Clinical and functional studies of autoimmune disorders of neuromuscular transmission

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A thesis submitted to University College London for the degree of Doctor of Philosophy

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September 2014

Declaration

I, Jennifer Spillane confirm that the work presented in this thesis is my original research work. Where contributions of others are involved, this has been clearly indicated in the text.

Abstract

Inherited and acquired disorders of the neuromuscular junction are an important cause of muscle weakness and fatigability.

In this thesis I focus on the autoimmune disorders of neuromuscular transmission. Myasthenia Gravis (MG) is the most common of these diseases and is typically caused by antibodies against the post-synaptic acetylcholine receptor. Lambert Eaton Myasthenic Syndrome (LEMS) is a pre-synaptic disorder typically caused by antibodies against voltage gated calcium channels (VGCC).

With regard to LEMS, my main aim was to gain a more complete understanding of the pathomechanisms of the disease. To date, the direct effect of LEMS IgG on presynaptic neurotransmitter release had not been investigated in detail. I examined how LEMS IgG affects neurotransmitter release by imaging action potential dependent vesicle exocytosis using a fluorescent dye. I found that LEMS IgG significantly inhibited the rate of synaptic vesicle release but this effect was lost in synapses from a *Cacna1a* knockout mouse. These data provide direct evidence that LEMS is caused by impaired neurotransmitter release due to an effect on P/Q-type VGCCs.

With regard to MG, I studied the long-term outcome of patients with thymomatous and non-thymomatous MG after thymectomy and found that in general the outcome was favourable in the majority of patients with 34% of patients achieving complete stable remission. I also reviewed the long-term outcome of patients after a severe exacerbation of MG requiring ITU admission. Despite the significant mortality associated with severe exacerbations of MG, it was found that specialised neuro-intensive care was associated with a good long-term prognosis in the majority of

patients. There were no significant differences in outcome in those with early or late onset MG.

Overall the data presented in this thesis provide new insights into the pathomechanisms of LEMS IgG and provide new information regarding the long-term outcome of patients with MG.

Acknowledgments

I would like to thank a number of people who have helped me in various ways during my PhD.

First of all I would like to extend my deepest thanks to my supervisor, Professor Dimitri Kullmann. I am indebted to him for his enthusiastic support, patient advice and unfailing commitment to my research. I was privileged to have him as a supervisor.

I would also like to thank my secondary supervisor, Professor Michael Hanna for his support and ongoing encouragement. I am deeply grateful to Dr Kirill Volynski who took a special interest in my research. I was very fortunate to be able to work with him and make use of his extensive knowledge and expertise and much of my PhD would not have been possible without his help. Thank you to Dr Robin Howard for his help with the clinical aspects of my research. It was a privilege to be able to see patients with him and I learnt an incredible amount from our Thursday afternoons in the NHNN. I would like to thank Professor Angela Vincent for her invaluable support, advice and interest in this project. I am very grateful to Dr Bethan Lang for providing and preparing the IgG samples used in this thesis.

There are countless people who helped me in the Dept. of Clinical and Experimental Epilepsy and UCL. I would like to thank Dr Stuart Martin for his help with genotyping. Heartfelt thanks go to Dr Yarik Ermolyuk who provided kind and patient help in the

laboratory on an almost daily basis. I must thank Dr Marife Cano for her help in making neuronal cultures, her advice regarding immunohistochemistry experiments but most especially for her enthusiasm and encouragement.

I would like to thank many of the other PhD students and the post-doctoral researchers in the lab. but particular thanks go to Drs Sarah Crisp, Umesh Vivekananda and Yamina Bakiri for their helpful and insightful comments on my thesis. Dr Iris Oren and Dr Felicity Alder provided invaluable help and support, particularly when I was "new" in the lab. I am grateful to Dr Alex Moreau who shared a desk with me for his daily IT help. I must also thank Juliet Solomon for her calm efficiency and warm support.

Dr Brian Sweeney, Dr Aisling Ryan and Dr Sean O'Sullivan all deserve thanks for encouraging me during my early stages in the field of Neurology and being excellent mentors.

I am deeply grateful to the Myasthenia Gravis Association for funding my PhD. Their generous support and unfailing commitment to research in this area has made this work possible.

I would like to thank the patients who donated serum samples that I used in my experiments. I have been fortunate enough to meet many patients with MG and LEMS during my research and have been inspired and amazed by their knowledge and interest in scientific research.

I have met many wonderful people during my PhD. In particular, I am lucky to call Dr Rahima Begum, Dr Stjepana Kovac and Dr Karen Doherty friends. They all provided so much support to me both in and outside the lab and I am truly grateful for this.

I was fortunate to have two fantastic flatmates during my PhD, Julie and Blaithin who listened to endless lab anecdotes with humour and patience and I am so glad for their friendship. I would like to give thanks to Aidan who provided so much encouragment and advice. Thank you to Ria and Fiona for their long distance support and friendship from Ireland and Australia.

Special thanks go to my wonderful family to whom I owe so much. My parents, Jerh and Avril, have been a constant source of support and encouragement in everything I have ever done. I have always been so proud of my sister, Susan and her own research successes have been a source of inspiration for me. Finally, to James, my fiancé, I was incredibly lucky to meet you during my PhD, thank you for everything.

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List of abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
AMPA(R)	α-Amino-3-hydroxy-5-methyl4-isoxazolepripionic acid (receptor)
AP	Action potential
ATP	Adenosine-5'-triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetracetic acid
BSA	Bovine Serum Albumin
BiPAP	Bilevel Positive Airways Pressure
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNS	Central Nervous System
CCD	Charge coupled device
ChAT	Choline acetyltransferase
Cm	Membrane capacitance
CMAP	Compound muscle action potential
CMF-HBSS	Ca^{2+}, Mg^{2+}, free Hanks balanced salt solution

CMS	Congenital Myasthenic Syndrome(s)
CNS	Central Nervous System
COLQ	Acetylcholinesterase collagenic tail peptide
CSR	Complete Stable Remission
CT	Computed tomography
3,4, DAP	3,4, Diaminopyridine
DBSS	Dulbecco's phosphate buffered saline
DELTA-P	Dutch English LEMS Tumour Association Predictor
DHP	Dihydropyridine
DIC	Differential interference contrast microscopy
$DiOC_2$	3,3' –diethyloxadicarbocyanine iodide
DIV	Days in vitro
DMSO	Dimethyl sulfoxoide
DNAase	Deoxyribonuclease
DPAGT	UDP-N-acetylglucosamine-dolichyl-phosphate N acetylgluocsaminephosphotransferase
DOK	Downstream of (Tyrosine) Kinase
EA2	Episodic Ataxia Type 2
ECG	Electrocardiograph
EDTA	Ethylenediaminetetraacetic acid
EFNS	European Federation of Neurological Sciences

EGTA	Ethyleneglycoltetracetic acid
EMCCD	Electron multiplying charge coupled device
EMG	Electromyography
EPP	End plate potential
(s)EPSC	(Spontaneous) Excitatory postsynaptic current
ERC1	ELKS/RAB6-interacting/CAST family member 1
FBS	Fetal bovine serum
FHM	Familial hemiplegic migraine
FM	Fei Mao dye
FVC	Forced vital capacity
GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
GFPT1	Glutamine-fructose-6-transaminase 1
GM	Glial medium
GPT	Guanine-5'-triphosphate
HBSS	Hanks balanced salt solution
HEK 293	Human embryonic kidney 293 cell line
HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
HET	Heterozygous
HOM	Homozygous
HRP	Horse Radish Peroxidase

HVA	High voltage activated
HypoPP	Hypokalemic Periodic Paralysis
ICU	Intensive Care Unit
^{125}I-α DTX	Iodinated α dendrotoxin
IgG	Immunoglobulin G
(s)IPSC	(spontaneous) Inhibitory post synaptic current
IS	Immunosuppression
ITRP1	Inositol 1 4,5 triphosphate receptor
IVIG	Intravenous Immunoglobulin
k_{AP}	AP-evoked destaining rate
K_d	Dissociation constant
k_{ev}	Evoked destaining rate
KO	Knock-out
k_{SP}	Spontaneous destaining rate
LEMS	Lambert Eaton Myasthenic Syndrome
LRP-4	Low-density lipoprotein receptor related protein 4
LVA	Low voltage activated
MC	Myasthenic Crisis
MEM/N2	Minimal essential medium: nutrient mixture N2
mEPP	Miniature end plate potential
MG	Myasthenia Gravis

MGFA	Myasthenia Gravis Foundation of America
MM	Minimal manifestations
MRI	Magnetic Resonance Imaging
MuSK	Muscle specific tyrosine kinase
NB	Neurobasal complete
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benxo[f]quinoxaline-2,3- dione
NCS	Nerve conduction studies
NHNN	National Hospital for Neurology and Neurosurgery
N-ICU	Neurological ICU
NMDA	N-methyl-D-aspartate
NMJ	Neuromuscular junction
NMOSD	Neuromyelitis optica spectrum disorder
NSF	N-etylmaleimide-sensitive factor
NT	Neurotransmitter
NT-LEMS	Non-tumour associated LEMS
P0	Postnatal day zero
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDL	Poly-D-Lysine
PFA	Paraformaldehyde
PLEX	Plasma Exchange

PLL	Poly-L-Lysine
PR	Pharmacological Remission
P_{rel}	Probability of neurotransmitter release in response to an AP
P_v	Average fusion probability of an individual vesicle
QMG	Quantitative Myasthenia Score
RA	Rheumatoid arthritis
RCT	Randomised controlled trial
RNS	Repetitive nerve stimulation
RP	Recycling Pool
RPM	Revolutions per minute
R_{rel}	Release rate
RRP	Readily releasable pool
SCA6	Spinocrebellar ataxia type 6
SCLC	Small cell lung carcinoma
SFCA	Surfactant free cellulose acetate
SFEMG	Single Fibre EMG
SLE	Systemic Lupus Erythematosus
SM	Sec1/Munc18 like
SNAP 25	Synaptosomal-associated protein 25
SNARE	Soluble N-ethlmaleimide sensitive factor attachment protein receptor
SOX-1	Sry like high mobility group box 1

SRC1	SynaptoRed C1
T-LEMS	Tumour associated LEMS
TPMT	Thiopurine methyl transferase
TTA-P2	3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide
TTX	Tetrodotoxin
VAMP	Vesicle associated membrane protein
VGCC	Voltage gated calcium channel
VGKC	Voltage gated potassium channel
ω-aga	ω-agatoxin IVA
ω-ctx	ω-conotoxin GVIA
WHO	World Health Organization
WT	Wild type
(3),4 DAP	(3), 4, Diaminopyridine

1 Introduction

In this thesis I focus on two specific autoimmune diseases of the neuromuscular junction, Myasthenia Gravis (MG) and Lambert Eaton Myasthenic Syndrome (LEMS). MG is the more common of the two disorders with a prevalence of approximately 7-15 per 100,000 (Robertson et al., 1998, Carr et al., 2010), whilst LEMS is much less common with an incidence of approximately 0.048 per 100,000 and a prevalence of 0.2 per 100,000 (Wirtz et al., 2003). Both disorders present with muscle weakness due to impaired synaptic transmission at the neuromuscular junction.

MG is a well-characterised autoimmune disease, most commonly due to antibodies directed against the postsynaptic acetylcholine receptor (AChR). Antibodies against other antigens including Muscle Specific Tyrosine Kinase (MuSK) and low-density lipoprotein receptor related protein 4 (LRP-4) have also been described in MG patients. Approximately 10-15% of patients have no identifiable autoantibodies using standard radioimmunoprecipitation assays. Anti-AChR antibodies can however be identified in up to 66% of these "seronegative" patients when a specific cell based assay using rapsyn clustered AChRs is performed (Leite et al., 2008). MG typically presents with painless fatigable muscle weakness and in 15% of cases this is associated with a thymoma, a tumour of the thymus gland (Tsinzerling et al., 2007).

LEMS, in contrast is typically associated with antibodies directed against presynaptic voltage-gated calcium channels (VGCCs) (Motomura et al., 1995, Takamori et al., 2000) and presents with proximal muscle weakness and autonomic symptoms (Titulaer et al., 2011). Approximately 50% of cases are associated with a malignancy, most often a small cell carcinoma of the lung (SCLC) (Titualer et al., 2011).

The goals of this thesis were two-fold. First, to obtain a more direct insight into the mechanisms by which LEMS antibodies affect synaptic transmission; in particular to probe both the effect of LEMS Immunoglobulin G (IgG) on synaptic vesicle release and the specificity of LEMS antibodies for individual calcium channel subtypes. Second to examine the long-term effects and outcome of patients with MG from the large cohort we have access to at the NHNN. I was particularly interested in outcome after thymectomy and also wished to assess the progress of MG patients after a severe exacerbation of MG requiring Intensive Care Unit (ICU) admission.

Firstly, I give an overview on the mechanisms of synaptic transmission, the clinical and pathological features of both MG and LEMS and outline the major unsolved questions that underlie this thesis.

1.1 Synaptic transmission

1.1.1 Electrical and chemical synapses

Synaptic transmission forms the basis of information transfer in the nervous system. Until the late nineteenth century there was a belief that all neurons were directly connected and that an impulse from one nerve was directly carried to another through a physical connection (reviewed by Holz and Fisher, 1999). Such synapses have subsequently been confirmed and are known as electrical synapses. They are composed of direct intercellular connections or gap junctions that allow the direct passive flow of

electrons between cells. The main function of electrical synapses is to synchronize electrical activity among populations of neurons (Bennett and Zukin, 2004).

We now recognise that most synapses are chemical, and, unlike electrical synapses although close together, are not continuous. Chemical neurotransmission involves the release of neurotransmitters or neuropeptides from the pre-synaptic cell, and their diffusion across a space or synaptic cleft, which typically measures between 15 and 25 nm to act on postsynaptic receptors (Südhof, 1995). The chemical neurotransmitters that are released from the presynaptic cell are packaged into synaptic vesicles in the presynaptic membrane. These vesicles fuse with the presynaptic membrane, releasing the neurotransmitter into the synaptic cleft. Various different types of chemical neurotransmitter exist; they can be divided up in terms of their structure- into amino acids (such as glutamate, γ-aminobutyric acid (GABA) and glycine), monoamines (including dopamine, noradrenaline and serotonin), peptides (including somatostatin, substance P and opioids) and others including acetylcholine, or in terms of their action – whether they are primarily excitatory or inhibitory. Glutamate is the most important excitatory neurotransmitter in the central nervous system (CNS) (Purves et al., 2001), binding to multiple different receptors including N- methyl –D-asparte (NMDA) receptors, α-Amino-3-hydroxy-5-methyl4-isoxazolepripionic acid (AMP) receptors and kainate receptors – these are excitatory receptors that allow influx of positively charged ions and subsequent depolarisation of the postsynaptic neuron when opened. Acetylcholine (ACh) is the neurotransmitter responsible for excitatory neurotransmission at the neuromuscular junction and so has particular relevance to the study of LEMS and MG. It also provides excitatory neurotransmission at various

autonomic synapses including the vagus nerve, cardiac muscle and various visceral motor synapses and also at several sites in the CNS (Purves et al., 2001).

Once a chemical neurotransmitter is released from the presynaptic membrane, it diffuses across the synaptic cleft and binds to specific receptors on the postsynaptic membrane. There are two major classes of postsynaptic receptors –ionotropic and metabotropic receptors. Ionotropic receptors are linked directly to ion channels – these receptors have two functional domains; an extracellular site that binds the chemical neurotransmitter and the membrane spanning ion domain. Binding of the neurotransmitter induces a conformational change in the postsynaptic receptor allowing ions to flow into the post synaptic cell causing postsynaptic current. If this current is depolarizing and sufficiently large, it may trigger an action potential in the postsynaptic neuron. Neurotransmitters also act at metabotropic (G-protein coupled) receptors. These receptors do not have ion channels as part of their structure; rather binding of the neurotransmitter induces activation of G proteins which then in turn leads to the opening or closing of ion channels (Purves et al., 2001)

1.1.2 History of our understanding of chemical synaptic transmission

The process of chemical neurotransmission was first described by the Austrian scientist Loewi in 1921 when he demonstrated that a chemical, released by the vagus nerve was able to alter heart rate- he termed this unknown chemical "vagusstoff" (Lowei et al., 1921). The nature of this "vagus substance" was characterised in a series of successive papers. Lowei demonstrated that the transmitter appeared subsequent to nerve

stimulation and was antagonised by cholinesterase (Loewi et al., 1926), thus identifying acetylcholine (ACh) as the first neurotransmitter.

The process of chemical neurotransmission was subsequently recorded at the neuromuscular junction in the 1930s when it was shown that acetylcholine was released in response to a presynaptic nerve impulse and that this resulted in postsynaptic depolarization and muscle contraction (Dale et al., 1936). In the 1950s, Bernard Katz and his colleagues used the frog neuromuscular junction to delineate the principles of neuromuscular transmission further by recording the response (the end plate potential – EPP) of a postsynaptic cell to the release of ACh using intracellular microelectrode recording (Fatt and Katz, 1951), allowing EPPs to be used as a sensitive monitor of ACh release from the presynaptic motor neuron (Augustine and Kasai 2007). Further work identified small spontaneous depolarisations of the post synaptic membrane that occurred even when the motor neuron was not stimulated (Fatt and Katz, 1952) – these became to be known as "miniature end plate potentials" or "minis" (mEPPs) and are due to the spontaneous release of ACh from the presynaptic motor neuron (Fatt and Katz, 1952).

Statistical analysis of fluctuations in EPP amplitude and mEPPS allowed del Castillo and Katz to conclude that the EPP consists of multiple quanta of ACh that are released in response to a depolarisation in the nerve terminal (del Castillo and Katz, 1954). They viewed the presynaptic nerve terminal as possessing a pool of such quanta – a proportion of which are released with each presynaptic nerve stimulation (Augustine and Kasai, 2007).

