containing AMPA receptors.

4.4.1. NR1.NTD IgG was generated from protein immunisation in rats

High purity IgG was recovered from the pooled serum sample of the best responding NR1.NTD-immunised rats in the same way as other IgG preparations (see Chapter 2) and was shown to have retained reactivity to the full-length NR1.NTD protein. However, epitope mapping conducted prior to IgG purification showed that pooled NR1.NTD serum did not react to peptide #37 as we had originally hoped in order to mimic antibodies from anti-NMDA receptor encephalitis patients (Gleichman et al., 2012), but instead displayed polyclonal reactivity to peptides #5, 10, 20, 33, 34 and 71 of the GluN1 N-terminus.
4.4.2. Basal excitatory synaptic transmission is unchanged following recNR1 IgG treatment but NR1.NTD IgG reduced mEPSC event frequency

We initiated our electrophysiological profiling of anti-GluN1 IgG-treated hippocampal neurons by recording mEPSC activity to assess basal excitatory synaptic transmission. GABAergic miniature IPSCs were eliminated by the addition of picrotoxin into the recording solution. Due to activity blockade by TTX, mEPSCs occurred from action potential-independent spontaneous release of glutamatergic vesicles from the presynaptic terminal (Katz, 1969). Although the AMPA and NMDA receptor components of mEPSCs can be temporally separated based on differences in their kinetics (Hestrin et al., 1990; Umemiya et al., 1999; Watt et al., 2000), the activity we observed were predominantly mediated by AMPA receptors because recordings were performed at a holding potential of -65 mV in the presence of magnesium ions. This was confirmed by the similarities in the decay time course between our mEPSC events and that of pure AMPAR-mediated mEPSCs (Watt et al., 2000). The lack of change in mEPSC decay time following recNR1 IgG or NR1.NTD IgG treatment suggests that AMPA receptor subunit and splice variant compositions were unchanged (Jonas, 2000; Sommer et al., 1990). Treatment with recNR1 IgG did not alter mEPSC event frequency or amplitude, suggesting that basal synaptic transmission was unchanged in the neurons. On the other hand, NR1.NTD IgG treatment did not change mEPSC amplitude but decreased event frequency in hippocampal neurons. It was also noted that NR1.NTD IgG-treated neurons had a smaller maximum mEPSC amplitude than those treated with Luc IgG or recNR1 IgG, which may suggest diminished postsynaptic capacity for AMPA receptors. Since the mEPSC amplitude is usually a representation of the density or overall conductance of postsynaptic receptors at individual synapses (O’Brien et al., 1998; Turrigiano et al., 1998), it was unexpected that the increase in synaptic AMPA receptor expression observed in NR1.NTD IgG-treated neurons did not correspond to an augmentation in amplitude. Changes in mEPSC frequency are generally interpreted as modifications in presynaptic properties. Possible presynaptic explanations for a reduction in frequency include decreases in the probability of presynaptic neurotransmitter release (El-Husseini et al., 2000; Prange & Murphy, 1999), the size of vesicle RRP docked to the active zone, or vesicle turnover rates (Dobrunz & Stevens, 1997; Murthy et al., 2001; Murthy et al., 1997). This is interesting because VGLUT1 plays an important role in synaptic glutamate release (Fremeau et al., 2004; Wojcik et al., 2004) and yet we did not detect any reduction in its expression in hippocampal neurons treated with NR1.NTD IgG. However, the mEPSC frequency could
also be influenced by the number of neurotransmitter release sites or functional excitatory synapses which, in this case, contain AMPA receptors (Liao et al., 2001). In addition, due to the small size of mEPSCs, the presence of events below the detection threshold may impede accurate measurements of frequency. Holding potentials could potentially be manipulated to amplify mEPSC amplitudes to ensure detection of all events (El-Husseini et al., 2000).

Various studies have investigated the effects of NMDA receptor modulation on basal synaptic transmission. Chronic blockade of NMDA receptor activity in cultured hippocampal neurons by APV increases AMPAR-mediated mEPSC amplitude but does not affect event frequency or decay kinetics (Kato et al., 2007). Similarly, chronic treatment with memantine in slice cultures has also been to shown to increase mEPSC amplitude without any changes in kinetics but also results in a slight reduction in frequency (He & Bausch, 2014). However, the activation of synaptic NMDA receptors via glycine application increases both the amplitude and frequency of AMPAR-mediated mEPSC components in cultured neurons even after washout (Lu et al., 2001). It is difficult to determine if our anti-GluN1 antibodies possess agonistic or antagonistic activity from making comparisons with these studies due to the mixed findings. In the case of anti-NMDA receptor encephalitis, Hughes et al. (2010) investigated changes in mEPSC in cultured hippocampal neurons treated with patient IgG which resulted in an attenuation of synaptic NMDAR-mediated mEPSCs that directly corresponds to a reduction in surface receptor expression. However, they did not observe any differences in AMPAR-mediated mEPSCs. This suggests that NR1.NTD IgG may behave differently to those patient IgG due to differences in their N-terminal domain target epitopes. Proper comparisons could not be made with recNR1 IgG because we did not measure NMDAR-mediated mEPSCs, which could be a variable of interest for future studies.

4.4.3. NMDAR- but not AMPAR-mediated synaptic response is enhanced by recNR1 IgG treatment

To further characterise changes in synaptic function following anti-GluN1 IgG treatment, paired cell recordings were conducted on hippocampal neurons in culture which displayed monosynaptic excitatory connections. Paired cell recording is one of the electrophysiology techniques, along with minimal stimulation, that has been developed for investigating single synapse connections between cells and allows the analysis of evoked synaptic transmission in a small population of synapses (Montgomery et al., 2001). There were no differences in the failure rates of evoked synaptic transmission following treatment with recNR1 IgG or
NR1.NTD IgG. The failure rates in all treatment groups were very low, which was indicative of a reliable release of neurotransmitters from the presynaptic terminal upon induction of action potential (Arons et al., 2012).

We reported no differences in the peak amplitude of AMPAR- or NMDAR-mediated EPSCs in NR1.NTD IgG-treated neurons, which suggests no changes in number of synapses that contain AMPA or NMDA receptors, or no alterations in glutamate release. These responses are partly reflected in the immunocytochemical characterisation of NR1.NTD IgG-treated neurons where no changes were found in excitatory synapse numbers or synaptic NMDA receptor expression. However, similar to the mEPSC analysis conducted, the lack of change in AMPAR-mediated synaptic response contradicts the observation where significant increases in both synaptic AMPA receptor expression level and the number of AMPAR-containing synapses were detected after NR1.NTD IgG treatment. The morphological data suggest that AMPA receptors were recruited to silent synapses in NR1.NTD IgG-treated neurons during their time in culture, possibly as an effect of NMDA receptor activation (Isaac et al., 1995; Liao et al., 2001; Liao et al., 1999). The dynamic nature of AMPA receptor trafficking could potentially explain the contradicting electrophysiological findings (Sheng & Lee, 2001). In addition to LTP, AMPA receptors are known to redistribute during LTD by rapid endocytosis in response to activation of various glutamate receptors including AMPA, NMDA and metabotropic glutamate receptors (Beattie et al., 2000; Carroll et al., 1999; Lisman et al., 2012; Snyder et al., 2001). Receptor internalisation can occur within minutes upon acute stimulation (Carroll et al., 1999; Lin et al., 2000). Although the actual rate is unknown, the constant flow of ACSF in the recording chamber may accelerate the dissociation of anti-GluN1 antibodies from neuronal NMDA receptors. Therefore, the removal of cells from their original NR1.NTD IgG-containing culture medium and the subsequent transfer into recording ACSF may have triggered AMPA receptor endocytosis in the short space of time by unknown mechanisms. This hypothesis can be easily tested by conducting immunohistochemistry on NR1.NTD IgG-treated neurons after they have been incubated in ACSF for a period of time.