It was later shown that synaptic transmission was heavily dependent on external Ca^{2+}. Studies of the squid stellate ganglion showed that inward movement of Ca^{2+} was essential for synaptic vesicle release (Katz and Miledi, 1967). Similar findings were seen at the frog (Dodge and Rahamimoff, 1967, Andreu and Barrett, 1980) and rat (Hubbard et al., 1968) neuromuscular junctions.

1.1.3 Process of synaptic transmission

The process of synaptic transmission has been described in detail and involves several steps (figure 1.1). Firstly, the neurotransmitter is synthesised in the cell body or at the presynaptic terminal. Neurotransmitters are then loaded into synaptic vesicles via active transport – an electrochemical gradient is created by a proton pump which transports H^{+} ions which in turn forces neurotransmitter uptake (Ahnert-Hilger, 2003). The synaptic vesicles containing neurotransmitter then dock near the synaptic terminal adjacent to presynaptic Ca^{2+} channels, in a process that involves the various key proteins known as the Soluble NSF Attachment protein receptor (SNARE) proteins (Bennett et al., 1992) (see below). The area where the synaptic vesicles cluster is known as the "active zone" an electron dense region directly opposite the postsynaptic density. Typically 8-10 vesicles are docked at small central glutamatergic synapses (Schikorski and Stevens, 2001; Xu-Friedman et al., 2001)

The depolarising current from the action potential at the presynaptic nerve terminal membrane triggers a conformational change in VGCCs, which allows entry of Ca^{2+} from the extracellular space (Katz and Miledi 1967; Miledi, 1973; Schneggenburger et

al., 2005). The subsequent increase in intracellular Ca^{2+} which, at the release site, reaches a concentration in the order of tens of micromoles per litre, activates sensors that subsequently trigger synaptic vesicle fusion with the presynaptic membrane and the release of neurotransmitter into the synaptic cleft (Katz, 1971) through exocytosis. Once the synaptic vesicles have fused with the presynaptic nerve terminal membrane, the neurotransmitters can diffuse across the synaptic cleft and subsequently bind to specific receptors on the postsynaptic membrane.

In response to neurotransmitter binding, postsynaptic receptors undergo a conformational change that leads to a transmembrane channel opening directly or via a G protein signalling pathway. Depending on the ion channel selectivity of these channels, certain ions can move into the channel along their electrochemical gradient. If the current is depolarising and of large enough magnitude, it will induce an action potential in the postsynaptic cell.

After exocytosis vesicles undergo endocytosis and recycle and refill with neurotransmitter for a new round of exocytosis in a “trafficking cycle” (Südhof, 2004). This recycling process is important as presynaptic nerve terminals only contain a few hundred vesicles on average and an efficient recycling process is necessary to preserve synaptic transmission and synaptic morphology (Klingauf et al., 1998). A number of different mechanisms of synaptic vesicle recycling have been proposed. These include (1) so-called “kiss and stay” whereby vesicles are re-acidified and refilled with neurotransmitter without undocking (van Kempen et al., 2011), (2) “kiss and run” where vesicles undock and recycle locally to re-acidify and refill with neurotransmitter via a

transient pore between the synaptic membrane and the vesicle (Ceccarelli et al., 1973; Klingauf et al., 1998; Richards et al., 2005; Haratata, 2006) and (3) a process by which vesicles endocytose via clathrin –coated pits and then refill with neurotransmitters either immediately or via an endosomal intermediate (Südhof , 2004).

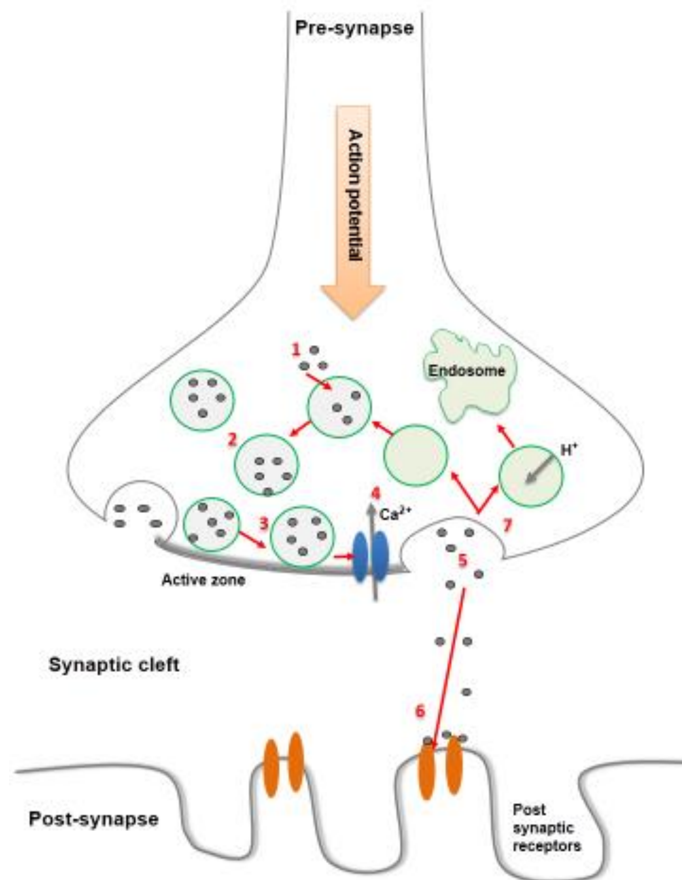


Figure 1.1 The Synaptic vesicle cycle: (1) Synaptic vesicles (green circles) are filled with neurotransmitter (grey particles) by active transport. (2) Synaptic vesicles cluster near the active zone. (3)Vesicles dock near the active zone in the vicinity of VGCCs. (4) Depolarisation of the plasma membrane activates VGCCs allowing influx of Ca^{2+}. (5) Vesicles fuse with the plasma membrane allowing release of neurotransmitter. (6) Neurotransmitter diffuses across synaptic cleft where it can activate postsynaptic receptors. (7) After fusion vesicles undergo endocytosis via either, kiss and run, clathrin mediated endocytosis with recycling via endosomes, or local reuse via kiss and stay

1.1.4 SNARE proteins

The synaptic vesicle cycle is the fastest and most tightly regulated cellular transport pathway (Südhof, 1995). There are a number of important protein–protein interactions that control and facilitate the synaptic vesicle cycle and neurotransmitter release. The SNARE proteins are required for docking of the vesicle at the active zone and fusion of the synaptic vesicle to the synaptic membrane. The SNARE complex is composed of three membrane proteins – two of which, syntaxin-1 and SNAP-25 (synaptosomal associated protein 25), are located on the synaptic membrane – and VAMP (vesicle associated membrane protein or synaptobrevin) which is located on the vesicle membrane (Südhof, and Rothman, 2009; Groffen et al., 2010; Pang et al., 2006). The SNARE proteins form a stable helical bundle composed of two helices from SNAP-25 and one each from syntaxin-1 and VAMP that brings the synaptic vesicle membranes together, allowing them to fuse when triggered by Ca^{2+} influx (Südhof, 1995, 2004; Hanson et al., 1997; Weber et al., 1998).

Synaptotagmin 1 is a vesicle protein that can bind SNARE proteins (Bennett et al., 1992; Chapman et al., 1995) and it serves as the major Ca^{2+} sensor for regulated exocytosis. It is required for Ca^{2+} triggering of synchronous neurotransmitter release (Geppert et al., 1994) and synaptic transmission is severely impaired in mice with synaptotagmin mutations (Geppert et al., 1994).

Many hundreds of other proteins are involved in vesicular exocytosis forming a complex network of interactions (Südhof, 2004), for example, SM (Sec1.Munc18-like)

proteins also have an important role in vesicular fusion – they bind to SNARE complexes and direct their fusogenic action (Südhof and Rothman, 2009).

1.1.5 Pools of synaptic vesicles

Synaptic vesicles in synaptic boutons are arranged into a number of different groups or "pools", including the readily releasable pool (RRP), the recycling pool (RP) and the reserve pool (Rizzoli and Betz, 2005) (See figure 1.2). The stimulation frequency at the presynaptic nerve terminal determines which pool of vesicles is released at any particular time. When a nerve terminal is stimulated repeatedly at high frequency the release rate of synaptic vesicles will initially drop and eventually reach a lower steady state level. This initial depression reflects the depletion of vesicles in the readily releasable pool (RRP). The steady state level of release corresponds to the rate at which vesicles are replenished by recycling or are recruited from the reserve pool.

The readily releasable pool (RRP) contains vesicles, which are immediately available for release on Ca^{2+} entry to the cell. It is generally assumed that they correspond to vesicles that are docked in the active zone, in close proximity to the VGCCs and are primed for release (Schikorski and Stevens, 2001; Rizzoli and Betz, 2005). At hippocampal boutons, the RRP is composed of 5-20 vesicles that can be can be depleted in 10s by high frequency (30 Hz) stimulation and can refill in one minute (Richards et al., 2003; Rosenmund and Stevens, 1996; Murthy and Stevens, 1999). At the frog neuromuscular junction approximately 20% of the vesicles reside in the RRP (Richards et al., 2003).

The recycling pool (RP) vesicles maintain continuous synaptic vesicle release under moderate physiological stimulation (Richards et al., 2003; Kuromi and Kidokoro, 2003). It comprises 10-20% of the total number of vesicles although this is variable (Harata et al., 2001; Rizzoli and Betz 2005). It is recycled continuously during physiological frequencies of stimulation and is refilled by newly recycled vesicles (Rizzoli and Betz 2005; Kuromi and Kidokoro, 2003; Harata et al., 2001; de Lange et al., 2003)

The reserve pool is only recruited to release synaptic vesicles with high frequency stimulation. It constitutes 80-90% of the total number of vesicles in the synaptic bouton (Rizzoli and Betz, 2005) but vesicles are seldom recruited from the reserve pool under physiological conditions (Rizzoli and Betz 2005). Although reserve pool vesicles are mixed with those from the recycling pool, they tend to be located further from the active zone as shown by Fei Mao (FM) dye staining of synaptic vesicles and electron microscopy (EM) (Schikorski and Stevens, 2001).

Vesicles can mix between different pools but it has been argued that they are preferentially recycled to their own pools (Pyle et al., 2000). The size of the synapse, active zone and total number of vesicles can be altered under certain conditions. For example, the number of docked vesicles, the total number of vesicles per synapse and the size of the synapse have all been shown to increase after hippocampal synapses were pharmacologically silenced for several days (Murthy et al., 2001).

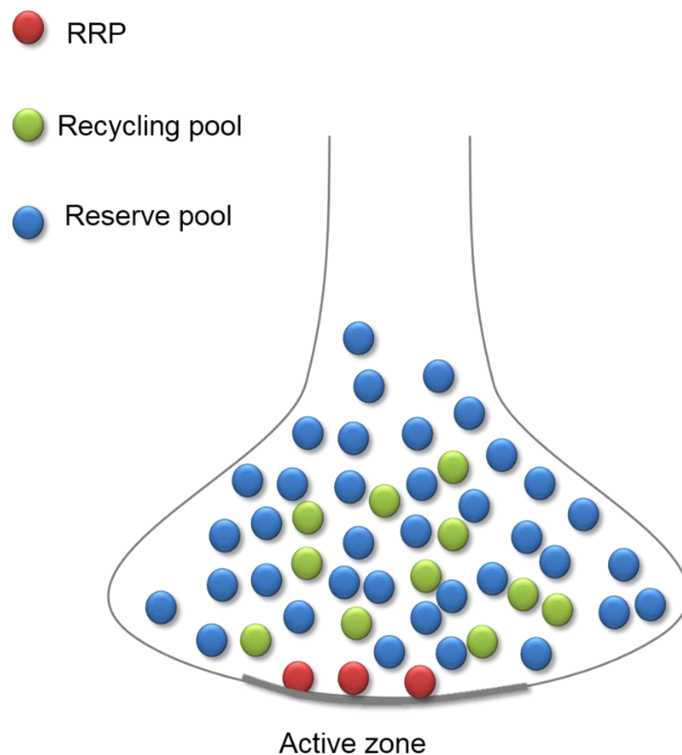


Figure 1.2 Pools of synaptic vesicles. Distribution and proportion of vesicle pools in a hippocampal presynaptic terminal. Readily releasable pool (RRP) in red ~ 5%, recycling pool (RP) ~20% (green), reserve pool ~75% (blue).

1.1.6 Spontaneous neurotransmission

The focus up to now has been on on evoked neurotransmission; synaptic vesicle release that occurs in response to a presynaptic nerve action potential. However it is known that neurotransmission also occurs in the absence of presynaptic stimulation. Fatt and Katz described small spontaneous depolarisations of the postsynaptic membrane potential that occurred even when the motor neuron was not stimulated – these miniature end plate potentials were found to result from spontaneous release of ACh from the presynaptic motor neuron (Fatt and Katz, 1952). Action potential-independent neurotransmitter release occurs at low frequency at all synapses (Xu et al, 2009). This

synaptic vesicle release is maintained in the presence of tetrodotoxin (TTX), a sodium channel blocker that abolishes action potentials and inhibits evoked synaptic vesicle release (Xu et al., 2009).

Spontaneous synaptic vesicle release was originally observed in muscle fibres as miniature end plate potentials (mEPPS) in 1952 (Fatt and Katz, 1952) but subsequently was recorded in central neurons as miniature inhibitory or excitatory postsynaptic currents (mIPSCs or mEPSCs) on patch clamping post synaptic cells (Simkus and Stricker 2002; 2002b). These miniature potentials or "minis" represent the fusion of a single synaptic vesicle with the presynaptic membrane. MEPP amplitude is typically about 1mV and the frequency at the NMJ is approximately 1/second (Lambert and Elmqvist, 1971).

The physiological significance of spontaneous neurotransmitter release was initially questioned but it is now accepted that it does serve certain biological functions including maintenance of dendritic spines (McKinney et al., 1999), regulation of neuronal firing (Carter and Regher, 2002) and dendritic protein synthesis which has a role in stabilising synaptic function (Sutton et al., 2006).

As described above, the process of action potential evoked synaptic vesice release is heavily dependent on the influx of Ca^{2+} through VGCCs. It is also now recognised that the frequency of spontaneous vesicular release is reduced under conditions of low Ca^{2+} (Groffen et al., 2010; Xu et al., 2009) and that Ca^{2+} triggers a significant proportion of spontaneous neurotransmission (Xu et al., 2009; Groffen et al., 2010; Ermolyuk et al.,

2013), although spontaneous vesicular release that is Ca^{2+} independent is also recognised (Maximov et al., 2007).

The mechanism by which Ca^{2+} plays a role in spontaneous synaptic vesicle release is incompletely understood. Synaptotagmin 1 is known to play a role in evoked synaptic vesicle release but is also thought to act as a Ca^{2+} sensor in spontaneous release (Xu et al., 2009). The synaptotagmin analogue, Doc2B has also been suggested to act as a Ca^{2+} sensor for spontaneous synaptic vesicle release (Groffen et al., 2006) although this is controversial (Pang et al., 2011).

It has also been suggested that spontaneous neurotransmitter release may, in part, be triggered by the release of Ca^{2+} from internal stores via Ryanodine receptors and other mechanisms (Llano et al., 2000; Emptage et al., 2001; Koizumi et al., 1999). More recently it has been shown that VGCC can open spontaneously at resting membrane potentials (Awatramani et al., 2005; Yu et al., 2010). Thehe opening of a single VGCC has been shown to be sufficient for a quantum of neurotransmitter to be released (Bennett et al., 2000).

Previously, in our laboratory, it was shown that opening of VGCCs accounts for approximately 50% of all spontaneous glutamate release at rat hippocampal synapses (Ermolyuk et al., 2013). The sensitivity of VGCC-dependent mEPSCs to the Ca^{2+} chelators EGTA and BAPTA is similar to that of action potential evoked exocytosis, suggesting that both evoked and spontaneous synaptic vesicle release are triggered by

similar Ca^{2+} micro and nano domains which are sensed by the same Ca^{2+} sensor – synaptotagmin -1 (Ermolyuk et al., 2013).

1.1.7. Study of synaptic transmission

Synaptic transmission has been studied in a number of model systems including the neuromuscular junction (Katz and Miledi, 1969b), the calyx of Held (Schneggenburger and Forsythe, 2006), the chick ciliary ganglion (Stanley and Goping, 1991) synaptosomal preparations (Nicholls, and Sihra 1986), retinal bipolar cells (Neves et al., 2001) and hippocampal mossy fibre terminals (Geiger and Jonas, 2000).

Direct patch-clamp recordings have been performed at a number of larger synapses: hippocampal mossy fibre synapses, the calyx of Held and retinal bipolar cells (Geiger and Jonas 2000. Schneggenburger and Forsythe 2006; Forsythe 1994; Palmer 2010). However, it has been hitherto more difficult to study transmission at the small central synapses of the hippocampus (Branco et al., 2008) and other techniques such as synaptic vesicle imaging have been used. I will discuss the use of FM imaging in the study of synaptic vesicle release in Chapter 3.

Many studies evaluating neurotransmission have used the postsynaptic response such as the EPP as a marker of presynaptic vesicle release. However, the “quantal content”, the number of quanta released in response to a presynaptic stimulus, cannot always be accurately estimated from end plate potentials. Occasionally neurotransmitter release does not activate postsynaptic receptors; so called ‘silent synapses’ (Kullmann, 1994;

Isaac et al., 1995). Moreover, not every action potential that arrives at a nerve terminal is converted into a secretory signal – at hippocampal excitatory terminals, it has been estimated that only 10-20% of action potentials trigger release (Goda and Südhof, 1997; Sudhof 2004).

1.2 Voltage Gated Calcium Channels

1.2.1 Role of VGCCs in synaptic transmission

Voltage gated Ca^{2+} channels (VGCCs) play an integral role in both evoked and spontaneous neurotransmission; the influx of Ca^{2+} through VGCCs is an obligatory step for excitation –secretion coupling during neurotransmission (Reid et al., 2003; Luebke et al., 1993). As described above, an action potential invading the presynaptic terminal causes a conformational change in the VGCC voltage sensor – this opens the channel allowing an influx of Ca^{2+} ions. The influx of Ca^{2+} ions remains quite local and very transient - a concentration of 5-20 µM is obtained at the release site but only briefly (4-500 µs) due to effective endogenous buffering and diffusion (Neher 1998; Meinrenken et al., 2002; Bollmann et al., 2000). Spontaneous neurotransmitter release has a Ca^{2+} dependent component (Xu et al. 2009; Groffen et al 2010) but can also occur in the absence of extracellular Ca^{2+} (Maximov et al., 2007).

The influx of Ca^{2+} to the presynaptic nerve terminal triggers at least two components of neurotransmitter release that are mechanistically distinct – the first is a fast synchronous phasic component, induced 50 µs after a Ca^{2+} transient (Sabatini and Regher, 1996).

The second component is a slower, more sustained elevation of release probability (Atluri and Regehr, 1998; Barrett and Stevens, 1972). This slower release can in turn be separated into two components- one lasting for tens of milliseconds that is steeply calcium-dependent and one lasting for hundreds of milliseconds that is driven by low levels of calcium (Atluri and Regehr 1998).

The proteins involved in synaptic vesicle exocytosis interact closely with VGCCs. The synprint (synaptic protein interaction) motif on the II-III linker on P/Q- and N- type VGCCs physically interacts with the synaptic vesicle release proteins (including syntaxin1A and 1B, SNAP-25, synaptobrevin, synaptotagmin) (Jarvis and Zamponi, 2005), a process proposed to allow for tight coupling between Ca^{2+} entry and synaptic vesicle exocytosis. VGCC activity is in turn regulated by interaction with the presynaptic release machinery (Jarvis and Zamponi, 2005).

VGCCs are ubiquitously expressed throughout the nervous system and have been identified in all excitable cells (Hagiwara and Byerly, 1981; Dolphin et al., 2006). VGCCs were first identified in crustacean muscle in the 1950s (Dolphin, 2003a; Fatt and Katz, 1953) and current through these channels was first recorded in cardiac myoctyes in the 1960s (Reuter, 1967). Initially, it was assumed that Ca^{2+} channels were all quite similar. However, we now recognise that VGCCs are a diverse family of proteins composed of pharmacologically distinct subtypes. VGCCs are broadly classified into two main subgroups on the basis of their threshold of activation; high and low voltage activated (HVA and LVA) (Carbone and Lux, 1984; Benarroch, 2010). These groups are further divided into the following sub-types depending on their

pharmacological profile; T-, L-, R-, N-, P- and Q- type (Wheeler et al., 1994; Tsien et al., 1988, Llinas et al., 1989; Dolphin et al., 2006).

1.2.2 Structure of VGCCs

The structure of voltage gated ion channels, including VGCCs is highly conserved. Biochemical purification has revealed that high voltage gated calcium channels are composed of four subunits including $\alpha1$, $\alpha_2\delta$, β and γ (Catterall, 2000) (Figure 1.3).

The α_1 subunit is the pore forming unit and determines the biophysical and pharmacological properties of the channel. The α_1 subunit composed of 2000 amino acid residues with a transmembrane sequence organised into four repeated domains (I-IV) which are each composed of six transmembrane sequences (S1-6) with a membrane associated loop between segments S5 and S6 (Catterall, 2000) which forms the pore. The external pore is lined by the pore loop, which contains a pair of glutamate residues that are required for Ca^{2+} selectivity. Some pharmacological antagonists have receptor sites on the inner pore, which is lined by the S6 segment whereas other antagonists bind to different sites of the α subunit – such as ω-agatoxin IVA (ω-aga) which binds to the S3-S4 linker domain (Winterfield and Swartz, 2000).

There are three major families of α_1 subunit; Ca_v1, Ca_v2 and Ca_v3 (Benarroch, 2010). Each family is further divided into several subtypes. Mutations in the α1 subunit of different VGCC have been associated with human disease (see below) and have been described in various spontaneous murine mutations.

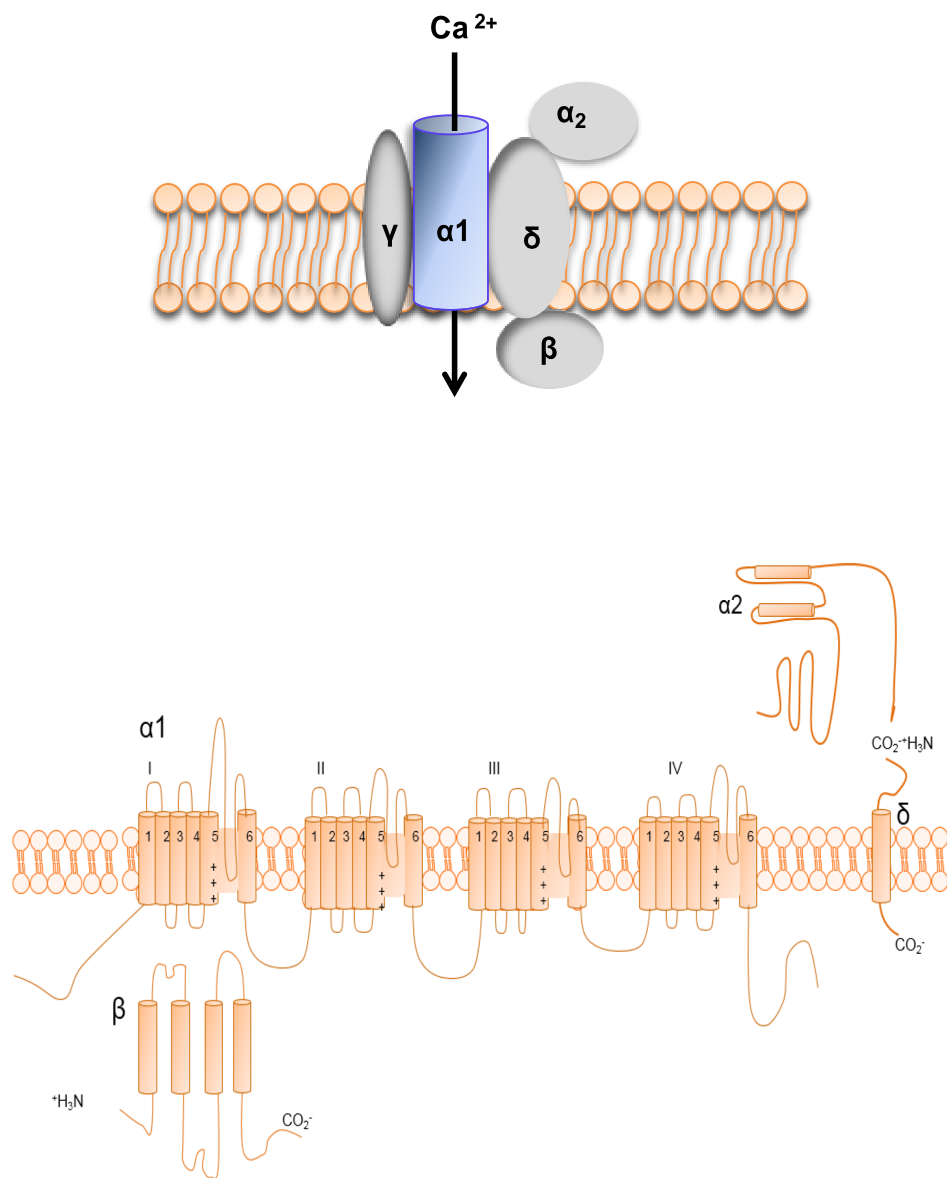


Figure 1.3. Structure of a VGCC. VGCCs are composed of several subunits. The α_1 subunit is the main pore forming protein composed of four domains (I-IV) each divided into six transmembrane segments (S1-S6). The β and $\alpha_2\delta$ are auxiliary subunits. The γ subunit (not shown) is known to associate with skeletal muscle calcium channels (Arrikath and Campbell, 2003).

1.2.3 Different subtypes of VGCCS – the α_1 subunits

The pharmacological features of VGCCs are determined by the pore forming α_1 subunit. The nomenclature of Ca^{2+} channels has changed a number of times but the VGCCs are grouped based on the α subunit with the accepted designation being $Ca_vX.X$ (Catterall et al., 2005). (See table 1.1)

The **Ca_v1** family encodes for L-type or "long-lasting" VGCCs that activate and inactivate slowly. They were first identified using dihydropyridines (DHPs) as Ca^{2+} channel blockers and are expressed in skeletal muscle cardiac muscle, endocrine cells and in neurons (Hess et al., 1984; Milani et al., 1990; Bean et al., 1989). There are four different subtypes of this VGCC family. The $Ca_v1.1$ subunit is expressed in the transverse tubules of skeletal muscle where it has an important role in excitation contraction coupling (Benarroch, 2010). Mutations of this gene manifest as the condition of hypokalemic periodic paralysis (HypoPP) (Venance et al., 2006). $Ca_v1.2$ is expressed in heart, smooth muscle and endocrine organs as well as dendrites and cell bodies throughout the CNS and has a role in long-term potentiation in the hippocampus and cortex (Ertel et al., 2000; Benarroch et al., 2010). $Ca_v1.3$ is expressed in cochlear hair cells, the medium spiny neurons of the striatum and the substantia nigra pars compacta (Benarroch et al., 2010). $Ca_v1.4$ VGCCs are the predominant type of calcium channel involved in glutamate release from rod photoreceptors in the retina and mutations in the gene can cause Congenital Stationary Night Blindness (Barnes and Kelly, 2002; Strom et al., 1998).

The **Cav 2 family** has three different isoforms that encode P/Q-, N and R type channels (Ca_v 2.1, Ca_v2.2 and Ca_v2.3 respectively) and these VGCCs are primarily expressed in neurons (Striessnig and Koschak, 2008). N (for "neuronal" or non-L) type channels were identified using a peptide extracted from the Conus geographus snail; ω-conotoxin GV1A (Nowycky et al., 1985; McCleskey et al., 1987; Tsien et al., 1988). Along with P/Q-type channels (see below), N- type channels play a role in triggering neurotransmitter release. N-channels have roles in sensory and autonomic function and mouse knockout models have revealed a role for these channels in neuropathic pain (Striessnig and Koschak, 2008).

The P- type VGCC was originally described in cerebellar Purkinje cells and is blocked by the peptide ω-agatoxin IVA, which comes from the funnel web spider Agelenopsis aperta (Llinas et al., 1989; Mintz et al., 1992). Ca^{2+} currents recorded from cerebellar granule cells are also blocked by Agatoxin IVA, albeit at lower affinity (Randall and Tsien, 1995) and these channels were originally termed Q-type channels. It is now thought that the pore forming subunit of both P- and Q-type channels are encoded by the same gene (Bourinet et al., 1999; Richards et al., 2007) and these channels are generally referred to as P/Q- type VGCCs. P/Q-type channels are the primary VGCC responsible for presynaptic Ca^{2+} entry and, consequently neurotransmitter release at the mammalian neuromuscular junction (NMJ) (Protti and Uchitel 1993, Uchitel et al., 1992). Synaptic transmission at central synapses is mediated mainly by P/Q-type channels with N-type channels playing a less prominent role (Cao and Tsien, 2010; Luebke et al., 1993; Takahashi and Momiyama 1993; Wheeler et al., 1994).

R-type VGCC were so named because they were “resistant” to DHP, conotoxin and agatoxin. Since then, however, these channels have been found to be sensitive to the toxin SNX -482 (Newcomb et al., 1998; Giessel and Sabatini 2011) and have been found to have a major role in presynaptic long-term potentiation in the mossy fibre CA3 synapse in the hippocampus (Benarroch 2010). R-type channels also contribute to spontaneous exocytosis (Ermolyuk et al., 2013).

The Ca_v3 family encodes for T type (tiny and/or transient) Ca^{2+} channels, which have a smaller channel conductance, are activated at more negative potentials and inactivate more quickly than other Ca^{2+} channels (Perez-Reyes et al., 1998). T type VGCCs are present in a wide variety of cell types. They play a role in pacemaker activity in the thalamus and are also involved controlling Ca^{2+} concentration in cells, which in turn acts as an important 2nd messenger in a variety of cellular processes (Hoffman et al., 1994; Perez-Reyes, 2003).

Disruptions of Ca^{2+} channel function either by genetic mutation, pharmacological or autoimmune alteration can cause a profound effect on the various Ca^{2+} signalling pathways including synaptic transmission. Studies *in vitro* or in knockout mice have provided insights into the function of the different VGCCs (Benarroch, 2010; Striessnig and Koschak, 2008). The different VGCC subtypes, the genes that encode α subunits, the predominant channel function, the human diseases associated with them and their antagonists are summarised in Table 1.1.

HVA/ LVA	Current type	Alpha subunit	Gene	Localization	Main function	Blocker	Human Disease
HVA	L	Ca_v 1.1	CACNA1S	Skeletal muscle	Excitation contraction coupling	DHP	HypoPP
		Ca_v1.2	CACNA1C	Cardiac Smooth muscle Endocrine CNS	Excitation contraction coupling Hormone secretion Plasticity (LTP)		Timothy syndrome
		Ca_v1.3	CACNA1D	Cochlea Striatum SNPars Compacta Kidney Ovary	Sensory transduction Pacemaker activity		Deafness and arrhythmia …
		Ca_v 1.4	CACNA1F	Retina	Sensory Transduction		Congenital Stationary Night Blindness
	P/Q	Ca_v 2.1	CACNA1S	Neuronal (presynaptic terminal at CNS and NMJ) Purkinje cells	Neurotransmitter release	ω-aga	SCA6 FHM EA2 LEMS
	N	Ca_v 2.2	CACNA1B	Neuronal (presynaptic terminal at CNS) Nociceptive sensory neurons	Neurotransmitter release	ω-ctx	
	R	Ca_v2.3	CACNA1E	CNS Cochlea Cardiac Pituitary	Neurotransmitter release	SNX-482	
LVA	T	Ca_v 3.1	CACNA1G	Neuronal Cardiac	Pacemaker activity Repetitive firing	TTAP2	
		Ca_v 3.2	CACNA1H	Neuronal Cardiac Kidney Liver	Pacemaker Repetitive firing	TTAP2	
		Ca_v 3.3	CACNA1I	Neuronal	Pacemaker Repetitive firing	TTAP2	

Table 1.1 VGCC subtypes

(ω-aga; ω-Agatoxin IVA, ω-ctx; ω-Conotoxin GVIA, CNS; Central Nervous System, DHP, dihydropyridine; EA2; Episodic Ataxia Type2, FHM; Familial Hemiplegic

Migraine; HypoPP; Hypokalemic Periodic Paralysis, LTP; Long term potentiation, PC; Pars Compacta; SN; Substantia Nigra, SCA-6; Spinocerebellar Ataxia Type 6, SNX; TTA P2; 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide).

Different types of Ca^{2+} channels support neurotransmitter release at different synapses (Nudler et al., 2003; Catterall et al., 1998). At mature rat, mouse and human NMJ, neurotransmitter release is almost completely blocked by ω-aga IVA indicating that P/Q type Ca^{2+} channels are the main VGCC subtype that support synaptic transmission (Protti and Uchitel, 1993; Uchitel et al., 1992; Katz et al., 1997). However at the neonatal rat NMJ, neurotransmitter release is dependent on both P/Q- and N-type channels with L type VGCCs also playing a role (Rosato-Siri and Uchitel, 1999; Katz et al., 1996). P/Q and N-type channels both play a role in synaptic vesicle release at the hippocampus (Cao and Tsien, 2010; Luebke et al., 1993; Takahashi and Momiyama 1993; Wheeler et al., 1994) with the former playing the dominant role.

1.2.4 VGCC accessory subunits

As well as the pore forming α_1 subunit, there are a number of accessory subunits associated with VGCCs including $\alpha_2\delta$, β and γ. These subunits have various roles in the trafficking of the channel to the cell membrane as well as channel kinetics.

1.2.4 $\alpha_2\delta$ subunit

Four genetically distinct $\alpha_2\delta$ subunits have been described (Arikkath and Campbell, 2003). The subunits are the product of a single gene that is post-translationally cleaved into two peptides which are then linked by disulphide bridges (Arikkath and Campbell, 2003). The δ portion is anchored in the membrane whilst the α_2 portion interacts with the α_1 subunit (Gurnett et al., 1997) and is entirely extracellular (Dolphin, 2009). The $\alpha_2\delta$ subunit has a role in the membrane trafficking of α_1 and also has an effect on channel kinetics (Felix et al., 1997; Arrikath and Campbell, 2003). The ducky (du/du) mouse has a mutation in the $\alpha_2\delta_2$ gene resulting in a protein that is not anchored in the plasma membrane – this mutation causes a severe reduction in P-type VGCC current density (Brodbeck et al., 2002; Barclay et al., 2001) with a phenotype of epilepsy and ataxia (Barclay et al., 2001).

1.2.4.2 β subunit

Four different genes code for the β subunit and various splice forms exist (Helton and Horne, 2002). It is entirely cytosolic (Arrikath and Campbell, 2003) and is composed of four α helices. The β subunit aids in the trafficking of the α_1 subunit to the plasma membrane (Gregg et al., 1996) and also can modulate the biophysical properties of the channel (Dolphin, 2003; Arrikath and Campbell, 2003).