On the other hand, there is a direct correlation between the paired cell recording data and morphological characteristics of recNR1 IgG-treated neurons described in Chapter 3. We reported previously that recNR1 IgG treatment in hippocampal neurons does not result in changes in synaptic AMPA receptor expression. This finding corresponds with the absence of change in AMPAR-mediated synaptic response. Similarly, the heightened NMDAR-mediated
synaptic response in recNR1 IgG-treated neurons is consistent with the increase in synaptic NMDA receptor expression observed previously and demonstrates the functionality of the receptors. Interestingly, functional changes in hippocampal neurons treated with recNR1 IgG were detectable in electrophysiological recordings unlike NR1.NTD IgG. This is in agreement with the idea that NMDA receptors are more stable than AMPA receptors in the synaptic membrane. The association of NMDA receptors with various scaffolding proteins such as PSD-95 makes them less susceptible to endocytosis (Allison et al., 1998; Lin et al., 2000; Roche et al., 2001). Taken together, these findings provide further support of the presence and increased number of functionally silent synapses in hippocampal neurons following recNR1 IgG treatment. This opens up the possibility of exploring changes in synaptic plasticity and remodelling processes in hippocampal neurons treated with IgG to see if LTP and LTD can be experimentally induced. We were, however, unable to identify and draw a conclusion on the pharmacological action of recNR1 IgG, which may be related to its neuroprotective properties observed in a previous study (During et al., 2000).

4.4.4. Limitations and future directions

Unlike slice cultures, dissociated hippocampal neurons do not belong to an intact hippocampal circuitry of properly defined cell layers, making it difficult to distinguish pyramidal neurons and interneurons by cell morphology. Although the excitatory phenotype of presynaptic neurons in pair recordings was confirmed electrophysiologically, the use of caesium ions in the postsynaptic recording solution prevented the identification of inhibitory interneurons by action potentials due to blockade of voltage-gated potassium channels (Chandler & Meves, 1965). Therefore, similar to immunocytochemical analyses, we are unable to say with certainty that all the neurons investigated in the study thus far were of the pyramidal type we aimed for. Although this may have contributed to variability in some results, it is still an adequate first approach to investigating the overall effects of anti-GluN1 antibodies on hippocampal cell populations. There is also insufficient information regarding the binding kinetics of anti-GluN1 IgG. We do not know if surface NMDA receptors on neurons were still bound by anti-GluN1 IgG during electrophysiological recordings, which is essential for the determination of pharmacological action. Ideally, electrophysiological recordings should be conducted on neurons while they are bathed in IgG-containing solutions to properly examine the pharmacological action of anti-GluN1 antibodies without the concern of dissociation. However, this set up is impractical due to the large amounts of IgG required.
It may potentially be applied to future studies where properties of monoclonal antibodies are investigated after the identification of epitopes crucial for anti-GluN1 IgG-mediated effects.
Chapter 5.
A pilot study investigating the effects of anti-GluN1 IgG on neurons expressing ASD-associated Shank3 mutations
5.1. Introduction

The primary aim of this chapter was to examine the potential therapeutic application of recNR1 IgG in primary rat hippocampal neurons expressing ASD-associated Shank3 mutations or with Shank3 knockdown where excitatory synaptic dysfunctions have been identified. We established in Chapter 3 that recNR1 IgG treatment increases excitatory synapse density by analysing VGLUT1 and Homer expressions in hippocampal neurons. Now we are interested to see if the treatment could normalise deficits in neurons with defective Shank3 functions.

Shank3 is a molecular scaffold protein in the PSD. With its multiple protein-binding domains, Shank3 physically links components in the PSD to facilitate signalling through glutamate receptors including NMDA and AMPA receptors (Naisbitt et al., 1999; Sheng & Kim, 2000). Therefore, it is not surprising that mutations in or loss of Shank3 function leads to alterations in synaptic protein expression and impairs synaptic transmission and plasticity in both in vitro and in vivo models of ASD (Arons et al., 2012; Bozdagi et al., 2010; Kouser et al., 2013; Peça et al., 2011; Wang et al., 2011). Disruption in the Shank3 gene is a major genetic cause of ASD, with mutations found in approximately 1% of patients (Moessner et al., 2007). While overexpression of wild type Shank3 (WT) leads to upregulation of various synaptic proteins including VGLUT1 and Homer, and results in an increase in synaptic density, Shank3 with ASD-associated point mutations do not have the capacity to exert the same effect and behave more similarly to Shank3 knockdown (Arons et al., 2012; Wang et al., 2011). Therefore, we decided to investigate the effects of recNR1 IgG in primary rat hippocampal neurons expressing ASD-associated Shank3 mutations or with Shank3 knockdown to see if the treatment could produce compensatory effects against the deficits in excitatory synapse density. Hippocampal neurons were transfected to express short hairpin RNA (shRNA) to knock down endogenous Shank3 or one of three Shank3 mutants. R87C and R375C are point mutations identified as inherited variations in ASD patients while InsG is a de novo frameshift mutation that produces a premature STOP codon and a truncated protein (Durand et al., 2011).

We hypothesised that recNR1 IgG treatment may structurally normalise excitatory synapse expression in primary rat hippocampal neurons expressing ASD-associated Shank3 mutations or with shRNA-mediated knockdown to those overexpressing Shank3 WT.
5.2. Materials and methods

5.2.1. Plasmids

Plasmid constructs expressing Shank3 WT, shRNA directed against Shank3, and three Shank3 mutants with ASD-associated point mutations (R87C, R375C and InsG) (Figure 5.1) were obtained from Associate Professor Johanna M. Montgomery’s laboratory at University of Auckland (Arons et al., 2012). The pEGFP-C1 vector (Clontech) (Figure 5.2 A) was used for the cloning of all Shank3 expression constructs. Target sequences of Shank3 were used as described previously (Grabrucker et al., 2011; Roussignol et al., 2005). In brief, the three Shank3 point mutations were cloned into the pEGFP C-1 vector containing the full-length rat Shank3 WT cDNA (Figure 5.2 B) using site-directed mutagenesis based on the human mutations (R12C, R300C, and InsG) as described previously (Durand et al., 2011). Also, shRNA oligonucleotides were synthesised and subsequently cloned into pSUPER and pFUGW H1 with a pZOFF vector (Leal-Ortiz et al., 2008). All plasmid constructs used in this section contained a kanamycin resistance gene for bacterial selection.

![Figure 5.1 ASD-associated Shank3 mutations](image)

Schematic diagram showing the approximate location of the ASD-associated point mutations on the Shank3 protein.

5.2.2. Bacterial transformation

*E. coli* DH5α competent cells were removed from -80 °C storage and thawed on ice before 1 µg of plasmid DNA was added to the cells. The tube was mixed gently by tapping and incubated on ice for 30 min. Heat shock transformation was performed by incubating the cells at 37 °C for 5 min. Cells were then immediately returned on ice for 1 min. Next, 500 µL of LB was added to the cells and incubated at 37 °C for 30 min. Finally, 100 µL of transformation sample was spread onto LB agar plates containing 25 µg/mL kanamycin (Sigma, K-4000) and cultured overnight at 37 °C for up to 16 hours.
Figure 5.2 pEGFP-C1 vector and pEGFP-Shank3 plasmid construct

Plasmid maps of the pEGFP-C1 vector (A) and pEGFP-Shank3 after insertion of the full-length rat Shank3 WT cDNA (B). The position of the EGFP-tag is in the N-terminus of the Shank3 cDNA sequence.
5.2.3. Mini plasmid preparation

Mini plasmid preparations were performed using the High Pure Plasmid Isolation Kit (Roche, 11754785001) with slight modifications to its protocol. A single transformant colony was isolated from the LB agar plate and used to inoculate 10 mL of LB containing 25 µg/mL kanamycin in a 15 mL tube. The culture was grown overnight at 37 °C with shaking at 220 RPM. The cell suspension was centrifuged the next day at 8,000 x g, RT for 3 min. The supernatant was discarded and the pellet was gently resuspended with 200 µL of Suspension Buffer containing RNase A. The suspension was transferred into a 1.5 mL microfuge tube before 200 µL of Lysis Buffer was added and immediately mixed five times by gentle inversion. The tube was left to incubate for 5 min at RT before it was spun briefly using a small benchtop microfuge. Then, 200 µL of Binding Buffer was added and immediately mixed five times by inversion. After a 5 min incubation period on ice, the tube was centrifuged at 20,800 x g, 2 °C for 30 min. The supernatant was loaded into the reservoir of a High Pure Filter Tube, which was inserted into a collection tube and centrifuged at 17,000 x g, RT for 30 s. The flowthrough was discarded and 400 µL of Wash Buffer was added to the reservoir and centrifuged at 17,000 x g, RT for 30 s. The column was washed again with 250 µL of Wash Buffer. The Filter Tube was removed and inserted into a 1.5 mL microfuge tube for DNA recovery. Finally, 60 µL of Elution Buffer was added to the reservoir and centrifuged at 17,000 x g, RT for 30 s. DNA concentration was determined according to Section 5.2.3 before storage at -20 °C.