1.2.4.3 γ subunit

The γ_1 subunit is known to associate with skeletal muscle VGCCs (Arrikath and Campbell, 2003) but the evidence for neuronal γ subunits is much less certain (Catterall,

2003). γ subunits do not affect VGCC surface expression but may affect the biophysical properties of the channel (Arikkath and Campbell, 2003).

1.2.5 Calcium Channel Toxins

As alluded to above, a number of different peptide toxins are derived from marine snails (conotoxins) and spiders (such as agatoxins) are known to potently inhibit the activity of specific VGCCs (Adams et al., 1993). The high selectivity of these toxins has made them very useful physiological tools in studying VGCC function. I made use of some of these toxins in the electrophysiology experiments in this thesis and will describe them briefly.

1.2.5.1 P/Q-type VGCC antagonists

1.2.5.1.1 Agatoxins

Agatoxins are a class of peptide toxins named after the funnel web spider Agelenopsis aperta, which produces a venom containing toxins that can target three different types of ion channels including VGCCs, voltage gated sodium channels and transmitter activated cation channels. The ω-agatoxins are subdivided into four classes based on their structure and calcium channel specificity. ω-Aga IIIA blocks various different VGCCs whereas ω-Aga IVA as a high specificity for P/Q type VGCCs (Mintz et al., 1992; Adams, 2004) and does not affect N-, L- or R-type channels (Mintz et al., 1992).

1.2.5.1.2 ω-Conotoxins

The ω-Conotoxins- MVIIC and MVIID from the C.magus snail (Hillyard et al., 1992) block P/Q-calcium channels but also N-type VGCCs, indicating that the ω-Conotoxin binding macrosites for the N and P/Q VGCCs are most likely related. Blocking of N-type channels is rapid and reversible whereas blocking of P-type channels is potent, slow and only very slowly reversible (McDonough et al., 1996).

1.2.5.2 N – type VGCCs antagonists

ω-Conotoxin GVIA from the Conus Geographus (Olivera et al., 1984) potently and selectively inhibits N-type VGCCs (McCleseky et al., 1987; Nowycky et al., 1985).

McDonough et al. studied differences in the characteristics of inhibition of various toxins including voltage dependence, reversal kinetics and fractional inhibition of currents to study how toxins affected VGCCs function (McDonough et al., 2002). VGCC characterisation studies demonstrated at least two distinct binding sites on N-type channels. ω-CgTx-GVIA, ω-Aga-IIIA and ω-CTx MVIIC were all shown to block the channel pore whereas grammotoxin was found to bind to a separate region coupled to channel gating. At P/Q type VGCCs ω-CTx MVIIC and ω-Aga-IIIA both had binding sites near the pore but ω-Aga-IVA bound to a different site associated with channel gating.

1.2.5.3 L type VGCC antagonists

Toxins from several species of snail and snake block L-type VGCCs but dihydropyridines are most commonly used both in the laboratory and in medical practice to block L-type VGCC. This class of drugs includes agents such as amlodipine, and nimodipine.

1.2.5.4 R type VGCC antagonists

SNX-482 is a 41 amino acid peptide isolated from the venom of the African tarantula Hystaeraocrates gigas (Newcomb et al., 1998). It completely and irreversibly inhibits R-type VGCCs (Schroeder et al., 2000).

1.2.5.5 T type VGCC antagonists

TTA-P2, (3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide)a novel piperidine derivative was initially found to inhibit recombinant T-type VGCCs (Shipe et al., 2008). Subsequently it was found to also potently, specifically and reversibly inhibit native T- type Calcium channels (Choe et al., 2011; Drefus et al., 2010)

1.2.6 Calcium channel subtypes involved in synaptic transmission

It is known that neurotransmission at both central and peripheral synapses relies on a combination of VGCC subtypes. At hippocampal synapses, for example, L type VGCCs are not involved under normal conditions but both P/Q- and N-type and to a lesser extent, R-type channels are all involved in synaptic transmission (Wheeler et al.,

1994, Cao and Tsein, 2010; Takahashi and Momiyama, 1993). Blockage of P/Q- N- and R-type VGCCs with specific toxins completely obliterates the EPSC in hippocampal neurons (Cao and Tsien, 2010). Blockage of either P/Q- or N-type channels individually reduces the amplitude of the EPSC by 45% each (Cao and Tsein, 2010). However, P/Q-channels play the predominant role as is evidenced by the more rapid onset of a reduction in block after application of ω-Aga IVa and the relative difficulty of overcoming inhibition of P/Q-type channels through increased stimulus strength (Luebke et al., 1993). P/Q-type channels are also the predominant VGCC involved in synaptic transmission at other central synapses including cortical and striatal synaptosomes (Turner et al., 1992; 1993) and at the nucleus accumbens (Horne and Kemp, 1991).

P/Q-type VGCCs are also the predominant mediator of neurotransmitter release at the neuromuscular junction in both humans (Protti et al., 1996) and in rodents (Uchitel et al., 1992). Mammalian neuromuscular transmission is relatively insensitive to blockage of N-type channels but blockage of P/Q-type channels causes an 80-90% reduction in neurotransmitter release (Katz et al., 1997).

1.3 *CACNA1A* subunit

The α_{1A} subunit is the pore forming subunit in P/Q-type VGCCs and is the most abundant α_1 subunit in the vertebrate brain (Ludwig et al., 1997). It was the first

representative of its class to be isolated by cDNA cloning (Mori et al., 1991; Starr et al., 1991) and is encoded by the *CACNA1A* gene.

The α_{1A} subunit is expressed densely in the cerebellum, particularly in Purkinje and granule cells (Kulik et al 2004; Westenbroek et al., 1995) but transcripts of α_{1A} are found throughout the human and rodent brain with immunoreactivity seen in cell bodies, dendrites and presynaptic terminals (Dolphin, 2009; Westenbroek et al., 1995).

Such is the importance of the P/Q-type channel for neurotransmission, it is not surprising that mutation or knockout of the *Cacna1a* gene of this channel causes a severe phenotype. Several mouse models of spontaneously occurring *Cacna1a* mutations exist including the *rolling Nagoya, tottering* and *leaner* mice (Rhyu et al., 1999; Tstuji and Meier 1971; Plomp et al., 2009). These mutations are recessively inherited and mice all display a severe ataxic phenotype. In addition motor and absence seizures with spike and wave discharges on EEG and paroxysmal dyskinesias can be observed in the *leaner* and *tottering* strains (Nobels and Sidman, 1979; Plomp et al., 2009, Noebels, 1984).

1.3.1 Cacna1a knockout mice

Studies of mice completely lacking the α_{1A} subunit, *Canca1a*$^{-/-}$ mice, have contributed greatly to our knowledge of P/Q- type VGCC function. Embryonic development is initially normal in *Canca1a*$^{-/-}$ mice (Jun et al., 1999). However, by postnatal day 10, they develop a severe neurological phenotype with ataxia, dystonia and weakness (Jun et al., 1999; Fletcher et al., 2001). In addition as KO mice get older they develop prolonged episodes of sustained twisting movements of the trunk and limbs with

extensor spasms of the hind limbs (Fletcher et al., 2001) which are thought to represent seizures. *Canca1a*$^{-/-}$ mice die three to four weeks after birth despite removing littermates and hand-feeding (Jun et al., 1999; Fletcher et al., 2001). Young mutant mice have normal brain architecture but aged mice have progressive cerebellar degeneration (Fletcher et al., 2001). The NMJs of *Cacna1a* knockout mice are morphologically similar to WT animals although they are smaller in size under light microscopy (Urbano et al., 2003).

Electrophysiological studies have shown that P/Q-type currents at central synapses are completely absent in *Cacna1a* knockout mice, thus indicating that a single gene encodes P/Q- channel activity (Fletcher et al., 2001). However synaptic transmission persists indicating that vesicle recycling remains functional despite loss of the α_1 subunit (Jun et al., 1999). With elimination of P/Q type currents, there is a greater reliance on Ca^{2+} entry through N- and R-type channels (Jun et al., 1999). The frequency of spontaneous excitatory postsynaptic currents and inhibitory postsynaptic currenst (sEPSC and sIPSCs) is reduced in the soma of Purkinje cells from *Cacna1a* KO mice (Lonchamp et al., 2009). Analysis of IPSCs in KO mice has suggested that as well as contributing to triggering of glutamate release in the cerebellum, P/Q-type VGCCs play a role in GABAergic release also (Lonchamp et al., 2009)

Similar findings are seen at the neuromuscular junction of *Cacna1a* knockout mice where Ca^{2+} entry through both N- and R- type channels is crucial for synaptic transmission (Urbano et al., 2003). MEPP amplitudes are normal but EPP amplitude is reduced and there is a reduction in postsynaptic AChR clusters at neuromuscular end

plates. (Urbano et al., 2003). Analysis of short-term plasticity has shown that there is little or no paired pulse facilitation (PPF) at the NMJ of KO mice (Urbano et al., 2003) and the synchrony of neurotransmission is altered, with increased jitter (Depetris et al., 2008).

At the KO synapse both N- and R-type channels become involved in neurotransmission. N-type channels are more abundant but R type channels are located closer to the release machinery (Urbano et al., 2003). It is possible that a smaller number of R-type channels which have closer but less optimal interaction with the presynaptic release machinery mediate a more asynchronous form of synaptic transmission than would be seen with the more distant but more abundant N-type channels (Depretris et al., 2008).

Mutations in *CACNA1A* in humans are associated with three different dominantly inherited disease; Episodic Ataxia Type 2, Familial Hemiplegic Migraine and Spinocerebellar Ataxia Type 6.

1.3.2 Human diseases associated with CACNA1A mutations

1.3.2.1 Episodic Ataxia Type 2

Episodic Ataxia Type 2 (EA2) typically presents in the second decade with episodes of ataxia that last hours to days. These symptoms may be accompanied by vertigo, vomiting and oscillopsia (Gancher and Nutt, 1986). Episodes may be triggered by stress, alcohol, infection and exertion (Jen et al., 2007). Patients are typically asymptomatic in between episodes but approximately 30-50% of patients develop a

mild progressive cerebellar ataxia and more than half report migrainous symptoms (Jen et al., 2007; Spacey et al., 2005). EA2 is caused by mutations in the *CACNA1A* gene (Ophoff et al., 1996) - over 80 different mutations including nonsense, frame shift, splice site and missense mutations have been described (Denier et al., 2001; Denier et al., 1999). Functional analysis has revealed that mutations causing EA2 can have different effects; including altered channel function with reduced calcium current, as well as effects on protein folding and trafficking which can reduce channel expression and alter channel function (Guida et al., 2001; Spacey et al., 2004; Wan et al., 2005). It remains unclear why P/Q- channel dysfunction causes paroxysmal cerebellar dysfunction. It has been proposed that mutations causing EA2 exert their effect through the interaction of P/Q-type channels with inositol 1,4,5-triphosphate receptor type 1 (ITRP1) (Schorge et al., 2010). It is likely that there is complete loss of P/Q channel function in EA2 (Guida et al., 2001). Given that an autosomal dominant mutation in *CACNA1A* leading to haploinsufficiency of one allele causes disease in humans, it would be expected that heterozygous *Cacna1a* knockout mice (*Cacna1a* $^{+/-}$) would provide a mouse model of EA2. However, these mice are not overtly ataxic although they do have impairment of motor learning (Katoh et al., 2007).

The ataxia in EA2 is treated with acetazolamide- a carbonic anhydrase inhibitor (Baloh, 2012). Patients display variable responses to treatment, likely due to the characteristics of the individual mutation (Jen et al., 2007; Strupp et al., 2007). Magnetic resonance spectroscopy in patients with EA2 has revealed some evidence for cerebellar alkalosis, which may be reversed by acetazolamide (Bain et al., 1992; Sappey-Marinier et al., 1999). The drug 4-aminopyridine has also been reported to have a prophylactic effect on ataxia in EA2 (Strupp et al., 2011).

A higher proportion of patients with EA2 have epilepsy than expected in the general public (Rajkulendran et al., 2010; Tomlinson et al., 2009). This has led to speculation that P/Q-type channel mutations may be linked to human epilepsy (Jouvenceau et al., 2001; Imbrici et al., 2005; Jen et al., 2007), which is supported by the observation of spike and wave discharges and epilepsy in *Cacna1a* mutant mice (Noebels and Sidman, 1979).

1.3.2.2 Spinocerebellar Ataxia Type 6

Spinocerebellar Ataxia Type 6 (SCA 6) is allelic with EA2 and is caused by expansions of the CAG repeat sequence in the 3' terminus of the *CACNA1A* gene (Zhuchenko et al., 1997). SCA6 presents as a late onset slowly progressive cerebellar syndrome that is characterised by dysarthria, limb incoordination, poor balance and cerebellar atrophy. Occasionally there can be extra-cerebellar features such as opthalmoplegia, spasticity and peripheral neuropathy although these are usually less prominent than in other forms of SCA (Schols et al., 1998). Pathological studies have shown evidence of Purkinje cell loss in the cerebellum consistent with a toxic gain of function (Pietrobon, 2010). Mouse models develop progressive motor impairment and aggregation of mutant $Ca_v2.1$ channels. The disease is thought to be due to dysregulation of the ITPR1 signalling caused by a polyglutamine repeat (Durr, 2010). Alterations in the voltage sensitivity of activation/inactivation and differences in surface expression of the channel have also been suggested as mechanisms (Matsuyama et al., 1997; Restituito et al., 2000; Kullmann et al., 2010; Piedras-Renteria et al., 2001). Some patients with EA2 have also

been found to have small CAG expansions in *CACNA1A* thus leading to suggestions that SCA6 and EA2 are a clinical continuum (Jodice et al., 1997).

1.3.2.3 Familial Hemiplegic Migraine

Migraine is an episodic disorder that affects approximately 10% of the general population (Pietrobon, 2005; Goadsby, 2009). Familial Hemiplegic Migraine (FHM) is a subtype of severe migraine that is inherited in an autosomal dominant fashion. Patients have severe auras that include unilateral weakness, as well as visual, somatosensory or dysphasic symptoms, typically followed or accompanied by migrainous headache. Most patients do not have additional neurological findings but a progressive cerebellar ataxia can occur in up to 20% of patients (Terwindt et al., 1996; Ducros, 2002). FHM is genetically heterogeneous and is classified into three types that are genetically distinct but cannot be distinguished clinically (Pelzer et al., 2013). FHM1 accounts for 75% of cases and is caused by missense mutations in *CACNA1A* (Ophoff et al., 1996). Eighteen different missense mutations have been described, most of which substitute conserved amino acids in the voltage sensor (S4) or pore lining (S5and S6 and the S5-6 linker) regions of the channels (Pietrobon, 2007; Barrett et al., 2005; van den Maagenberg et al., 2004). Functional expression studies in *Xenopus* oocytes, Human Embryonic Kidney (HEK) 293 cells and transgenic mouse studies including two knock-in mouse models have been used to argue that FHM1 mutations result in various gain of function effects including increased $Ca_v2.1$ current density in cerebellar neurons and enhanced neurotransmitter release (van den Maagdenberg et al., 2004). Knock-in mice also show a reduced threshold and increased velocity of cortical

spreading depression which is thought to underlie the aura of migraine (van den Maagdenberg et al., 2004).

LEMS is associated with antibodies against the P/Q-type VGCC. Perhaps surprisingly, patients with the inherited mutations described above are not well known to have a neuromuscular phenotype although some patients may have occasional episodes of weakness (Tomlinson et al., 2009). However electrophysiological studies have revealed subtle abnormalities in single fibre electromyography in patients with EA2 and FHM (Ambrosini et al., 2001; Jen et al., 2001; Maselli 2003; Tomlinson et al., 2009 suggesting that evidence of neuromuscular instability may have a role in supporting the clinical diagnosis of these disorders (Tomlinson et al., 2009).

1.4 Structure of the Neuromuscular Junction

The NMJ is a highly specialised synapse that has evolved to ensure efficient transmission of the motor nerve action potential into a muscle depolarization (Hoch et al., 2001). It has a complex structural and functional organisation involving many structural and signalling proteins, several of which are involved in acquired and inherited diseases of neuromuscular transmission.

The presynaptic nerve terminal contains a large number of synaptic vesicles which contain ACh. The vesicles are concentrated in the nerve terminal in an area known as the active zone. Freeze fracture microscopy has shown that the active zones consist of

ordered arrays of particles 10-12 nm in diameter arranged in double parallel lines known as active zone particles (Fukuoka et al., 1987 Engel et al., 1991). These particles are thought to correspond to VGCCs (Fukuoka et al., 1987). As discussed above, the VGCCs are closely involved with proteins such as the SNARE proteins that are essential for the Ca^{2+} induced exocytosis of synaptic vesicles.

The basal lamina between the nerve and the post synaptic membrane contains several important proteins that are essential for synaptic transmission. Proteins such as collagens, lamins, fibronectin and perlecan anchor some of the proteins involved in NMJ development and function. Acetylcholinesterase (AChE) is tethered to the basal lamina via a covalent interaction with ColQ. Agrin and other nerve secreted proteins interact with postsynaptic proteins and help regulate gene expression (Hall and Sanes, 1993; Hoch. 1999)

The postsynaptic membrane is organised into a series of deep folds, with the AChRs found densely clustered at the top of the folds and the voltage gated sodium channels concentrated in the troughs and peri-junctional membranes (Flucher and Daniels, 1989; Vincent, 2008). Several neuromuscular junction specific cytoskeletal proteins co-localise with AChRs- rapsyn in particular is essential for AChR clustering. Rapsyn is immobilised by Muscle Specific Tyrosine Kinase (MuSK) linked proteins and contributes to MuSK-induced phosphorylation of AChR subunits (Lee et al., 2009). MuSK is expressed by skeletal muscle and is essential for the formation and maintenance of neuromuscular synapses (Burden et al., 2013). It is stimulated by an adaptor protein, Dok7 (Downstream of Tyrosine Kinase) from inside the muscle cell

(Yamanashi et al., 2012; Hallock et al., 2010) and by lipoprotein receptor related protein 4 (Lrp4), a receptor for the nerve secreted protein agrin, from outside the cell (Weatherbee et al., 2006; Zhang et al., 2008; Kim et al., 2008). MuSK contributes to AChR stabilisation and clustering through various intracellular kinase cascades (Wu et al., 2010) and phosphorylation of the AChR β subunit (Finn et al., 2003)

MuSK also has the receptor for Wnts, a family of secretory glycoproteins (Wu et al., 2010; Koles and Budnik, 2012). Wnt proteins are best known for roles in cell patterning and are essential in embryonic development (Koles and Budnik, 2012) but also contribute to AChR clustering.

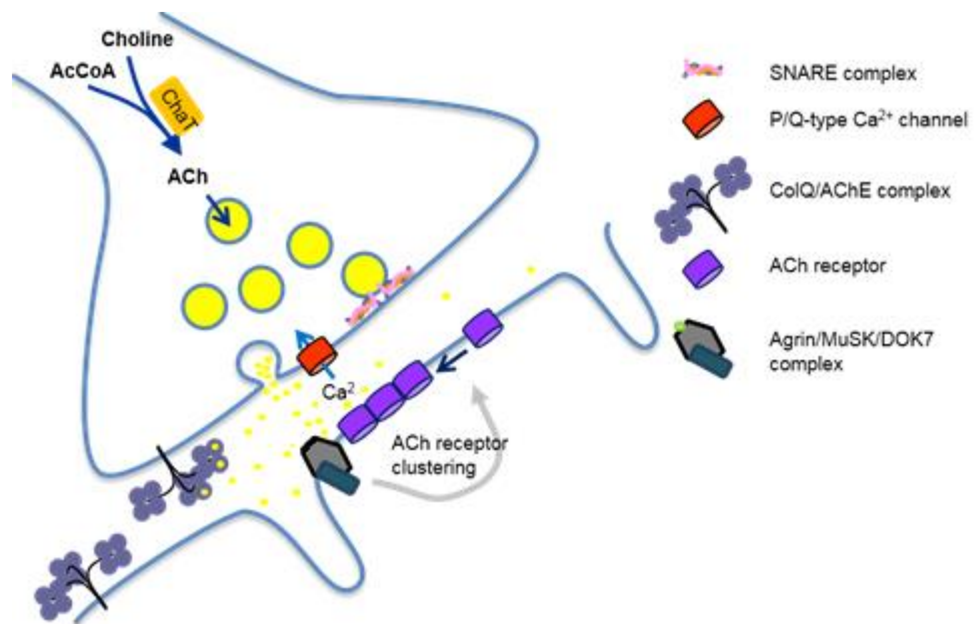


Figure 1.4 Structure of the neuromuscular junction

1.5 Disorders of Neuromuscular Transmission

Under normal conditions the end plate potential (EPP) that occurs at the neuromuscular junction in response to the binding of ACh and the subsequent opening of voltage gated Na^+ channels is more than sufficient to trigger an action potential – the extent to which the EPP exceeds that necessary to initiate the action potential is called the “safety factor” of neuromuscular transmission (Wood and Slater, 2001). Usually the degree of depolarisation exceeds the threshold by a factor of at least three (Finlayson et al., 2013). Under conditions of impaired neuromuscular transmission due to insufficient presynaptic ACh release or due to a defect of postsynaptic receptors, the EPP can become sub-threshold leading to failure of the EPP to generate an action potential and muscle weakness. Both inherited and acquired disorders can affect neuromuscular transmission.

1.6 Inherited disorders of Neuromuscular Transmission – the congenital myasthenic syndromes

A heterogeneous group of rare genetic disorders can affect neuromuscular transmission and these are termed the congenital myasthenic syndromes (CMS). Mutations have been identified in several of the genes encoding key proteins involved in development and maintenance of the neuromuscular junction and signal transmission. These disorders are typically inherited in an autosomal recessive fashion and can be classified

on the basis of the site of defective neuromuscular transmission – presynaptic, synaptic and postsynaptic. They are rare, with a prevalence of genetically confirmed cases in the UK of 3.8 per million (Finlayson et al., 2013).

Most patients with CMS present with symptoms early in life but some subtypes, such as DOK7 may present in childhood with walking difficulties (Finlayson et al., 2013). Symptoms of CMS include oculofacial weakness, limb weakness and bulbar and respiratory muscle weakness.

1.6.1 Presynaptic CMS

ChAT deficiency

Choline Acetyltransferase (ChaT) deficiency is due to impaired ACh synthesis at the presynaptic nerve terminal. It is characterised by sudden episodes of respiratory distress and apnoea (Ohno et al., 2001) and may also be associated with more generalised weakness (Finlayson et al., 2013).

1.6.2 Synaptic CMS

COLQ

Mutations in the gene encoding for the acetylcholinesterase (AChE) collagenic tail peptide, COLQ result in a loss of AChE- resulting in a prolonged lifetime of ACh in the synaptic cleft which in turn leads to prolonged EPPs, AChR desensitisation and an excitotoxic end plate myopathy (Ohno et al., 1998; Donger et al., 1998). Patients typically present with severe weakness from early infancy with frequent respiratory

involvement but some later onset milder phenotypes have also been described (Mihaylova et al., 2008). Acetylcholiesterases and 3,4 diaminopyridine can worsen symptoms and treatment is with salbutamol or ephedrine (Finlayson et al., 2013)

1.6.3 Post-Synaptic CMS

Postsynaptic mutations can affect the AChR subunits, leading to reduced channel expression or abnormal channel gating, or the proteins involved in AChR clustering such as rapsyn and DOK7.

1.6.3.1 AChR deficiency

AChR deficiency syndromes are the most common type of CMS (Finlayson et al., 2013) and are most frequently due to mutations in the gene encoding the ε subunit. The ε subunit of the AChR replaces the foetal subunit late in gestation; a continued low level of expression of the foetal γ subunit explains why null mutations of the ε subunit are compatible with life and often not very severe (Finlayson et al., 2013). Patients invariably have ophthalmoplegia and their neuromuscular weakness can worsen with intercurrent infection. The clinical severity can vary greatly, even within families (Barisic et al., 2011). Treatment is typically with pyridosgimine with 3,4 Diaminopyridine.

1.6.3.2 Escobar Syndrome

Escobar syndrome is an antenatal CMS due to a mutation in the CHRNG gene that codes for the foetal γ subunit. As it is a foetal syndrome myasthenic symptoms do not

persist after birth but it can cause a variety of developmental effects such as arthrogyroposis multiplex congenita with joint contractures (Hoffman et al., 2006; Barisic et al., 2011).

1.6.3.3 Slow channel syndrome

This is the only dominantly inherited CMS and is due to gain of function mutations that can involve any of the AChR subunits (Bairisc et al., 2011). Prolonged AChR opening leads to desensitisation block of the receptor and an excitotoxic endplate myopathy (Chaouch et al., 2012). It presents in childhood with evidence of delayed motor milestones and ocular muscle weakness (Barisic et al., 2011). Treatment is based on the use of open channel blockers such as fluoxetine or quinine (Harper and Engel 1998; Harper et al., 2003) as, similar to AChE deficiency syndromes, pyridostigmine and 3,4 Diaminipyridine can worsen symptoms.

1.6.3.4 Fast channel syndrome

Fast channel syndrome is a very severe form of CMS frequently causing acute crises on a background of severe weakness (Palace et al., 2012). There is an abnormally fast decay of the synaptic response to nerve impulses (Engel and Sine, 2005). This can be due to an abnormally rapid closing of the ion channel or a reduced probability of the channel opening or a combination of the two (Palace et al., 2012; Engel and Sine, 2005).

1.6.3.5 RAPSYN deficiency

Mutations in the rapsyn gene cause a deficiency of AChRs at the motor end plate. Both early and late onset cases have been reported (Burke et al., 2003). Early onset cases can be associated with respiratory and feeding difficulties but symptoms often improve in adult life. The late onset subtype may mimic autoimmune MG (Burke et al., 2003).

1.6.3.6 DOK 7

As mentioned above, DOK-7 binds to MuSK and amplifies the MuSK signal to downstream pathways that are important for AChR clustering and maintenance of the NMJ. DOK 7 associated CMS presents with a limb girdle pattern of weakness- typically as a deterioration in a child who has previously achieved normal motor milestones (Beeson et al., 2006; Palace et al., 2007). DOK 7 is a slowly progressive syndrome – likely because of a secondary myopathy (Finlayson et al., 2013). Ephedrine, given for many months, can have a longstanding beneficial effect in DOK7 CMS, most likely due to a downsteam effect from the stimulation of β2 adrenergic receptors (Lashley et al., 2010).

1.6.3.7 MuSK deficiency

As discussed above, MuSK lays a key role in the organisation of the postsynaptic cytoskeleton and AChR clustering. MuSK deficiency is a rare form of CMS with three different kinships described to date (Chevessier et al 2004; Mihaylova et al., 2009; Maselli et al., 2010)

1.6.3.8 GFPT1 and DPAGT1 mutations

Two recently described subtypes of CMS result from mutations in genes that encode glycosylation pathway enzymes – GPT1 and DPAGT1 – which encode gluocosamine-fructose-6-phosphate aminotransferase1 and UDP-N-acetylglucosamine-dolichyl-phosphate N-acetylglucosaminephosphotransferase respectively (Guergueltcheva et al., 2012; Belaya et al 2012). These disorders are associated with limb girdle weakness and often have tubular aggregates on muscle biopsy (Guergueltcheva et al., 2012; Belaya et al., 2012).

Other rarer CMS syndromes include those caused by mutations in SCN4A, the post synaptic sodium channel and other post synaptic proteins such as LAMB2 and PLEC1 (Maselli et al., 2009; Maselli et al., 2010 Barnwell et al., 1999). Rarely mutations in the gene encoding Agrin have been described in CMS (Maselli et al., 2012).

The CMS subtypes, the genes involved, their pathology and treatment are summarised in Table 1.2.

Site	Syndrome	Gene	Main pathology	Treatment
Presynaptic				
	ChAT deficiency	CHAT	Reduced ACh synthesis	Pyridostigmine, 3,4 DAP
Synaptic				
	AChE deficiency./ COLQ	COLQ	Paucity of AChE – increased lifetime of ACh in the synaptic cleft with AChR desensitisation and an end plate myopathy	Salbutamol/ Ephedrine Pyridostigmine can worsen symptoms
Postsynaptic				
Receptor deficiencies or abnormal synapse formation	Receptor deficiency	CHRNE CHRNA CHRNB CHRND	Reduced AChR expression	Pyridostigmine, 3,4 DAP
	Rapsyn	RAPSN	Impaired clustering of AChR	Pyridostigmine, 3,4 DAP
	DOK7	DOK7	Synaptopathy – small and simplified pre and post synaptic structure	Ephedrine and salbutamol
	GFPT1	GFPT1	Not ascertained, abnormal glycosylation of synaptic components	Pyridostigmine
	DPAGT1	DPAGT1		
	Escobar syndrome	CHRNG	Loss of foetal AChR	-
Abnormal kinetics	Slow channel	CHRNE CHRNA CHRNB CHRND	Prolonged AChR channel opening. Excitotoxic end plate myopathy	Fluoxetine, Quinidine
	Fast channel	CHRNE, CHRNA CHRND	Abnormally brief channel opening	Pyridostigmine 3,4 DAP
Rare subtypes		LAMB2	Reduced β2 laminin,- contributes to alignment of nerve terminal with postsynaptic regions	Ephedrine
		PLEC1	Reduced plectin, cytoskeletal linking protein	3,4DAP
		MuSK	Impaired MuSK – important for synapse formation and AChR clustering	Pyridostigmine 3,4 DAP
		SCN4A	Altered postsynaptic sodium channel function	Unclear
		AGRN	Agrin deficiency	Ephedrine

Table 1.2 Congenital myasthenic syndrome subtypes, associated genes, main pathology and treatment. Adapted from Finlayson et al., 2013

1.7 Acquired Disorders of Neuromuscular Transmission

The study of acquired autoimmune disorders of neuromuscular transmission is the main focus of this thesis. The most common acquired disorder of neuromuscular transmission is Myasthenia Gravis (MG). This is an autoimmune disorder, characterised in its most common form by IgG1 and IgG3 antibodies that are directed against the postsynaptic AChR receptor. Antibodies against other postsynaptic proteins including MuSK and LRP4 are found in a variable proportion of patients who typically do not have AChR antibodies. LEMS is another acquired disorder of neuromuscular transmission. Unlike MG, it is a presynaptic disorder with antibodies directed against VGCCs. Both of these disorders are discussed in detail below.

Botulism is a rare infectious disease that can also impair neuromuscular transmission. It is caused by exposure to neurotoxins produced by the anaerobe clostridium botulinum that is found in soil and aquatic sediment. There are three toxin subtypes, A B and E and they all act by cleaving SNARE proteins, preventing the close apposition of vesicles to the presynaptic membrane leading to a failure of synaptic vesicle release (Sobel, 2005; Shapiro et al., 1998). There are four types of botulism classified according to the mode of exposure- foodborne, wound, intestinal and iatrogenic (Shapiro et al., 1998, McLauchlin et al., 2006, Crowner et al., 2007, Chertow et al., 2006). It typically presents with descending paralysis causing ptosis, opthalmoplegia, bulbar weakness and proximal muscle weakness. Management is based on supportive care and the early administration of antitoxin.

1.7.1 Synaptic transmission in LEMS and MG

Synaptic transmission at the neuromuscular junction is abnormal in both MG and LEMS but the precise defect is quite different in the two disorders. In MG, the number of ACh quanta released from the presynaptic nerve terminal is unaffected, however each package of ACh that is released causes a smaller end plate depolarisation than normal (Elmqvist et al., 1964). There is also a reduction in MEPP amplitude (Elmqvist et al., 1964) and a loss of AChR binding sites (Fambrough et al., 1973) with reduced sensitivity of the end plate to ACh (Cull-Candy et al., 1978; Ito et al., 1978).

In contrast, in LEMs, the sensitivity of the end plate to ACh is preserved and cholinergic vesicle size and contents are normal as is evidenced by the normal MEPPs recorded in muscle samples from patients with LEMS (Elmqvist et al., 1964; Lang et al., 1984; Molenaar et al., 1982). However, there is a reduction in the number ACh quanta released in response to a single nerve impulse (Lambert and Elmqvist, 1971). In turn, the amplitude of the EPP evoked by nerve stimulation is reduced and can be sub-threshold to elicit a muscle action potential. Active zone particles, which represent VGCCs, are disorganised and fewer in number at LEMS affected NMJs (Fukuoka et al., 1987; Flink and Atchison. 2003).

1.8 LEMS

1.8.1 History of LEMS

The syndrome that subsequently became known as LEMS was first described in London in 1953 when a 47 year old man with lung carcinoma was found to have prolonged

apnoea after receiving the depolarising paralytic agent succinylcholine during surgery (Anderson et al., 1953). The patient's weakness improved markedly after removal of the lung tumour. Three years later a case series of six patients was described by Lambert and Eaton, after whom the disease was named (Lambert et al., 1956). These patients all had an obvious defect of neuromuscular transmission in the context of a malignancy and all had distinctive findings on neurophysiology consistent with reduced quantal release of acetylcholine from the presynaptic nerve terminal.

The initial case reports all described in LEMS in the context of a malignancy. The largest case series have revealed that between 50 and 60% of patients with LEMS have an underlying malignancy, most often a small cell lung cancer (Titulaer et al., 2008; O'Neill et al., 1988; Wirtz et al., 2003). The remaining patients 40-50% develop LEMS as an idiopathic autoimmune disease.

1.8.2 Epidemiology

LEMS is rare with a prevalence of 2.3 per million and an annual incidence of 0.5 per million (Wirtz et al., 2003). The relatively low prevalence to incidence ratio partially reflects the poor survival of patients with the paraneoplastic form of the disease. Paraneoplastic LEMS is more common in males and the median age of onset in this group is older than in patients with the non-paraneoplastic form of the disease (Titulaer et al., 2008). The age and sex distribution for non-paraneoplastic LEMS is similar to that in Myasthenia Gravis and there is also a genetic association with HLA-B8-DR3 in the two disorders (Titulaer et al., 2011).

1.8.3 Clinical Features

LEMS usually presents with proximal muscle weakness, affecting the legs initially in 80% of cases but upper limb weakness usually develops soon after (Titulaer et al., 2008). Weakness typically spreads distally and the speed of progression tends to be faster in patients with a malignancy (Wirtz et al., 2005). Although LEMS and MG are both autoimmune disorders of the neuromuscular junction, there are important distinctions that can be made clinically that help to distinguish the two diseases. The fluctuation of symptoms and fatigability, so typical of MG, is not as prominent in patients with LEMS. Moreover, facial and extraocular muscle weakness are not such a prominent feature of the disease in LEMS as they are in MG (Titulaer et al., 2011).

Although ocular symptoms can and do occur in LEMS, in contrast to MG, they are rarely seen in isolation (Titulaer et al., 2008). Respiratory failure in LEMS is uncommon but is recognised and can occasionally be the presenting symptom (Smith and Wald 1996). It is more commonly seen in association with a malignancy and frequently has been reported to occur after administration of a neuromuscular blocking agent (O'Neill et al., 1988; Smith and Wald 1996; Sanders et al., 1980; Gracey and Southorn 1987).