5.2.4. Determination of DNA concentration

DNA concentration was quantified using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific) and TE buffer as the blank. Since the absorbance maxima of nucleic acids and proteins are at 260 and 280 nm, respectively, the ratio between the absorbances at these two wavelengths (A260/280) was used for the assessment of DNA purity. A ratio of ~1.8 indicated high purity of DNA, while ratios lower than 1.8 indicated possible contamination by proteins or residual reagents.
5.2.5. Sequencing

Plasmid DNA were sequenced by Massey Genome Service, Massey University, Palmerston North, New Zealand. Primers were designed to sequence and detect each ASD-associated Shank3 point mutation (Table 5.1). Shank3 WT plasmid was also sequenced using all three primers to confirm the absence of point mutations. Samples were prepared for sequencing by mixing 300-600 ng of template DNA, 1 µL of 5 µM sequencing primer and PCR-grade water up to 20 µL in a 0.2 mL PCR tube. Sequencing results were analysed using the online Basic Local Alignment Search Tool (BLAST®, National Center for Biotechnology Information, www.ncbi.nlm.nih.gov). See the Appendix for full sequencing results.

Table 5.1 Primers used for sequencing Shank3 plasmids

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>R87C</td>
<td>Reverse sequencing primer 1 (R1)</td>
<td>5’-TCCTGTAAGAGCGCGCTTC-3’</td>
</tr>
<tr>
<td>R375C</td>
<td>Forward sequencing primer 1 (F1)</td>
<td>5’-AGATCCATCAGGCTGTCGC-3’</td>
</tr>
<tr>
<td>InsG</td>
<td>Forward sequencing primer 2 (F2)</td>
<td>5’-TGGATTCCAGTGCTGCTCG-3’</td>
</tr>
</tbody>
</table>

5.2.6. Maxi plasmid preparation

Maxi plasmid preparations were performed using the PureLink® HiPure Plasmid Maxiprep Kit (Invitrogen, K2100-07) with slight modifications to its protocol. A starter culture of 10 mL of LB containing 25 µg/mL kanamycin was inoculated with a single transformant colony. The culture was grown overnight at 37 °C with shaking at 220 RPM. The starter culture was added to 80 mL of LB containing 25 µg/mL kanamycin in a 1 L conical flask the following day and cultured overnight at 37 °C with shaking at 220 RPM. The overnight culture was divided into two 50 mL centrifuge tubes. Next, 30 mL of Equilibration Buffer (EQ1) was applied to the HiPure Maxi Column and allowed to drain. At the same time, the bacterial culture tubes were centrifuged at 7,000 x g, RT for 5 min to pellet cells. The supernatant was discarded and pellets were gently resuspended with 5 of mL Resuspension Buffer (R3)
containing RNase A. Cells were lysed by adding 5 mL of Lysis Buffer (L7) to each tube and mixed gently by inversion five times. Tubes were left to incubate at RT for 5 min. Next, 5 mL of Precipitation Buffer (N3) was added to each tube, which were mixed immediately by inversion. After the mixture became homogeneous from mixing, lysates were centrifuged at 7,000 x g, RT for 30 min. The supernatant was loaded onto the equilibrated HiPure Maxi Column and allowed to drain. The column was washed twice with 30 mL of Wash Buffer (W8) and the flowthrough was discarded. A sterile 50 mL centrifuge tube was used to collect the eluate after 15 mL of Elution Buffer (E4) was added to the column. The eluate was mixed well with 10.5 mL of isopropanol to precipitate the plasmid DNA and the tube was centrifuged at 7,000 x g, RT for 30 min. The supernatant was discarded before 0.4 mL of TE buffer was added to the DNA pellet and left for 30 min at RT for resuspension. DNA suspension was transferred to a 1.5 mL microfuge tube before 20 µL of 3 M NaAC and 1 mL of 100% ethanol were added. The tube was mixed well and centrifuged at 17,000 x g, RT for 3 min. The supernatant was discarded and 0.7 mL of 70% ethanol was added, followed by a final centrifugation at 17,000 x g, RT for 10 min. The supernatant was discarded and 250 µL of TE buffer was added. DNA concentration was determined according to Section 5.2.3 before storage at -20 °C.

5.2.7. Primary culture of rat hippocampal neurons

Primary rat hippocampal neuronal cultures were prepared and maintained as described in Section 3.2.1.

5.2.8. Calcium phosphate transfection

Primary hippocampal neurons were transfected on DIV 6 (Figure 5.3) with the following constructs: pEGFP-C1 (EGFP); pEGFP-Shank3 WT (WT); pEGFP-Shank3 shRNA (shRNA); pEGFP-Shank3 R87C (R87C); pEGFP-Shank3 R375C (R375C); pEGFP-Shank3 InsG (InsG). Before transfection, cells were refed by replacing 0.5 mL of culture medium with fresh complete NBM. Polystyrene centrifuge tubes (15 mL) (BD, 352095) were used for the preparation of transfection mixes. For each well of a 6-well plate, 60 µL of 2X HEPES-buffered saline (HBS) (274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 15 mM D-glucose, 42
mM HEPES, pH 7.05 - 7.15 with NaOH) was added to a 15 mL tube. In another 15 mL tube, 5 µg of plasmid DNA was added to 7.6 µL of 2 M CaCl₂, and MilliQ water to a final volume of 60 µL before the tube was gently mixed. The reagents were added in the following order: MilliQ water, CaCl₂, and plasmid DNA. The DNA-Ca²⁺ solution was added to the 2X HBS solution, 1/8 of the volume at a time in a dropwise manner, and mixed vigorously between each addition. The transfection mix was then incubated in the dark for 20-30 min. Towards the end of this incubation period, culture medium was removed and each well was replaced with 1 mL of pre-warmed NBM containing 25 µM D-APV (Sigma, A8054) and 10 µM CNQX (Sigma, C239) to prevent excitotoxicity from excessive calcium influx through NMDA and AMPA receptors. The original culture medium was transferred into a 50 mL centrifuge tube, and kept warm in the cell culture incubator. For each well, 120 µL of DNA-calcium phosphate suspension was applied to the coverslip in a dropwise manner and cells were returned to the cell culture incubator without mixing. The incubation time required for sufficient uptake of DNA-calcium phosphate precipitates differed for each plasmid construct used. EGFP and Shank3 shRNA were incubated for 20 min, Shank3 WT, R87C and R375C for 35 min, and Shank3 InsG for 45 min. Plates were examined for the presence of fine, homogenous DNA-calcium phosphate precipitates before transfection was terminated by thoroughly washing each well three times with 2 mL pre-warmed HBSS. The original culture medium was added back into the plates before cells were returned to the incubator.

Figure 5.3 Timeline of primary culture experiment where cells were transfected and treated with IgG
5.2.9. Quantification of synaptic protein expression following IgG treatment in transfected cells.

Transfected primary hippocampal neurons were treated with IgG on DIV 8 as described in Section 3.2.2 and fixed on DIV 15 with 4% (w/v) PFA. Immunocytochemistry was performed according to Section 3.2.3. Primary and secondary antibodies used are detailed in Table 5.2 and Table 5.3, respectively. Images were acquired and analysed as described in Sections 3.2.5.1 and 3.2.5.2, respectively. Imaging conditions for the ASD Shank3 experiment are detailed in Table 5.4. However, only synaptic puncta fluorescence intensities and densities were measured due to the expression of Shank3 proteins. A total of five individual cultures were used for this immunostaining experiment.