Autonomic dysfunction is found in 80-96% of patients with LEMS and can precede the onset of neuromuscular weakness (Waterman, 2001). Dry mouth is the most common symptom followed by erectile dysfunction in men and constipation. Orthostatic dysfunction, micturition difficulties and dry eyes are seen less frequently (Titulaer et al., 2011). Patients occasionally report a metallic taste (Mareska and Gutmann 2004).

The clinical examination of a patient with LEMS typically reveals proximal muscle weakness and the patient may have depressed or absent deep tendon reflexes – a reappearance of previously absent reflexes after exercise can be demonstrated in approximately 40% of LEMS patients (Odabasi et al., 2002).

	MG	LEMS
Presenting symptom	Ocular in 85%	Proximal lower limb in 85%
Fatigability	Common	Usually not demonstrable, weakness may improve after exercise
Reflexes	Normal	In 40% reflexes are absent but reappear after exercise
Electrophysiology	Normal CMAPS, decremental response to low frequency repetitive nerve stimulation	CMAPS have reduced amplitude, decremental response to low frequency stimulation but there is potentiation to high frequency stimulation or maximal voluntary contraction
Serology	85% of patients are positive for anti-acetylcholine receptor antibodies. A variable proportion of the remainder have anti-Muscle Specific Tyrosine Kinase antibodies (MuSK)	85-90% are positive for antibodies against the P/Q-VGCC
Association with malignancy/ tumour	15% of patients have an underlying thymoma	50-60% of patients have an underlying malignancy, most often small cell lung carcinoma
Symptomatic treatment	Acetylcholinesterase inhibitors (pyridostigmine)	3,4 Diaminopyridine

Table 1.3 Clinical features of LEMS and MG

1.8.4 Diagnosis of LEMS

Given its rarity and the often quite subtle physical signs, the diagnosis of LEMs can be difficult – the mean delay from first symptoms to correct diagnosis ranges from four months for paraneoplastic LEMS to 19 months for non paraneoplastic LEMS (Titulaer

et al., 2008). The delay from symptom onset to diagnosis has been reported to be as high as 4.2 years in non-paraneoplastic LEMS in non-specialist centres (Pellkofer et al., 2008). The classical triad of proximal muscle weakness, areflexia and autonomic dysfunction should prompt a high suspicion of LEMS but not all patients will have all three features (O'Neill et al 1988).

1.8.4.1 Neurophysiology

Clinical neurophysiology is the cornerstone of diagnosis in LEMS with the repetitive nerve stimulation test (RNS) being the main test used.

Compound muscle action potential (CMAP) amplitudes are typically low at rest and a decremental response to low frequency stimulation (2-5Hz) is observed (AAEM Quality Assurance Committee 2001). A decrement of 10% is considered abnormal and the majority of patients with LEMS will show a substantial decrement (Oh et al., 2005; Tim et al., 2000). This is not a specific feature for LEMS as it is also seen in MG. A more specific finding for LEMS is a short-lived incremental response after maximal exercise or high frequency stimulation (50Hz) (AAEM Quality Assurance Committee, 2001). (Figure 1.5). An increment of 100% has traditionally been used as the gold standard in LEMS and is very specific. However a lower increment of 60% has been shown to have a sensitivity of 97% in diagnosing LEMS and a specificity of 100% in excluding disorders such as MG (Oh et al., 2005), although other disorders that affect presynaptic neuromuscular transmission such as botulism can cause similar findings. This facilitatory response to high frequency stimulation has been postulated to be due to build-up of Ca^{2+} in the nerve terminal (Flink and Atchison 2003). Single fibre EMG is

usually abnormal in LEMS with increased jitter and block – however, this finding is not specific and does not help distinguish LEMS from MG. In mild cases of LEMS, the findings on neurophysiology can resemble those in MG, with normal CMAP amplitude, decrement at low frequency stimulation and little facilitation (Sanders, 2003).

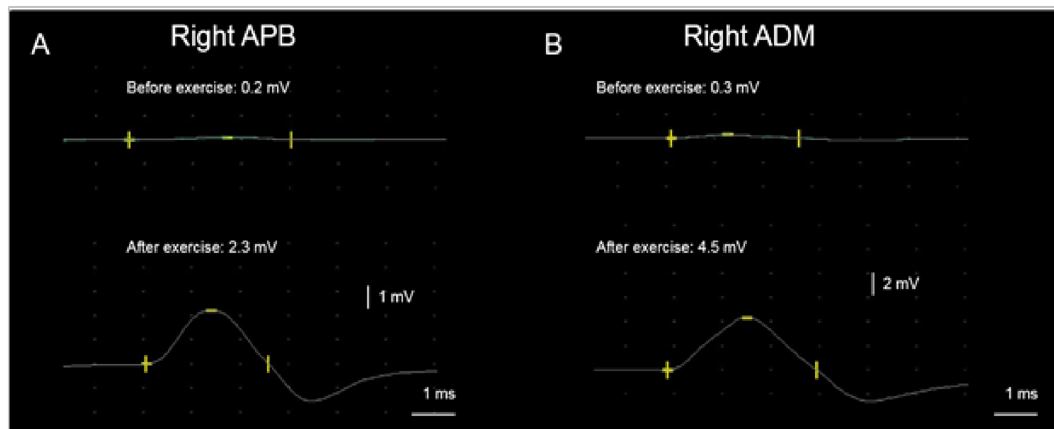


Figure 1.5 Electrophysiological features of LEMS taken from a patient seen at the National Hospital for Neurology and Neurosurgery. Stimulation of nerves supplying either the Abductor pollicis brevis (A) or abductor digiti minimi (B) was performed before (upper panel) and after 10 s of exercise (lower panel). Note the amplitude increased significantly after exercise indicating presynaptic dysfunction.

1.8.4.2 Serological Tests in LEMS

As was briefly mentioned in the introduction, antibodies to presynaptic voltage gated calcium channels are detected in approximately 90% of patients with LEMS and in almost 100% of patients with LEMS and SCLC (Lennon et al., 1995; Motomura et al., 1997). These antibodies are predominantly of the P/Q- subtype although antibodies to N-type VGCCs are also found in up to 33% of patients with LEMS (Motomura et al., 1997) and are detected by a radioimmunoprecipitation assay (Motomura et al., 1997).

Up to 3-5% of patients with SCLC that do not have clinical or electrophysiological features of LEMS may also have antibodies to presynaptic VGCCs (Titulaer et al., 2009). In particular patients with paraneoplastic cerebellar degeneration in association with small cell lung cancer may have positive LEMS serology in the absence of clinical evidence of neuromuscular dysfunction and VGCC antibodies have also occasionally been described in pure cerebellar ataxia (Gillhus, 2011).

Approximately 10-15% of patients with LEMS have no antibodies detectable against presynaptic VGCCs and are thus defined as "seronegative". However these patients also appear to have an antibody mediated disease as the clinical features of the disease and the response to immunomodulation are similar to seropositive patients (Nakao et al., 2002). Furthermore, passive transfer of "seronegative" LEMS IgG can transfer the electrophysiological features of the disease to mice (Burges et al., 1994). Antibodies against various other proteins at the neuromuscular junction have been found in a small proportion of seronegative LEMS patients (see below).

1.8.5 Screening for malignancy in LEMS

Given the strong association with malignancy, a diagnosis of LEMS should spark a thorough and systematic search for an underlying neoplasm. LEMS represents the most common paraneoplastic manifestation of SCLC and occurs in 1-3% of patients with this tumour (Titulaer et al., 2008). However, other tumours including non-small cell lung cancers, as well breast, bladder, renal and lymphoproliferative malignancies, have all been reported in association with LEMS. The neurological disorder typically presents

before the underlying malignancy, which may not be detectable initially- meaning that repeated screening for malignancy is required once a diagnosis of LEMS is made.

Screening by chest radiograph is insufficient and patients require CT and FDG PET scans and often also require bronchoscopy. If the initial search is negative, investigations should be repeated. Most malignancies are, however detected within 12 months and discovery of a tumour after 24 months is very unusual (Titulaer et al., 2008). Certain clinical clues can make the presence of an underlying tumour more likely; these include an associated cerebellar ataxia, a rapid speed of progression of neurological symptoms and the presence of other paraneoplastic antibodies (Titulaer et al., 2008). Antibodies against SOX-1 proteins (Sry like high mobility group box 1) have been shown to be a serological marker of SCLC in LEMS patients with a 95% specificity and 65% sensitivity (Sabater et al., 2008). Data from two national cohorts of LEMS patients was recently used to develop and validate a screening tool for predicting the presence of an underlying SCLC in LEMS patients. The Dutch English LEMS Tumour Association Predictor (DELTA-P) score is calculated as a sum score according to different categories as listed in Table 1.4, depending on certain clinical features at the onset of the disease including the presence of bulbar features, weight loss, erectile dysfunction, smoking history at onset and performance score. The Karnofsky performance score is used which calculated the extent to which people need assistance with the activities of daily living. The DELTA-P score is a sum score and ranges from 0-6. A score of 0-1 corresponds to a chance of underlying SCLC below 3% whereas a score of four or more corresponds to a greater than 90% chance of underlying SCLC (Titulaer et al., 2011). It is recommended that all newly diagnosed patients with LEMS undergo a thoracic CT followed by a FDG PET if the CT is normal. If this is also

normal, the DELTA-P score is calculated and the urgency and duration of further screening is determined by this (Fig 1.6).

	< 3months since symptom onset	Score
D	Dysarthria, dysphagia, bulbar and neck weakness	1
E	Erectile dysfunction	1
L	Loss of weight > 5%	1
T	Tobacco use at onset	1
A	Age of onset > 50 yrs.	
P	Karnofsky performance score < 60 (patients need at least some assistance with activities of daily living)	1

Table 1.4. DELTA P-score – (from Titulaer et al., 2011)

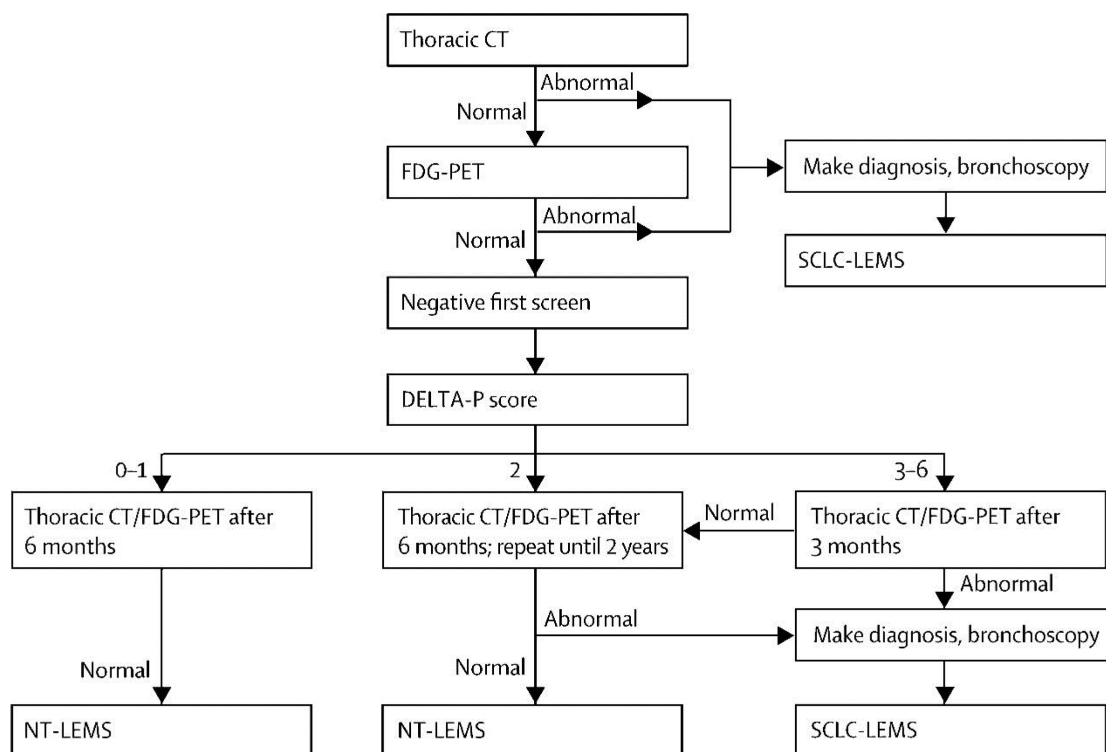


Figure 1.6 Screening for malignancy in LEMS (from Titulaer et al., 2011)

1.8.6 Treatment of LEMS

The treatment of LEMS depends on the presence of an underlying tumour. If a SCLC is present, treatment must be directed towards this. The neurological symptoms typically improve if the underlying tumour that is "driving" the immune process is removed (Chalk et al., 1990). SCLC treatment typically involves combination chemotherapy such as cisplatin and etoposide rather than surgery. Anthracycline based regiments with cyclophosphamide, doxorubicin and vincristine have also been widely used (Verschuuren et al., 2006). Occasionally, patients are also treated with radiotherapy.

Interestingly there are reports that patients with paraneoplastic LEMS have an improved tumour prognosis compared to patients with a malignancy but without the neurological deficit. Preliminary results of an ongoing prospective study showed that antibody positive SCLC patients with LEMS have a longer median survival than patients without LEMS (Maddison and Lang, 2008). However, larger numbers of patients are required to corroborate this finding.

Symptomatic treatment for LEMS includes drugs that prolong the nerve terminal action potential, allowing more acetylcholine to be released at the neuromuscular junction.

1.8.6.1. 3,4 Diaminopyridine

4-Aminopyridine has been used in LEMs but as it crosses the blood brain barrier, it can have unwanted CNS side effects (Keogh et al., 2011). 3,4 Diaminopyridine (3,4-DAP) is used more widely and is recommended by the Task Force of the European Federation

of Neurological Societies (EFNS) for the symptomatic treatment of both paraneoplastic and non-paraneoplastic LEMS (Titulaer et al., 2011). There have been four double blind randomized controlled trials investigating the effectiveness of 3,4 DAP in LEMS in 54 patients in total (McEvoy et al., 1989; Sanders et al., 2000; Wirtz et al., 2009; Oh et al., 2009) and the evidence was recently reviewed in a Cochrane systematic review (Keogh et al 2011). 3,4 Diaminopyridine was shown to result in a significant improvement in muscle strength, CMAP amplitude, Quantitative Myasthenia Score (QMG), LEMS classification and subjective symptom score in comparison to placebo. Perioral paraesthesia is a frequent but often transient side effect. At higher doses, 3,4-DAP can cause seizures and it is contraindicated in patients with epilepsy. Prolongation of the QT interval on ECG is also occasionally mentioned as a side effect and clinical and ECG monitoring is recommended at initiation of the drug and yearly thereafter (Lindquist et al., 2011).

1.8.6.2 Guanidine Hydrochloride

Guanidine hydrochloride also blocks potassium channels and has been used for the symptomatic treatment of LEMS (Verschuuren et al., 2006). Severe side effects including bone marrow suppression and renal tubular acidosis, chronic interstitial nephritis, cardiac arrhythmias and liver toxicity have precluded its widespread use (Sanders 2003). A small open label study outlined the use of low dose guanidine in combination with pyridostigmine in nine LEMS patients (Oh et al., 1997). Although guanidine was effective in controlling neurological symptoms, one third of the patients discontinued it because of severe gastrointestinal side effects and it is generally not used in LEMS unless 3,4-DAP is unavailable or contraindicated.

1.8.6.3 Immunomodulatory treatment

If, 3,4-DAP satisfactorily controls symptoms, no further treatment may be needed. However, immunomodulatory therapy may be required if symptoms do not respond to symptomatic therapy. Prednisolone and azathioprine are the most frequently used immunomodulatory agents and were prescribed in combination in 70% of patients with autoimmune LEMS in one series (Newsom-Davis and Murray 1984). Although this combination was effective in a retrospective study, these agents have not been tested in prospective randomised controlled clinical trials (Newsom-Davis and Murray 1984; Titulaer et al., 2001). The rationale for use of azathioprine as a steroid-sparing agent is extrapolated from evidence in MG, where it reduces the number of treatment failures and allows lower steroid doses to be used than treatment with prednisolone alone (Palace et al., 1998). Successful use of rituximab has also been reported in a small number of LEMS patients with severe weakness (Maddison et al., 2011; Pellkofer et al., 2009). There is one case report describing the use of cyclosporin A in LEMS that was associated in a resolution of symptoms (Yuste et al., 1996).

Plasma exchange and IVIG are reserved for acute treatment of severe weakness in LEMS. Plasma exchange showed a short-term clinical and electromyographic benefit in a small group of paraneoplastic and autoimmune LEMS patients (Newsom Davis and Murray 1984). The clinical benefits may not be seen for up to 10 days, longer than is typical in MG (Newsom-Davis 1998). A randomized double-blind placebo-controlled crossover trial showed that IVIG resulted in significant improvement in muscle strength and a decline in VGCC antibody titres (Bain et al., 1996). The benefit peaked at 2-4 weeks and declined by 8 weeks. Other data regarding the benefit of IVIG are scant but

case reports have suggested that IVIG may be beneficial (Bird, 1992; Takano et al., 1994).

1.8.7 Pathomechanisms of LEMS

Studies of muscle biopsy samples from patients with LEMS provided some of the initial insights into the pathomechanisms of the disease. Electrophysiological studies showed findings consistent with those found using EMG; EPP amplitudes are small but increase in size following high frequency stimulation (Cull-Candy et al., 1980; Lambert and Elmqvist 1971) whereas MEPP amplitudes are not reduced (Elmqvist and Lambert 1968; Lambert and Elmqvist 1971; Cull-Candy et al., 1980).