Table 5.2 List of primary antibodies used for the ASD Shank3 experiment

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Host species</th>
<th>Company</th>
<th>Catalogue no.</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>Anti-Homer</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-15321</td>
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<tr>
<td>Anti-MAP2A,2B</td>
<td>Mouse</td>
<td>Chemicon</td>
<td>mab378</td>
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<tr>
<td>Anti-VGLUT1</td>
<td>Guinea pig</td>
<td>Millipore</td>
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Table 5.3 List of secondary antibodies used for the ASD Shank3 experiment

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<th>Catalogue no.</th>
<th>Dilution</th>
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<td>Donkey</td>
<td>Abcam</td>
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<tr>
<td>Anti-mouse IgG</td>
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<td></td>
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<tr>
<td>Alexa Fluor 594</td>
<td>Donkey</td>
<td>Jackson ImmunoResearch</td>
<td>706-585-148</td>
<td>1:500</td>
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<tr>
<td>Anti-guinea pig IgG</td>
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<tr>
<td>Alexa Fluor 647</td>
<td>Donkey</td>
<td>Invitrogen</td>
<td>A31573</td>
<td>1:500</td>
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<tr>
<td>Anti-rabbit IgG</td>
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Table 5.4 Imaging conditions used for the ASD Shank3 experiment

Details of target proteins, fluorophores, filter blocks, exposure times, and the number of ND filters used for each immunostaining experiment.

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Fluorophore</th>
<th>Filter block</th>
<th>Exposure time (ms)</th>
<th>ND filter(s)</th>
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<td>MAP2</td>
<td>Alexa Fluor 405</td>
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<tr>
<td>Native GFP</td>
<td>-</td>
<td>GFP</td>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td>VGLUT1</td>
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<tr>
<td>Homer</td>
<td>Alexa Fluor 647</td>
<td>Cy5</td>
<td>600</td>
<td>1</td>
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5.2.9.1. Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 6. All data were presented as mean ± SEM. The Kolmogorov-Smirnov test for normality was used to determine if data sets were parametric. Bartlett’s test and Levene's test were used to test for equality of variances in parametric and non-parametric data, respectively. Statistical differences were determined by one-way ANOVA with comparison to Shank3 WT using Dunnett’s multiple comparisons test if assumptions of the parametric method were satisfied. Otherwise, the non-parametric Kruskal-Wallis test was used with comparison to Shank3 WT by Dunn’s multiple comparisons test. Statistical tests were performed separately for Luc IgG and recNR1 IgG treatment groups.
5.3. Results

5.3.1. recNR1 IgG normalised excitatory synapse density in hippocampal neurons transfected with Shank3 shRNA or Shank3 InsG

To investigate excitatory synapse expression in transfected neurons, double-labelling with VGLUT1 and Homer was performed. Primary hippocampal neurons were transfected with plasmids expressing EGFP, Shank3 WT, Shank shRNA or one of three Shank3 mutants, R87C, R375C and InsG on DIV 6. After protein expression was confirmed 48 hours later by visualisation of EGFP fluorescence, transfected hippocampal neurons were treated with either Luc IgG or recNR1 IgG. To ensure accurate dendritic length measurements in image analysis, neurons were labelled for MAP2 to visualise dendrites in addition to VGLUT1 and Homer (Figure 5.4).

Native EGFP fluorescence was distributed throughout the dendrites and axon of neurons transfected with pEGFP-C1 (Figure 5.4 A, B). A similar observation was made with hippocampal neurons expressing Shank3 shRNA (Figure 5.4 E, F). On the other hand, Shank3 expression following transfection, as determined by EGFP expression, was distinctly punctate along the dendrites for Shank3 WT (Figure 5.4 C, D), R87C (Figure 5.4 I, J) and R375C (Figure 5.4 K, L) but not InsG (Figure 5.4 G, H). Intensity of the native EGFP fluorescence in neurons expressing Shank3 InsG mutant was noticeably weaker than all other transfection groups and the expression pattern was very diffuse compared to other Shank3 groups. For image analysis, only synaptic puncta which colocalised with native EGFP expression in transfected neurons were examined. Fluorescence intensity values in each image were normalised to the EGFP Luc IgG treatment group of the same culture.

For Luc IgG treatment, synaptic VGLUT1 fluorescence intensity was significantly higher in the Shank3 WT group (1.23 ± 0.06) compared to EGFP (one-way ANOVA, $F = 5.265$, $p < 0.001$; Dunnett’s post-hoc: 1.00 ± 0.03, $p = 0.002$) and shRNA (1.07 ± 0.03, $p = 0.044$) (Figure 5.5 A). Neurons expressing Shank3 InsG mutant displayed significantly lower total VGLUT1 fluorescence intensity (1.06 ± 0.04, $p = 0.027$) when compared to those overexpressing Shank3 WT. No significant differences were detected between Shank3 WT neurons and both the R87C (1.25 ± 0.06, $p = 0.998$) and R375C (1.17 ± 0.04, $p = 0.829$) mutants. Although one-way ANOVA showed an overall significance in synaptic VGLUT1 fluorescence intensity between recNR1 IgG groups (one-way ANOVA, $F = 3.634$, $p = 0.019$; Dunnett’s post-hoc: 1.06 ± 0.04, $p = 0.027$) compared to EGFP, these differences were not significant compared to Shank3 WT neurons (1.23 ± 0.06; $p = 0.002$).
0.004), no significant differences were found by Dunnett’s post-hoc test when comparisons were made with WT. Therefore, synaptic VGLUT1 fluorescence intensity was comparable between all transfection groups which received recNR1 IgG treatment.

Synaptic Homer fluorescence intensity in Luc IgG-treated transfected neurons was found to be significantly increased in the Shank3 WT group (1.42 ± 0.07) compared to both EGFP (one-way ANOVA, $F = 15.73, p < 0.0001$; Dunnett’s post hoc: $1.00 ± 0.03, p = 0.0001$) and shRNA groups (1.11 ± 0.05, $p = 0.007$) (Figure 5.5 B). Moreover, a significant decrease in synaptic Homer fluorescence intensity was detected in neurons expressing Shank3 InsG (1.13 ± 0.05, $p = 0.012$) but not R87C (1.66 ± 0.10, $p = 0.066$) or R375C (1.56 ± 0.08, $p = 0.463$) when comparisons were made with Shank3 WT. For recNR1 IgG treatment, synaptic Homer fluorescence intensity was significantly higher in neurons transfected with Shank3 WT (1.71 ± 0.1) than those with EGFP (one-way ANOVA, $F = 15.73, p < 0.0001$; Dunnett's post-hoc: 1.13 ± 0.05, $p = 0.0001$) or shRNA (1.12 ± 0.04, $p = 0.007$). Furthermore, synaptic Homer fluorescence intensity was significantly reduced in neurons expressing Shank3 InsG (1.157 ± 0.05, $p < 0.0001$) but not R87C (1.61 ± 0.10, $p = 0.825$) or R375C (1.64 ± 0.09, $p = 0.946$) when compared to Shank3 WT overexpression.

The number of VGLUT1/Homer colocalised puncta on transfected cells were analysed to determine excitatory synapse numbers. For Luc IgG treatment groups, overexpression of Shank3 WT significantly increased excitatory synapse density (4.70 ± 0.35) compared to EGFP (one-way ANOVA, $F = 3.615, p = 0.004$; Dunnett's post-hoc: 3.40 ± 0.30, $p = 0.028$) and knockdown of Shank3 by shRNA (3.03 ± 0.27, $p = 0.003$) (Figure 5.5 C). However, the number of excitatory synapses was significantly reduced when cells were transfected with Shank3 InsG (3.33 ± 0.32, $p = 0.018$) in comparison to the WT group. There were no differences with the Shank3 R87C (4.09 ± 0.31, $p = 0.574$) or R375C (4.15 ± 0.42, $p = 0.664$) mutants. On the other hand, excitatory synapse density was comparable between all transfection groups treated with recNR1 IgG (one-way ANOVA, $F = 3.800, p = 0.003$; Dunnett's post-hoc: 4.01 ± 0.37, $p = 0.126$).