Light microscopy analysis of tissue biopsies from LEMS patients does not reveal any gross abnormalities of the motor nerve terminal (Engel and Santa, 1971). There are conflicting reports of abnormalities of the postsynaptic membrane on electron microscopy in LEMS patients (Flink and Atchison 2003) with some reports suggesting hypertrophy of the postsynaptic region (Engel and Santa 1971) and others suggesting decreased areas and lengths of postsynaptic membrane folds (Hesselmans et al., 1992; Tsujihata et al., 1987). This discrepancy may reflect differences in the duration of LEMS in the patients studied (Flink and Athchison, 2003) – hypertrophy of the postsynaptic membrane may reflect a response to chronic impairment of neuromuscular transmission. Freeze-fracture studies show a paucity and disorganisation of presynaptic membrane active zones in biopsy samples from LEMS patients (Engel et al., 1991). As active zones are topographically related to the sites of synaptic vesicle exocytosis and are thought to represent the VGCCs on the presynaptic membrane (Fukuoka et al.,

1987; Engel et al., 1991), these findings originally suggested that the pathology in LEMS is due to dysfunction of presynaptic VGCCs.

The pathomechanisms of LEMS have been further elucidated by careful clinical observation of patients with the disease and by the study of the action of LEMS IgG on a variety of model systems in the laboratory. One of the first indications that LEMS was an autoimmune disease came from observations that patients with LEMS also had other autoimmune disorders such as pernicious anaemia, coeliac disease and Type I Diabetes Mellitus and that up to 45% of patients have other organ specific antibodies detectable on serological assay (Gutmann et al., 1972; Takamori et al., 1972; Lennon et al., 1982; O Neill et al., 1988). The favourable response of LEMS patients to immunomodulatory therapy also provided evidence regarding the autoimmune nature of the disease (Lang et al., 1981; Newsom-Davis et al., 1984).

1.8.7.1 Passive transfer experiments

It was passive transfer experiments that provided definitive proof that LEMS was autoimmune in nature. The electrophysiological features of LEMS; namely reduced end plate potentials and facilitation at high frequency stimulation can be transferred to mice in a dose-dependent fashion by injecting them with IgG from LEMS patients (Lang et al., 1981; Lang et al., 1983; Kim et al., 1986). The passive transfer of LEMS IgG is equally effective in C5 deficient mice, indicating that late complement components are not required for LEMS activity (Prior et al., 1985; Lambert and Lennon 1988).

Passive transfer also results in morphological changes to the presynaptic end plate with selective depletion of active zones and active zone particles seen on freeze fracture microscopy (Fukunaga et al., 1983). These findings are similar to those seen in biopsies from LEMS patients (Engel et al., 1989; Engel et al., 1991).

As mentioned above, autonomic symptoms are a significant feature in LEMS patients. Synaptic vesicle release from both parasympathetic and sympathetic neurons is also reduced by passive transfer of LEMS IgG to mice (Waterman et al., 1997).

1.8.7.2 Antigenic targets in LEMS – the Voltage Gated Calcium Channel

Initially the target of autoantibodies in LEMS was unknown. An effect on presynaptic Ca^{2+} entry or function was first thought to be a feature of LEMS when it was found that the amplitude of end plate potentials (EPP) of intercostal muscle preparations from LEMS patients increased during repetitive nerve stimulation and with an increase in external calcium concentration (Lambert et al., 1971). The rate of spontaneous release from LEMS motor terminals in the setting of reduced Ca^{2+} concentration is normal (Lang et al., 1987), suggesting that intraterminal processes involving Ca^{2+} are preserved and that it is the entry of Ca^{2+} into the presynaptic VGCCs that is affected by LEMS IgG (Flink and Atchison, 2003). The finding that calcium currents in nerve preparations from animals passively treated with LEMS IgG were reduced gave further weight to the theory that LEMS antibodies targeted presynaptic VGCCs (Xu et al., 1998; Smith et al., 1995).

A number of studies involving the incubation of LEMS IgG in a variety of cell types gave more direct evidence that the current through VGCCs is reduced by LEMS IgG. Approximately 50% of LEMS patients have an associated SCLC (O'Neill et al., 1988; Titulaer et al., 2011) and SCLC cells contain functional VGCCs (Roberts et al., 1985). Incubation with LEMS IgG has been shown to inhibit K^+ induced $^{45}Ca^{2+}$ flux through SCLC channels (Roberts et al 1985; de Aizpurura et al., 1988). Moreover, electrophysiological studies of SCLC cell lines have shown that LEMS IgG can reduce the amplitude of currents through VGCCs by 58-70 % in a dose dependent manner (Meriney et al., 1996; Viglione et al., 1995).

Calcium channel currents in other model systems including anterior pituitary cells, adrenal chromaffin cells and neuroblastoma cells have also been shown to be reduced after incubation with LEMS IgG (Login et al., 1987; Kim and Neher 1988; Grassi et al., 1994;) as has VGCC-mediated currents in motor neurones and cerebellar neurones (Garcia et al., 1996; Pinto et al., 1998). Ca^{2+} currents seem to be preferentially affected by LEMS IgG – current through other channels is not reduced (Garcia and Beam, 1996)

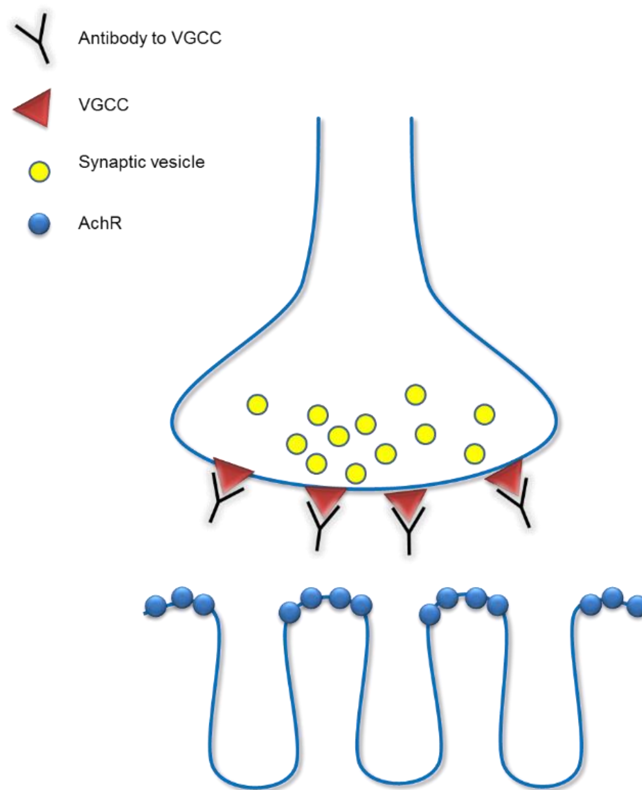


Figure 1.7 Schematic diagram highlighting antibody binding sites in LEMS

1.8.7.3 VGCC specificity in LEMS

The specific VGCC antibodies affected by LEMS IgG have been elucidated by analysing the effects of different neurotoxins that are specific for individual VGCC in cells treated with LEMS IgG. In general, it is thought that LEMS IgG predominantly reduces currents through P/Q- type VGCCs but there have been conflicting results in the literature.

In SCLC cells, ω-AgaIVA was much less effective in reducing currents mediated by P/Q-type channels after incubation with LEMS IgG, suggesting that current through these channels was reduced by LEMS antibodies (Viglione et al., 1995). Similar

findings have been seen in bovine adrenal chromaffin cells (Kim et al., 1998). Other studies have examined the effect of LEMS IgG on Ca^{2+} currents in cultured motor neurones (Garcia et al., 1996). Currents in motor neurons were reduced to a greater extent than those in sensory neurons. Given that the predominant type of VGCCs in sensory neurons is believed to be N-type channels whereas P/Q-type are believed to be the predominant type in motor neurones, these data were consistent with a preferential effect of LEMS IgG on P/Q-type channels (Garcia et al., 1996).

A study using electric ray organ synaptosomes showed that LEMS IgG inhibits K^+ evoked release of ACh, and the same IgG samples reduced P/Q- but not N-type currents in bovine adrenal chromaffin cells (Satoh et al., 1998). Furthermore, neurotransmitter release from autonomic nerves subserved by P/Q- but not by N-type VGCCs are reduced following exposure to LEMS IgG (Houzen et al., 1998).

More compelling evidence that LEMS IgG is directed against P/Q-type VGCCs came from the work of Pinto et al. This group examined the effect of overnight incubation of LEMs IgG on cell lines that were stably transfected with cDNAs encoding P/Q-or N-type channels (Pinto et al., 1998). Currents mediated by P/Q-type VGCCs (the 10-13 cell line) were reduced by LEMS IgG but there was no reduction in current through the N-type VGCCs (G1A1 cell line) even though some of the patient sera contained antibodies that precipitated N-type VGCCs. The same group examined the effect of LEMS IgG on calcium currents in cultured cerebellar neurones. The magnitude of the whole-cell calcium current was not significantly different in neurones incubated with LEMS IgG. However, pharmacological analysis with ω-Aga IVA and conotoxin GVIA,

toxins revealed that there was a large reduction in P/Q type current but current through N-type channels was unaffected (Pinto et al., 1998).

The effect of LEMS IgG on N-type VGCCs is however, far from certain with conflicting data in the literature. Although the studies described above have shown that N type currents are not affected by LEMS IgG other studies have suggested a reduction in N-type currents in SCLC and neuroblastoma cell lines after incubation with LEMS IgG (Grassi et al., 1994; Meriney et al., 1996).

The reason for these discrepancies is unknown. It is possible that an effect of LEMS on N-type channels was not seen in transfected HEK cells for various reasons including splice variations, different subunit combinations or altered density of transfected channels (Flink and Atchison 2003).

1.8.7.4 Upregulation of VGCCs not normally involved in neurotransmission in LEMS

Alterations in the contribution of different VGCCs to Ca^{2+} in LEMS treated neurons have been described in the literature. With current through P/Q-type channels diminished there is an increase in current through channels that are not normally involved in synaptic transmission.

An increase in the amplitude of R-type current has been reported by Pinto et al (Pinto et al., 1998). There are also reports that an L-type current is unmasked in LEMS treated nerve terminals (Giovannini et al., 2002; Smith et al., 1995; Xu et al., 1998). L-type

currents are not normally involved in neuromuscular transmission (Flink and Atchison 2002). Acute exposure of diaphragm phrenic nerve preparations to LEMS IgG does not increase the amplitude of L-type current but chronic exposure to LEMS IgG by passive transfer results in the emergence of a dihydropyridine sensitive L type current in nerve muscle preparations (Flink and Atchison 2002).

The prolonged period of time necessary for L type calcium currents to become involved in synaptic transmission suggests that the L-type channel involvement is not merely due to unmasking of usually silent channels that are already present on the nerve terminal; it is likely that formation, assembly and/or trafficking of new channel are required.

1.8.8 Immunoprecipitation assays in LEMS

Immunoprecipitation assays have also provided evidence regarding the VGCC specificity of LEMS IgG and are used clinically to aid with the diagnosis of LEMS. Although various immunoprecipitation assays are used – they all essentially rely on the same technique. Firstly, neuronal cell membranes are solubilised and crude fractions of membrane proteins (including VGCCs) are extracted. Subsequently a radiolabelled ligand that binds to specific VGCC is used to label the VGCC. The radiolabelled VGCCs are then incubated with IgG from LEMS patients or controls and the levels of radioactivity are calculated; this correlates with specific antibody binding to VGCC (See figure 1.8.).

1.8.8.1. Different immunoprecipitation assays used in LEMS

The first immunoprecipitation assay using LEMS serum described involved the use of ω-conotoxin – a 27 amino acid amino acid peptide derived from the marine snail Conus geographus that specifically blocks N-type calcium channels. Calcium channels from the IMR-32 neuroblastoma cell line, which expresses N-, L-, and T- type calcium channels were labelled with ω-conotoxin and were incubated with LEMS IgG (Sher et al., 1989; Grassi et al., 1994). This initial study showed that over 90% of IgG samples from LEMS patients bound to labelled ^{125}I- ω -Cg Tx-VGCC complexes. However, there was a high false positive rate of 9% (Sher et al., 1989). A study performed at the same time by a different group also used conotoxin but extracted VGCCs from human SCLC cells (Lennon and Lambert 1989). Using this method, antibodies that bound to calcium channels were found in 52% of LEMS patients, particularly in those who had an associated malignancy. A control extract of colonic carcinoma showed negative results, implying that LEMS antibodies targeted an antigen on SCLC cells (Lennon and Lambert 1989). This method, although less sensitive seemed more specific, as antibodies were not detected in healthy control samples or in patients with other neurological diseases. Radioimmunoprecipitation assays using ^{125}I- ω -Cg labelled VGCC from a human neuroblastoma cell lines yielded similar results with approximately 44% of LEMS patients, both tumour associated LEMS (T-LEMS) and and non tumour associated, (NT-LEMS) testing positive (Leys et al., 1989; Leys et al., 1991). The test was not particularly specific, however, as a relatively high proportion of patients with other autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematous also had a positive result on the radioimmunoprecipitation assay.

Toxins that are specific for P/Q- type calcium channels have been used more recently and have been found to have a much greater sensitivity and specificity in detecting antibodies in LEMS patients; -ω-conotoxin MVIIC from the Conus magus snail inhibits calcium currents through P/Q-type calcium channels at micromolar concentrations (Wheeler et al., 1994).

Lennon et al used ^{125}I-ω-conotoxin MVIIC to label calcium channels extracted from SCLC cells and from human cerebellar and cortical tissue. Over 90% of NT LEMS patients and 100% of T-LEMS patients had antibodies detected using this assay (Lennon et al., 1995). In addition, approximately half of the LEMS patients also had antibodies that precipitated N-type channels, a finding which may reflect the homology that exists between different α subunits. Patients with associated N-type antibodies were more likely to have an underlying malignancy (Lennon et al., 1995). However a small number of patients with amyotrophic lateral sclerosis (ALS) were also found to have antibodies against P/Q- and N-type calcium channels. Suenaga et al found that antibodies to P/Q-type channels were present in 74% of all LEMS patients and antibodies to N-type channels were detectable in 62% (Suenaga et al., 1996). Motomura et al reported the highest specificity assay for detecting antibodies to P/Q-type channels in LEMs patients (Motomura et al., 1997). In this study, 92% of LEMS patients had antibodies to P/Q – type channels. Antibodies to N type channels were detected in 33% but all of these patients also had P/Q- type antibodies detectable; suggesting that N- type antibody positivity reflected a shared epitope binding site. All controls including those with MG, RA and SLE were negative for VGCC antibodies.

The concentration of VGCC used in this assay was low (100 pM) and therefore only antibodies of high avidity could be measured.

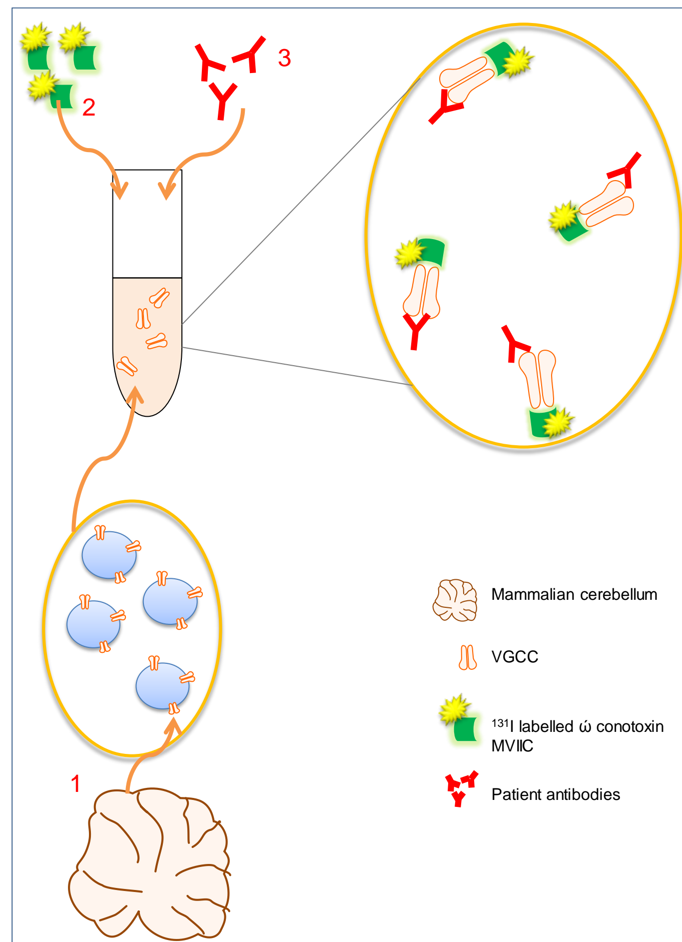


Figure 1.8 Radioimmunoprecipitation assay for LEMS (schematic diagram). (1) Neuronal cell membranes (such as primate cerebellar cells) are solubilised and crude fractions of membrane proteins (including VGCCs) are extracted. (2) A radiolabelled ligand (such as ^{131}I-MVIIC) that binds to specific VGCC is used to purify the VGCC form the other membrane proteins. (3)The radiolabelled VGCCs are then incubated with IgG from LEMS patients or controls and the levels of radioactivity are calculated; this correlates with specific antibody binding to VGCC

1.8.8.2 Synthetic peptides and recombinant proteins

Synthetic peptides and recombinant proteins have been used to demonstrate that the extracellular S5-S6 linker domain III of the P/Q-type VGCC is a major epitope recognised by autoantibodies in LEMS patients (Iwasa et al., 2000). Sera from other LEMS patients have shown antibodies positive for domains II and IV of the S5S6 linker domain (Takamori et al., 1997)

A polyclonal peptide antibody generated against the S5-S6 loop of Domain III of the P/Q- VGCC has been shown to inhibit Ca^{2+} current through P/Q- and N-type channels in cerebellar granular cells and also in HEK 293 cells expressing either P/Q- or N- type VGCCs subunits (Liao et al., 2008).