Compared to Luc IgG, the absence of significant differences in measures of synaptic VGLUT1 fluorescence intensity and excitatory synapse density following recNR1 IgG treatment demonstrated a potential therapeutic effect in neurons expressing Shank3 shRNA or the Shank3 InsG mutant.
Figure 5.4 VGLUT1 and Homer immunostaining in primary hippocampal neurons expressing ASD-associated Shank3 mutations following IgG treatment

Representative dendritic images of transfected hippocampal neurons labelled for MAP2 (Alexa Fluor 405, gray), VGLUT1 (Alexa Fluor 594, red) and Homer (Alexa Fluor 647, blue) following IgG treatment. Cells were transfected to express EGFP (A, B), Shank3 WT (C, D), shRNA (E, F), InsG (G, H), R87C (I, J) or R375C (K, L) before they were treated with Luc IgG or recNR1 IgG at 7.0 µg/mL. Native EGFP expression (green) was observed in all positively transfected cells. Boxed regions in merged images of native EGFP fluorescence, VGLUT1 and Homer immunostaining on the left are enlarged on the right and shown as individual channels and merged images. VGLUT1/Homer colocalised puncta are purple or white in colour (arrows) when there is strong colocalisation with native EGFP fluorescence.

Scale bars, 10 µm (left) and 5 µm (right).
Figure 5.4 (Part 1)

A  EGFP Luc

B  EGFP recNR1

C  Shank3 WT Luc

D  Shank3 WT recNR1
Figure 5.4 (Part 2)

E  Shank3 shRNA Luc

F  Shank3 shRNA recNR1

G  Shank3 InsG Luc

H  Shank3 InsG recNR1
Figure 5.4 (Part 3)

I. Shank3 R87C Luc

J. Shank3 R87C recNR1

K. Shank3 R375C Luc

L. Shank3 R375C recNR1
Figure 5.5 Effect of recNR1 IgG treatment on excitatory glutamatergic synapses in primary hippocampal neurons expressing ASD-associated Shank3 mutations.

Cells were imaged from five individual cultures and the same number of hippocampal neurons were imaged for each treatment group ($n = 22$). Images of hippocampal neurons transfected with EGFP, Shank3 WT, shRNA, InsG, R87C or R375C, and treated with Luc IgG or recNR1 IgG at 7.0 µg/mL were analysed to assess excitatory synaptic protein expression. Fluorescence intensity values were normalised to the EGFP Luc IgG group. Image analysis showed that synaptic VGLUT1 fluorescence intensity was significantly lower in EGFP-, shRNA- and InsG-expressing neuron compared to WT-expressing neurons following Luc IgG treatment but no differences were found with recNR1 IgG for any of the transfection groups (A). Synaptic Homer fluorescence intensity was significantly lower in EGFP-, shRNA- and InsG-expressing neurons compared to WT-expressing neurons in both Luc IgG and recNR1 IgG treatment groups (B). The number of excitatory synapses was significantly lower in EGFP-, shRNA- and InsG-expressing neurons compared to WT-expressing neurons following Luc IgG treatment but no differences were detected in recNR1 IgG-treated transfected neurons (C).

Data represent mean ± SEM.

*p < 0.05, **p < 0.01, ***p < 0.001, Luc IgG treatment groups against Shank3 WT Luc. 
****p < 0.0001, recNR1 IgG treatment groups against Shank3 WT recNR1.
Figure 5.5

A

Synaptic VGLUT1 fluorescence intensity

B

Synaptic Homer fluorescence intensity

C

Excitatory synapse density (number/10 μm)

Legend:
- Luc
- recNR1
5.4. Discussion

In this chapter, recNR1 IgG treatment was applied to primary rat hippocampal neurons expressing ASD-associated Shank3 mutations or shRNA for Shank3 knockdown in order to assess its therapeutic potential in a cell model of ASD. Deficits in excitatory synaptic protein expression and function, as compared to Shank3 WT, have been associated with the Shank3 point mutations and Shank3 knockdown in cultured neurons and rodent models (Arons et al., 2012; Bozdagi et al., 2010; Kouser et al., 2013; Peça et al., 2011; Wang et al., 2011). On this basis, we decided to investigate if recNR1 IgG treatment could normalise excitatory synapse density to compensate for the reduction observed with Shank3 knockdown or the inability of Shank3 mutants to increase expression. Unexpectedly, expression of Shank3 R87C or R375C increased synaptic VGLUT1 and Homer expression levels to around the same extent as Shank3 WT following Luc IgG or recNR1 IgG treatment. There were also no differences in excitatory synapse density in the neurons expressing these two Shank3 mutants compared to WT when comparisons were made within the same IgG treatment. On the other hand, synaptic VGLUT1 and Homer expression levels as well as excitatory synapse density were significantly and consistently lower in neurons expressing Shank3 shRNA or InsG compared to WT following Luc IgG treatment. However, recNR1 IgG treatment abolished the significant differences in VGLUT1 expression and excitatory density numbers but not Homer expression in these same groups, suggesting a partial normalisation of excitatory synaptic structure.

5.4.1. recNR1 IgG exhibited potential therapeutic effect in neurons transfected with Shank3 shRNA or Shank3 InsG

Following IgG treatment and immunocytochemistry against VGLUT1 and Homer, positively transfected hippocampal neurons were identified by native EGFP fluorescence. In concurrence with the study by Arons et al. (2012) who had used the same plasmids, robust levels of postsynaptic Shank3 expression was observed in hippocampal neurons expressing Shank3 WT, R87C and R375C. On the other hand, expression of the Shank3 InsG frameshift mutant was of a diffuse somatodendritic pattern with no punctate localisation, which could be explained by the C-terminal truncation in the protein and resultant loss of the synaptic targeting SAM domain (Durand et al., 2011; Grabrucker et al., 2011). As expected, overexpression of Shank3 WT in hippocampal neurons led to increases in synaptic VGLUT1 and Homer expressions as well as synaptic density (Arons et al., 2012; Roussignol et al.,
Coordinated changes in the presynaptic terminal are thought to be the result of transsynaptic signalling mediated by the interaction of the neurexin-neuroligin cell adhesion molecule complex, which may be impaired in neurons expressing Shank3 mutants (Arons et al., 2012). To our surprise and in disagreement with Arons et al. (2012), neurons expressing Shank3 mutants R87C or R375C displayed elevated levels of synaptic VGLUT1 and Homer expression on par with those expressing WT, regardless of the IgG treatment. Although the excitatory synapse densities for these two mutants were slightly lower with Luc IgG treatment, they were not significantly different from Shank3 WT, which made it difficult to assess the effects of recNR1 IgG. On the other hand, shRNA-mediated knockdown of Shank3 or the expression of Shank3 InsG mutant in hippocampal neurons with Luc IgG treatment both resulted in reductions in synaptic VGLUT1 and Homer expression levels as well as decreased synaptic density compared to WT. Unlike Shank3 R87C and R375C, these findings are in agreement with past studies (Arons et al., 2012; Roussignol et al., 2005; Verpelli et al., 2011). Treatment with recNR1 IgG in neurons expressing Shank3 shRNA or InsG eliminated the significant differences in synaptic VGLUT1 expression and excitatory synapse density but not Homer expression observed with Luc IgG, indicating a partial normalisation and structural restoration. It is uncertain why we have obtained conflicting data for the Shank3 R87C and R375C mutations. There were a few differences between this study and that of Arons et al. (2012). Arons et al. carried out transfections on primary hippocampal neurons on DIV 9 instead of DIV 6, which may have resulted in slightly different phenotypes. In addition, they had used confocal microscopy for image acquisition instead of wide field epifluorescence, which could have potentially affected the image analysis process due to higher resolving power. Needless to say, further investigation is required to verify and ascertain the reproducibility of our findings. Since there is evidence of synaptic transmission and plasticity impairments in neurons with Shank3 dysfunction, it may of interest to characterise synaptic glutamate receptor expression and signalling following recNR1 IgG in such cells.
Chapter 6.
Summary and conclusions
6.1. Summary

Our laboratory has described various potential therapeutic effects of anti-GluN1 IgG in previous studies. The administration of a novel genetic vaccine in rats generated high titres of anti-GluN1 IgG which offered protection from kainate-induced seizures and reduced stroke lesion sizes in the animals (During et al., 2000). Furthermore, cognitive enhancing properties have been demonstrated in both immunised and passively transferred animals. Anti-GluN1 IgG with high reactivity against the glycine-binding site was detected in the CSF of rats immunised with recombinant recNR1 protein which displayed improved learning and memory functions in various behavioural tests (Chen, 2010). Mice passively transferred with anti-GluN1 IgG purified from the sera of rats from the protein immunisation study exhibited similar behavioural improvements (Chen, 2015).

The overall aim of this present study was to elucidate the underlying mechanisms of these therapeutic effects by examining changes in protein expression and excitatory synaptic transmission in glutamatergic synapses following anti-GluN1 IgG treatment in primary rat hippocampal neurons. However, antibodies directed against the GluN1 subunit, specifically to its N-terminal domain, have also been associated with a severe neurological disorder, anti-NMDA receptor encephalitis (Dalmau et al., 2008; Gleichman et al., 2012; Hughes et al., 2010). Taking into consideration the differences in the epitopes recognised by these anti-GluN1 antibodies, we also aimed to identify possible epitope-dependent effects by utilising anti-GluN1 IgG targeted against different regions of the extracellular domains in our investigations. Lastly, we expanded on the therapeutic application of anti-GluN1 IgG in a pilot study by examining changes in the excitatory synapses of cultured neurons expressing ASD-associated Shank3 mutations or with shRNA-mediated Shank3 knockdown.

In Chapter 2, we described the production of recombinant proteins, their use in the immunisation of rats, and the successful generation of anti-GluN1 IgG. Two types of recombinant GluN1 proteins, recNR1 and NR1extra2 were produced at the initial stage of the study with the former comprising both extracellular domains and the latter comprising the extracellular M3-M4 loop. Protein preparations of reasonably high purity were purified from bacterial lysates. Immunisation and blood collection were undertaken in rats where most animals successfully developed IgG against their respective protein immunogen but with variable immune response. Most unexpected was the finding that the level of specific NR1extra2 IgG in immunised animals gradually declined in the latter half of the
immunisation period after peaking. As expected, epitope mapping of rat sera against extracellular GluN1 peptides demonstrated high polyclonal reactivity of recNR1 IgG to the glycine-binding site (Chen, 2010; During et al., 2000) and low polyclonal reactivity of NR1extra2 IgG to the M3-M4 region. These epitope mapping profiles suggest that anti-GluN1 IgG may potentially compete with the endogenous ligand glycine at its GluN1 binding site. IgG preparations of high purity for each target protein were very effectively isolated from pooled rat sera and confirmed to have retained binding capacity against their immunogens. This was followed by the successful preabsorption of recNR1 IgG with recNR1 protein preparation, which removed specific IgG from solution and attenuated its binding capacity to a negligible level. Future studies involving NR1extra2 protein immunisation should prioritise the optimisation of its immunogenicity, possibly through coupling to a potent carrier or the arrangement of epitopes in tandem repeats (Hsu et al., 2000; Kim et al., 2005; Moulton et al., 2002).

In Chapter 3, the effects of anti-GluN1 IgG treatment on protein expression in the glutamatergic excitatory synapses of primary hippocampal neurons were characterised. We found no indications of neurotoxicity in cultured neurons following treatment with anti-GluN1 IgG, which agrees with a previous animal study from our laboratory (Chen, 2015) but opposes the findings of anti-GluN1 antibody-mediated toxicity observed in anti-NMDA receptor encephalitis (Manto et al., 2010; Sansing et al., 2007). In the first screen to detect anti-GluN1 IgG-mediated synaptic changes, we found a concentration-dependent increase in PSD-95 puncta density as a result of recNR1 IgG treatment, which was confirmed to be a specific effect through the use of preabsorbed recNR1 IgG. Subsequent experiments showed that recNR1 IgG also increased excitatory synapse density and the number of NMDAR-containing synapses in a concentration-dependent manner after 7 days of treatment without any change in the number of AMPAR-containing synapses. Together, these results indicate a possible increase in morphologically silent synapses in hippocampal neurons following recNR1 IgG treatment which, if true, could be an explanation for its cognitive enhancing effects since the unsilencing of such synapses by recruitment of functional AMPA receptors is thought to underlie the mechanisms of LTP (Isaac et al., 1995; Kerchner & Nicoll, 2008; Liao et al., 1995). We failed to identify any changes in synaptic protein expression following treatment with NR1extra2 IgG, which may be attributed to low amounts of specific IgG. As a consequence, we were unable to discriminate epitope-dependent effects between recNR1 IgG and NR1extra2 IgG. However, our findings thus far suggest contrasting effects on synaptic
protein expression between our glycine-binding site-targeting recNR1 IgG and the N-terminal-targeting antibodies from anti-NMDA receptor encephalitis patients (Dalmau et al., 2008; Hughes et al., 2010).

In Chapter 4, we extended our characterisation of the effects of anti-GluN1 IgG treatment in primary hippocampal neurons by electrophysiological methods to determine if changes in glutamate receptor expression were reflected in alterations of excitatory synaptic transmission. The allosteric N-terminal-targeting NR1.NTD IgG was included to continue our exploration of possible epitope-dependent effects. Treatment with recNR1 IgG did not affect basal excitatory synaptic activity or AMPAR-mediated synaptic response but did increase evoked NMDAR-mediated synaptic response. This finding is in concert with the immunocytochemical data from Chapter 3, which showed an increase in NMDAR- but not AMPAR-containing synapses following recNR1 IgG treatment, and confirms the functionality of those receptors. The finding also adds to the support that recNR1 IgG mediates an increase in the number of functionally silent synapses in hippocampal neurons and opens up new avenues for further characterisation of the effect, namely changes in synaptic plasticity (Isaac et al., 1995; Liao et al., 1995). This could be carried out by chemically or synaptically inducing NMDAR-dependent LTP and LTD in cultured neurons during paired cell recordings (Li et al., 2011; Montgomery et al., 2005). On the other hand, we observed conflicting immunocytochemical and electrophysiological data in hippocampal neurons treated with NR1.NTD IgG. Presynaptic deficits were suggested by a decrease in mEPSC frequency following treatment with NR1.NTD IgG but we did not observed a coordinated reduction in the number of excitatory synapses or VGLUT1 expression (Dobrunz & Stevens, 1997; Liao et al., 2001). The reduced frequency may have also resulted from a change in vesicle release probability (Prange & Murphy, 1999) but the low failure rate of evoked synaptic transmission, which is frequently used as an indicator of release probability, suggested otherwise (Feldmeyer & Sakmann, 2000; Markram et al., 1997). Therefore, further investigation is needed to isolate the exact cause. Possible experiments to clarify presynaptic changes include examining paired pulse ratios or the progressive block of NMDAR-mediated synapse response by MK-801 upon glutamate release (Dobrunz & Stevens, 1997; Hessler et al., 1993; Rosenmund et al., 1993). In addition, NR1.NTD IgG treatment did not alter NMDA receptor expression but resulted in an overall augmentation of synaptic AMPA receptor expression in that both protein expression level and the number of AMPAR-containing synapses were increased. Surprisingly, this was not associated with a corresponding increase
in AMPAR-mediated synaptic response as we had expected. It is possible that rapid AMPA receptor trafficking had taken place prior to or during electrophysiological recordings after cells were removed from their IgG-rich culture environment (Carroll et al., 1999; Lin et al., 2000). Although the dissociation rate of the antibody-receptor complex is unknown, this could be an indication of a reversible anti-GluN1 IgG-mediated effect, much like the changes in NMDA receptor expression in anti-NMDA receptor encephalitis (Dalmau et al., 2008; Hughes et al., 2010). While still targeting the GluN1 N-terminal domain, NR1.NTD IgG does not exhibit reactivity to the N368/G369 amino acid residues pertinent for immunoreactivity in anti-NMDA receptor encephalitis, which suggests that antibodies binding to the same region may elicit very different responses depending on the epitope recognised (Gleichman et al., 2012). We were unable to ascertain the pharmacological actions of anti-GluN1 IgG purely by comparing immunocytochemical and electrophysiological data with past studies of NMDA receptor agonists and antagonists. This was partly due to the lack of information on whether antibodies remained bound to surface receptors during recordings, which would inevitably affect the interpretation of our results. Anti-GluN1 IgG may behave completely differently to conventional pharmacological modulators of NMDA receptor activity and thus warrants further investigation into pharmacological characterisation.

Finally in Chapter 5, we applied recNR1 IgG to primary hippocampal neurons expressing ASD-associated Shank3 mutations or with Shank3 knockdown in a pilot study to determine whether the treatment could rescue deficits in excitatory synapse density linked to Shank3 dysfunction. Despite being a heterogeneous group of disorders, cognitive impairment is a common co-morbidity in individuals with ASD (Bauman & Kemper, 2005). Furthermore, treatment with NMDA receptor partial agonist D-cycloserine or non-competitive antagonist memantine has demonstrated efficacy in reducing certain ASD symptoms (Chez et al., 2007; King et al., 2001; Posey et al., 2004). Thus, ASD may be a candidate disorder suitable for investigating the therapeutic potential of recNR1 IgG treatment. Given that recNR1 IgG increases excitatory synapse density, we decided to test its effects in hippocampal neurons with Shank3 dysfunction where there is evidence of deficits in excitatory synaptic protein expression and function (Arons et al., 2012; Kouser et al., 2013). Overexpression of Shank3 WT in hippocampal neurons increased the expression of VGLUT1 and Homer as well as excitatory synaptic protein expression and density (Arons et al., 2012), which prevented the
assessment of recNR1 IgG-mediated effects. However, recNR1 IgG treatment led to a partial structural normalisation of excitatory synapses in neurons expressing the Shank3 InsG mutant or Shank3 shRNA for knockdown where it restored synaptic VGLUT1 expression and excitatory synapse density to levels similar to Shank3 WT neurons. Although the mechanisms for pathogenesis are unknown, ASD is thought to arise from disruptions in the careful balance of excitatory and inhibitory synaptic transmission (Gogolla et al., 2009). Therefore, recNR1 IgG may potentially have a therapeutic application in ASD if it is able to modulate excitatory synapse transmission.

6.2. Limitations

There are a number of limitations to our study, in addition to the ones described in previous chapters. Firstly, our anti-GluN1 IgG preparations were polyclonal and reacted to a wide range of epitopes along the extracellular domains of GluN1, only restricted by the recombinant protein immunogen used for their generation. Although we sought to identify epitope-dependent effects, we could not isolate the efficacious antibodies and exact epitopes required for the changes induced by recNR1 IgG and NR1.NTD IgG treatments. It may also be possible that the effects we observed in this study were synergistic effects of antibodies with different epitopes working in concert. In addition, we had no control over the immunogenicity of individual epitopes within recombinant proteins in rats. A notable example was the lack of reactivity observed with NR1.NTD sera against peptide #37 which contained the N368/G369 residues associated with anti-NMDA receptor encephalitis (Gleichman et al., 2012).

Secondly, anti-GluN1 IgG treatment was applied to primary hippocampal neurons when they were still in development, as evidenced by the time course experiments. Cultured neurons typically have more silent synapses early in development, which progressively gain AMPA receptor expression (Liao et al., 1999). There is also a change in protein distribution during the formation and maturation of dendritic spines, which are thought to increase in number and size during LTP (Papa et al., 1995; Reimers et al., 2011; Yuste & Bonhoeffer, 2001). Therefore, the effects we observed in this study could potentially be age-dependent. Since the previous immunisation studies by our laboratory were undertaken in adult animals, it would
be important to determine whether anti-GluN1 IgG-mediated changes in the excitatory synapse can persist even with increased age of culture.

Lastly, the effects of anti-GluN1 IgG treatment were tested under basal conditions, which may not be the most suitable approach for identifying the underlying mechanisms of its neuroprotective properties. Such effects may only be revealed in hippocampal neurons upon the introduction of an insult such as hypoxia or excitotoxic levels of glutamate.

6.3. Future directions

As with most pharmacological therapies, a reversible drug effect is desirable in cases of incompatibility and adverse reactions. It would be important to investigate the reversibility of anti-GluN1 IgG-mediated changes in the hippocampal excitatory synapse in a manner similar to studies on anti-NMDA receptor encephalitis patient antibodies (Dalmau et al., 2008; Hughes et al., 2010). While hippocampal neurons were the focus of this study, NMDA receptors are expressed in almost all the neurons of the CNS (Monyer et al., 1994; Petralia et al., 1994). Given that the GluN1 subunit is ubiquitously expressed in all NMDA receptors (Moriyoshi et al., 1991), treatment with anti-GluN1 IgG in animals can essentially result in a global targeting of neurons in all brain regions. Therefore, the improvements in behavioural phenotype of animals observed in previous studies may be due to concerted action of anti-GluN1 IgG in different brain regions. There is growing evidence indicating gender differences in memory formation and synaptic plasticity, as well as the structural and signalling components underlying their mechanisms which include NMDA receptors (Ho & Lo, 1993; Wang et al., 2015). Contextual learning through fear conditioning and NMDAR-dependent LTP has been shown to be lower in both pre-pubescent and adult female rats when compared to males, suggesting limited activation of NMDA receptors in female animals (Maren et al., 1994). However, hippocampal NMDAR-dependent LTP in adult male rats declines with age while it remains intact in female rats (Monfort & Felipo, 2007). The decline in LTP is correlated with reductions in the hippocampal expressions of both GluN1 and GluN2A subunits in male animals. In addition, modulation of NMDA receptors in male and female animals may elicit very different, even opposite responses in synaptic plasticity (Shors et al., 2004). Gender differences in NMDA receptor expression and function are less characterised in vitro in the context of dissociated neuronal cultures and may be a factor of
consideration for future studies. Furthermore, NMDA receptors are known to be expressed at synaptic and extrasynaptic sites within individual neurons, and their location has been implicated in pro-death and pro-survival pathways, respectively (Hardingham & Bading, 2010; Hardingham et al., 2002). Interestingly, synaptic and extrasynaptic NMDA receptors differ in their GluN2 subunit content and exhibit preferential affinity for different endogenous co-agonists, D-serine and glycine, respectively, even though they all contain the GluN1 subunit (Papouin et al., 2012). This may lead to differential interactions between anti-GluN1 IgG and the glycine-binding sites of receptors in the two locations. Therefore, it would be interesting to dissect and compare synaptic and extrasynaptic NMDAR-mediated responses in hippocampal neurons following anti-GluN1 IgG treatment. Finally, NMDA receptors are also expressed presynaptically in the glutamatergic axon terminals in various parts of the brain including the hippocampus (Aoki et al., 1997; Jourdain et al., 2007). Presynaptic NMDA receptors have been shown to modulate synaptic efficacy and neurotransmitter release but the mechanism for their activation remains largely uncharacterised (Bouvier et al., 2015; Brasier & Feldman, 2008; Duguid & Smart, 2004). Future studies will need to address the aforementioned questions relating to the effects of anti-GluN1 IgG on NMDA receptors of different localisation.

### 6.4. Concluding remarks

This study aimed to elucidate the cellular mechanisms underlying the neuroprotective and cognitive enhancing properties of anti-GluN1 IgG previously observed in animal studies conducted by our laboratory. In support of the initial hypothesis, treatment with IgG targeted against different extracellular regions of the NMDA receptor subunit GluN1 resulted in divergent changes in synaptic glutamate receptor expression and function in primary rat hippocampal neurons. Although the pharmacological actions of anti-GluN1 IgG remain unclear, the main findings of this study suggest an increased presence of silent synapses on hippocampal neurons treated with recNR1 IgG, which could be implicated in plasticity changes relating to improvements in learning and memory. There is potential for therapeutic application of recNR1 IgG as a cognitive enhancer but further characterisation is warranted to evaluate its global effect on the CNS.
## Appendix

### Table A.1 Buffers and solutions for recombinant protein purification

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reagent</th>
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<tbody>
<tr>
<td>Bacterial lysis buffer</td>
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<tr>
<td></td>
<td>50 mM KH$_2$PO$_4$</td>
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<tr>
<td></td>
<td>pH 8.0</td>
</tr>
<tr>
<td>Denaturing IMAC Wash Buffer #1</td>
<td>300 mM KCl</td>
</tr>
<tr>
<td></td>
<td>50 mM KH$_2$PO$_4$</td>
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<tr>
<td></td>
<td>5 mM Imidazole</td>
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<tr>
<td></td>
<td>6 M Urea</td>
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<tr>
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<tr>
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<tr>
<td></td>
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<tr>
<td></td>
<td>10 mM Imidazole</td>
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<td></td>
<td>6 M Urea</td>
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<tr>
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<tr>
<td>Denaturing IMAC Elution Buffer</td>
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<td>Cleaning solution 2</td>
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<tr>
<td></td>
<td>pH 4.5 with Acetic acid</td>
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<td>Storage solution</td>
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<td>1.5 mM KCl</td>
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<td>pH 7.4</td>
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Note for wash buffers containing Urea: Make up to ~750 mL, adjust pH to 8.0 with 2.5 M NaOH (~18 mL) and add MilliQ water to 1 L.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Reagent</th>
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</thead>
</table>
| 2X reducing sample buffer | 125 mM Tris-HCl, pH 6.8  
10% (w/v) SDS  
20% (v/v) Glycerol  
0.02% (w/v) Bromophenol blue  
5% (v/v) β-mercaptoethanol |
| 12% acrylamide running gel | 375 mM Tris-HCl, pH 8.8  
0.1% (w/v) SDS  
0.1% (w/v) APS  
12% (w/v) Acrylamide  
0.04% (v/v) TEMED |
| Acrylamide stacking gel | 125 mM Tris-HCl, pH 6.7  
0.1% (w/v) SDS  
0.1% (w/v) APS  
3.75% (w/v) Acrylamide  
0.1% (v/v) TEMED |
| SDS-PAGE electrophoresis tank buffer | 25 mM Tris  
192 mM Glycine  
0.1% (w/v) SDS |
| Fixative | 40% (v/v) Methanol  
10% (v/v) Acetic acid |
| Coomassie blue staining solution | 0.05% (w/v) Coomassie Brilliant Blue R-250  
(Bio-Rad, #161-0400)  
50% (v/v) Methanol  
10% (v/v) Acetic acid |
| Destaining solution | 5% (v/v) Methanol  
7% (v/v) Acetic acid |
| Transfer buffer | 48 mM Tris  
39 mM Glycine  
20% (v/v) Methanol  
0.0375% (w/v) SDS |
| Tris-buffered saline + Tween 20 (TBS-T) | 20 mM Tris  
500 mM NaCl  
pH 7.5  
0.1% (v/v) Tween 20 |
| Tissue lysis buffer | 50 mM Tris-HCl, pH 7.5  
2 mM EDTA  
0.05% (v/v) Triton X-100 |
### Table A.3 Buffers and solutions for electrophysiology

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<tr>
<td></td>
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<tr>
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<td></td>
<td>26 mM NaHCO(_3)</td>
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<tr>
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<td>11 mM Glucose</td>
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<tr>
<td>Potassium gluconate internal solution</td>
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<tr>
<td></td>
<td>40 mM HEPES</td>
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<tr>
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<td>5 mM MgCl(_2)</td>
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<tr>
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<td>2 mM NaATP</td>
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<td></td>
<td>0.3 mM NaGTP</td>
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<td></td>
<td>pH 7.2</td>
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<td>298 mOsm</td>
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<tr>
<td>Caesium gluconate internal solution</td>
<td>120 mM Cs Gluconate</td>
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<td>40 mM HEPES</td>
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<td>5 mM MgCl(_2)</td>
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### Table A.4 Buffers and solutions for calcium phosphate transfection

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<td>pH 7.05 - 7.15 with NaOH</td>
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<td>Calcium chloride solution</td>
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Figure A.1 Western blot secondary antibody only controls

Recombinant Luc, recNR1 and NR1extra2 (A) as well as NR1.NTD (B) protein preparations were loaded at 1 µg per lane and probed with HRP-conjugated anti-rabbit, anti-mouse or anti-rat secondary antibodies only and exposed for 10 s, which was beyond the maximum length of time used to develop any blot shown in this thesis. Rat hippocampal brain lysate was loaded at 20 µg per lane and probed with HRP-conjugated anti-rat or anti-mouse secondary antibodies and exposed for 10 s (C).
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Table A.5 GluN1 peptides synthesised by Mimotopes
Table A.6 Image analysis settings for assessing immunofluorescent punctate staining using ImageJ Software.

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<th>Max. display value</th>
<th>Min. threshold</th>
<th>Particle size (pixel²)</th>
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Figure A.2 PSD-95 immunocytochemistry fluorescence bleed-through test

MAP2 immunoreactivity was detected with FITC filter under its imaging condition (A) but not with Rhodamine filter under the imaging condition of PSD-95 (B). PSD-95 immunoreactivity was detected with Rhodamine filter under its imaging condition (D) but not with FITC filter under the imaging condition of MAP2 (C).

Scale bar, 20 µm.
Figure A.3 VGLUT1/Homer immunocytochemistry fluorescence bleed-through test

MAP2 immunoreactivity was detected with FITC filter under its imaging condition (A) but not with Rhodamine filter under the imaging condition of VGLUT1 (B) or with Cy5 filter under the imaging condition of Homer (C). VGLUT1 immunoreactivity was detected with Rhodamine filter under its imaging condition (E) but not with FITC filter under the imaging condition of MAP2 (D) or with Cy5 filter under the imaging condition of Homer (F). Homer immunoreactivity was detected with Cy5 filter under its imaging condition (I) but not with FITC filter under the imaging condition of MAP2 (G) or with Rhodamine filter under the imaging condition of VGLUT1 (H).

Scale bar, 20 µm.
**Figure A.4 VGLUT1/GluN1 immunocytochemistry fluorescence bleed-through test**

GluN1 immunoreactivity was detected with GFP filter under its imaging condition (A) but not with Rhodamine filter under the imaging condition of VGLUT1 (B) or with Cy5 filter under the imaging condition of MAP2 (C). VGLUT1 immunoreactivity was detected with Rhodamine filter under its imaging condition (E) but not with GFP filter under the imaging condition of GluN1 (D) or with Cy5 filter under the imaging condition of MAP2 (F). MAP2 immunoreactivity was detected with Cy5 filter under its imaging condition (I) but not with GFP filter under the imaging condition of GluN1 (G) or with Rhodamine filter under the imaging condition of VGLUT1 (H).

Scale bar, 20 µm.
Figure A.5 VGLUT1/GluA1 immunocytochemistry fluorescence bleed-through test

GluA1 immunoreactivity was detected with GFP filter under its imaging condition (A) but not with Rhodamine filter under the imaging condition of VGLUT1 (B) or with Cy5 filter under the imaging condition of MAP2 (C). VGLUT1 immunoreactivity was detected with Rhodamine filter under its imaging condition (E) but not with GFP filter under the imaging condition of GluA1 (D) or with Cy5 filter under the imaging condition of MAP2 (F). MAP2 immunoreactivity was detected with Cy5 filter under its imaging condition (I) but not with GFP filter under the imaging condition of GluA1 (G) or with Rhodamine filter under the imaging condition of VGLUT1 (H).

Scale bar, 20 µm.
Sequencing results for ASD Shank3 plasmids

Sequencing pEGFP-Shank3 R87C with R1 primer (A) and pEGFP-Shank3 R375C with F1 primer (B) confirmed the presence of their respective missense mutation. Sequencing pEGFP-Shank3 InsG with F2 primer confirmed the presence of a single guanine nucleotide insertion mutation, which caused a frameshift and a premature STOP codon (C). Sequencing pEGFP-Shank3 WT with R1 (D), F1 (E) and F2 primers (F) confirmed the absence of the three ASD-associated point mutations R87C, R375C and InsG.
Figure A.6 B

**pEGFP-Shank3 R375C with F1 primer**

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Figure A.6 C

pEGFP-Shank3 InsG with F2 primer

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Sbjct 73 CACGTCTCTTGCAAGCCAGCCTGGCCTATGTTGTTCTCACTGCACAGCAAATGAGCGAAAGGA 132

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Sbjct 193 CCATGAGCGAGCTGCTGACCACTGCACACTGCTCTCACTGTCACGAGCTGGGAGCGAGGAGGCC 252

Query 4018 ACATCGAGCCACGATCCCGGGCCAGGCCAAGGCAGCTCAGAGGAGGAGCCAGAGCTGGTAT 4077

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Figure A.6 E

pEGFP-Shank3 WT with F1 primer

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251
**Figure A.6 F**

**pEGFP-Shank3 WT with F2 primer**

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References


Dicker, B. L. (2010). *Peripheral vaccination with brain proteins used as a tool to modulate central nervous system function.* (Unpublished doctoral thesis), University of Auckland, New Zealand.


Lorente de Nó, R. (1934). Studies on the structure of the cerebral cortex. II. Continuation of the study of the ammonic system. *Journal für Psychologie und Neurologie*