1.8.9 Mechanism of action of LEMS IgG

It has been proposed that LEMS IgG causes a reduction in VGCCs by antibody mediated cross-linking followed by internalization. As discussed above, LEMS IgG has been shown to reduce peak density of currents mediated by P/Q-type VGCCS in HEK cells (Pinto et al., 2002). The maximum current at any given potential is dependent on several factors including the single channel conductance, the voltage dependence of activation and the voltage dependence of inactivation (Pinto et al., 2002). LEMS IgG does not affect the voltage dependence of activation/inactivation (estimated from the current/voltage relationship; Pinto et al., 2002), nor is mean single channel conductance altered by LEMS IgG (Magnelli et al., 1996). These findings suggest that LEMS IgG reduces current through VGCCs by reducing the surface expression of VGCCS (Pinto et al., 2002).

Morphological studies of the effect of LEMS at the motor end plate have shown that the reduction in the number of active zone particles is preceded by a shortening of the distance between adjacent active zone particles as might be expected if cross linking occurs (Engel et al., 1989; Fukuoka et al., 1987). Moreover, monovalent IgG does not result in aggregation and depletion of active zone particles- giving further support to the theory that antibodies in LEMs cause crosslinking which in turn causes depletion of VGCC (Nagel et al., 1988)

1.8.10 Seronegative LEMS –other target antigens

Approximately 10-15% of patients with LEMS do not precipitate antibodies against VGCCs (Nakao et al., 2002; Motomura et al., 1997, Titulaer et al., 2011). Nevertheless, the pathology of "seronegative" LEMS is still thought to be autoimmune and various alternative antigenic targets have been suggested. There have been rare reports of antibodies to the β subunit of the VGCC detected in LEMS patients (Rosenfeld et al., 1993) but the pathogenicity of these antibodies is unknown as the β subunit is mainly intracellular.

Antibodies to the N terminus of synaptotagmin 1 have been detected in another group of LEMS patients including both VGCC antibody positive and seronegative LEMS patients (Takamori et al., 1995). Immunising animals against the extracellularly exposed segment of synaptotagmin can induce an animal model of LEMS (Takamori et al., 1994; 2000. It is therefore thought that synaptotagmin could be a target antigen in a small proportion of patients with LEMS (Takamori et al 2008).

The presynaptic muscarinic acetylcholine receptor (M1 AChR) has also been suggested as an alternative antigenic target in LEMS. This is a G protein coupled receptor that modulates ACh release at the adult neuromuscular junction. Stimulation of the M1 receptor enhances P/Q-type VGCC mediated ACh release (via a cascade involving phospholipase C, protein kinase C and the generation of diacylglycerol from phosphatidyl inositol 4,5 bisphosphate) (Santafe et al., 2006). The M1 mAChR is expressed in the nerve terminal and is partially exposed extracellularly (Garcia et al., 2005). Fourteen of 20 anti VGCC antibody positive LEMS patients and five "seronegative" LEMs patients were found to be positive for antibodies against the M1 AChR in one study (Takamori et al., 2007). IgG from patients positive for anti M1 mAChR antibodies and negative for anti VGCC antibodies was shown to transfer the electrophysiological features of LEMS to mice (Nakao et al., 2002; Takamori et al., 2007).

More recently antibodies against the protein ERC1 were identified by a recombinant ELISA assay and a cell based assay in a single patient with non-tumour LEMS (Huijbers et al., 2013) who also had antibodies to VGCC. ERC1 contributes to the formation of presynaptic active zones and has a supportive role in promoting ACh exocytosis (Hida and Ohtsuka, 2010). Additional testing of 58 other LEMS patients as well as disease and healthy controls revealed no other cases suggesting that ERC1 is a rare antigen in LEMS (Huijbers et al., 2013).

1.8.11 LEMS – further questions

Although the pathology of LEMS has been extensively investigated in the past, questions still remain and I intend to address a number of these in this thesis.

Firstly, I wished to visualise the binding of LEMS IgG to neurons in culture. As outlined above, a radioimmunoprecipitation assay is currently used for the detection of LEMS antibodies. Cell-based assays have been increasingly used in recent years to specifically detect antigenic targets in a variety of autoimmune diseases including limbic encephalitis, NMDA receptor encephalitis and in MG. I wanted to use immunohistochemical techniques to detect cell surface binding of IgG from LEMS patients to neurons in culture in an effort to more specifically define the antigenic target in LEMS.

I also wanted to clarify the effect of LEMS IgG on the presynaptic nerve terminal. Many of the studies examining the pathophysiology of LEMS to date have been indirect, using post-synaptic responses as a measure of presynaptic vesicle release. I decided to specifically analyse the effect of LEMS IgG on synaptic vesicle release. To do this I planned to use a technique whereby synaptic vesicles in cultured neurons could be fluorescently labelled and the rate of spontaneous and action potential (AP) evoked release could be directly assessed after exposure to LEMS IgG

Finally, I wished to define the VGCC specificity of LEMS IgG. Although the studies reviewed above provide strong evidence that LEMS IgG acts on P/Q-type VGCCs, and this provides a compelling explanation for a reduction in neurotransmitter release, a direct causal link has not been demonstrated. That is, it remains possible that LEMS

IgG has two separate actions, one on P/Q-type VGCCs, and another one on neurotransmitter release through another mechanism, mediated by another IgG species recognising an antigen yet to be identified. Moreover, the role of N-type antibodies in LEMS is incompletely understood. Up to 33% of LEMS patients have antibodies against N-type channels but currents through N-type VGCCs expressed in HEK cells have not been shown to be affected by LEMS IgG. There are discrepancies in the literature, with some studies showing a reduction in current through N-type VGCCs exposed to LEMS IgG. I therefore decided to probe the effect of LEMS IgG on synaptic vesicle release in cultures lacking P/Q type VGCCs from a *Cacna1a*$^{-/-}$ mouse. This experiment had two goals: first, to test directly the requirement for IgG binding to P/Q-type VGCCs to decrease neurotransmission; and second, to clarify whether LEMS antibodies affect neurotransmitter release via N-type VGCCs.

1.9 Myasthenia Gravis

Myasthenia Gravis is a more common autoimmune disorder of the neuromuscular junction, characterised by painless, fatigable muscle weakness. There is a large cohort of MG patients attending the NHNN and I wished to take advantage of this to tackle some of the unanswered questions regarding the clinical management of this disorder.

Firstly I will outline the clinical features of Myasthenia Gravis.

1.9.1 Epidemiology

The incidence of MG varies among age, gender and ethnic groups (Alshekhlee et al., 2009). MG is the commonest disorder of the NMJ with a prevalence estimated at 15 per 100,000 although it is still under diagnosed in the older population (Vincent et al., 2003). The incidence of MG is bimodal with different sex ratios in the two age groups in which it presents most commonly: young adults and elderly patients: a female to male ratio of approximately 2:1 is seen in young adults, and a reversed sex ratio in the older group that accounts for approximately 60% of cases (Roberston et al., 1998; Phillips, 2003). An increasing frequency of MG in the elderly has been noted in the past decade (Pakzad et al., 2011; Matsuda et al., 2005)

1.9.2 Classification of MG

Myasthenia Gravis is classified in a number of different ways: according to antibody specificity (AChR antibody MuSK antibody, LRP4 antibody), thymic histology (thymoma, non thymomatous – hyperplastic, atrophic or normal), age of onset (< or > 40 years) distribution of clinical symptoms (ocular or generalised), and severity. The Myasthenia Gravis Foundation of America (MGFA) clinical classification system (Jaretzki et al., 2000) classifies MG based on severity of symptoms.

Class	Description	Subtypes	
I	Ocular Muscle weakness		
II	Mild weakness affecting muscles other than ocular muscles (but may also have ocular muscle weakness of any severity)	IIa Predominantly affects limb, axial muscles or both*	IIb Predominantly affects oropharyngeal or respiratory muscles or both**
III	Moderate weakness affecting muscles other than ocular muscles (but may also have ocular muscle weakness of any severity)	IIIa Predominantly affects limb, axial muscles or both *	IIIB Predominantly affects oropharyngeal or respiratory muscles or both **
IV	Severe weakness affecting muscles other than ocular muscles (but may also have ocular muscle weakness of any severity)	IVb Predominantly affects limb, axial muscles or both *	IVb Predominantly affects oropharyngeal or respiratory muscles or both **
Class V	Intubation with or without mechanical ventilation except when for post-operative management The use of a feeding tube without intubation places the patient in Class IVb		

Table 1.5 MGFA classification of MG severity

** May also have a lesser involvement of oropharyngeal muscles*

*** May also have a lesser or equal involvement of limb or axial muscles*

1.9.3 History of MG

The first reported cases of MG date from the 1600s. The English physician Willis described a patient with “fatigable weakness” involving the ocular and bulbar muscles (Jayam-Trouth et al., 2012). The first paper to deal entirely with MG was published in 1878 by Wilhelm Erb, a German physician (Jayam-Trouth et al., 2012). The name “myasthenia gravis” dates from the description by Jolly at the Berlin society meeting in the 1890s of two cases under the name “myasthenia gravis pseudo paralytica” (Jolly, 1895).

1.9.4 Autoimmune nature of MG

MG is one of the best characterised autoimmune diseases. The autoimmune nature of the disease was proposed in the late 1950s/early 1960s (Nastuk et al., 1959) and the typical features of MG were successfully passively transferred by injection of IgG from an MG patient to mice in 1975 (Toyka et al., 1975).

As is the case with LEMS, the association of other autoimmune diseases with MG provides further support for its autoimmune nature. Thyroditis, rheumatoid arthritis and SLE are all seen more frequently in MG patients than in the general population (Sieb et al., 2013). More recently neuromyelitis optica spectrum disorder (NMOSD) has been described to occur in association with MG at least 70 times more frequently than would be expected by chance (Leite et al., 2012). There are currently four different antigenic targets described in MG.

1.9.4.1 AChR Antibodies in MG

Antibodies that bind to AChRs in the synaptic cleft are present in the serum of approximately 85% of patients (Vincent 2002; Fambrough et al., 1973; Toyka et al., 1977) and are detected by a radioimmunoprecipitation assay using ^{125}I-α- bungarotoxin (Lindstrom et al., 1976). Antibodies against the AChR are of IgG1 or IgG3 subtype and can activate complement which results in focal destruction of the post synaptic membrane by the membrane attack complex (Meriggioli and Sanders, 2009). Anti-AChR antibodies also cross link AChRs leading to an increase in their rate of destruction and may also inhibit the function of AChR directly (Vincent, 2002).

1.9.4.2 MuSK MG

Antibodies to Muscle Specific Tyrosine Kinase (MuSK) can be detected in approximately 15-20% of MG patients that test negative for antibodies to AChR.

MuSK is a transmembrane postsynaptic protein essential for clustering of AChRs at the post synaptic receptor during development (DeChiara et al., 1996) and also for the maintenance of the neuromuscular junction (Kong et al., 2004). The frequency of MuSK positive MG varies according to geographic location with the highest prevalence about 40° north of the equator (Evoli and Padua, 2013). It is also more common in women than men and it typically presents below the age of 40 (Oh, 2009).

MuSK antibody positive patients are more likely to have early bulbar and respiratory symptoms with less severe limb involvement (Evoli and Padua, 2013; Farrugia et al., 2006). The disease process can be rapidly progressive leading to myasthenic crisis (Guptill et al., 2011; Evoli and Padual, 2013). Muscle atrophy, particularly of the facial and tongue muscles is a well-recognised feature of MuSK MG (Farrugia et al., 2006).

Thymic pathology in MuSK positive patients usually does not show lymphocytic infiltrates or complement deposition and thymectomy has not been shown to be helpful in the treatment of the disease (Evoli and Padua, 2013). In contrast to the IgG1 and IgG3 subclass antibodies that are seen in AChR positive MG, the antibody subtype in MuSK MG is IgG4. This antibody subclass does not cause complement activation and does not crosslink antigens (Gomez et al., 2010).

The pathogenicity of anti- MuSK antibodies has been confirmed by passive transfer experiments but remains incompletely understood. Passive transfer of MuSK antibodies to mice has been shown to cause reductions in post synaptic AChR packing in a dose-dependent fashion, associated with depletion of MuSK from the post synaptic membrane (Cole et al., 2008). MuSK antibodies have also been shown to block binding of collagen Q to MuSK (Kawakami et al., 2011). It has also been suggested that the weakness seen in MuSK MG is not only due to a reduction in post synaptic AChR numbers but also because of a lack of presynaptic compensatory increase in quantal release of ACh, something that is seen in AChR ab-positive MG (Viegas et al., 2012) suggesting that inhibition of retrograde signalling may be an important aspect of the disease (Koneczny et al., 2013).

1.9.4.3 LRP 4 antibodies

Approximately 10% of patients with MG do not have antibodies to AChR or MuSK (Zouvelou et al., 2013). Recently, antibodies against Lipoprotein related protein 4 (LRP-4) have been detected in the serum of a varying proportion of patients with "double-seronegative" MG (Zhang et al., 2012; Higuchi et al., 2011; Pevzner et al., 2012, Zouvelou et al., 2013).

LRP-4 is a postsynaptic protein that is a receptor for agrin and is essential for neuromuscular junction formation (Wu et al., 2012). Binding of agrin to LRP4 stimulates myotubes to form clusters of AChRs linked via rapsyn (Kim et al., 2008; Weatherbee et al., 2006). Antibodies against LRP-4 are mainly of the IgG1 subtype and

have the potential to inhibit interaction between neural agrin and the extracellular portion of LRP-4 (Higuchi et al., 2011). Mice immunized with the extracellular domain of LRP-4 generate anti LRP4 antibodies and exhibit MG type symptoms indicating that these antibodies are likely to be pathogenic (Shen et al., 2013)

1.9.4.4 Autoantibodies to agrin

Very recently autoantibodies to agrin were detected in seven of a cohort of 93 patients with MG, including five patients who also had AChR antibodies and two who were negative for AChR, MuSK and LRP4 antibodies (Zhang et al., 2014). Sera from these patients was shown *in vitro* to inhibit agrin induced MuSK phosphorylation and AChR clustering (Zhang et al., 2014). The significance of antibodies to agrin is unknown and further, larger studies are required to determine the frequency of these antibodies in MG patients.

1.9.5 Seronegative MG

A small proportion of patients remain persistently negative for both AChR and MuSK antibodies. These "seronegative" patients are similar to patients positive for AChR antibodies with regards to clinical features and thymic histology. Many of these patients do in fact have antibodies detectable against AChR receptors that are expressed in a cell line with rapsyn which has a role in clustering of AChR (Leite et al., 2008).

1.9.6 Clinical presentation of MG

MG presents with painless fatigable muscle weakness. The pattern of muscle involvement varies but the ocular muscles are involved first in 85% of cases. Up to 20% of patients with MG have prominent bulbar symptoms early on in the disease (Grob et al., 1987). Limb weakness in MG can affect any muscle group although proximal muscles are most often affected. Symptoms worsen towards the end of the day and with exercise. Extreme heat, emotion, infection and menstruation can all exacerbate myasthenia as can many drugs including aminoglycoside antibiotics and penicillamine.

1.9.7 Course and Prognosis

Prior to the introduction of treatment in the form of acetylcholinesterases in the mid 1930s 70% of the recognised patients died of respiratory failure or pneumonia (Grob et al., 2008). However, due to improvements in the management of MG, and in particular the management of acute respiratory myasthenic crises, the mortality has now fallen to less than 5% (Barohn, 2008). The course of MG can be very variable between patients.

The natural history of the disease was characterised by a large long-term follow up study of 1976 MG patients over 60 years (Grob et al 2008). Typically the first symptoms are ocular in 85% of cases and generally 80% of these patients subsequently develop generalised symptoms. If the symptoms are limited to the eyes for more than two years however, there is a 90% probablility that MG will not become generalised after this (Grob et al., 2008). Typically the most severe weakness occurs in the first two years after diagnosis and 39% of patients are classified as having severe disease. There is a spontaneous rate of remission of approximately 30% (Grob et al., 2008).

1.9.8 Diagnosis and diagnostic tests

1.9.8.1 History and examination

A history of painless fatigable muscle weakness is critical to the diagnosis of MG. Relevant questions for the patient upon review include the pattern of muscle weakness, diurnal variation and any exacerbating factors. Fatigability can be demonstrated in the clinic by looking for the development of ptosis during prolonged up gaze or by testing shoulder abduction before and after unilateral repetitive movements.

1.9.8.2 Erdrophonium test

The edrophonium ("Tensilon") test is uncommonly performed but has a high sensitivity for generalised MG (Phillips and Melnick, 1990). The acetylcholinesterase inhibitor, edrophonium, is administered intravenously and the clinician observes for a transient improvement in muscle strength (such as the resolution of ptosis). The edrophonium given can cause life threatening bradycardia and atropine and resuscitation facilities must be available.

1.9.8.3 Ice test

A relatively sensitive and specific test for MG is the ice pack test. It is useful in patients who have ptosis and is performed by placing an ice pack over the eye for 2-3 minutes and assessing for an improvement in ptosis – it has a sensitivity approaching 90% (Reddy and Backhouse, 2007)

1.9.8.4 Neurophysiological tests

Neurophysiological tests are critical to the diagnosis of MG. Routine nerve conduction studies (NCS) and electromyography (EMG) are usually not informative but the compound muscle action potential (CMAP) amplitudes can be reduced in severe cases. However, repetitive nerve stimulation (RNS) typically elicits a decremental response at 3-10 Hz, with a decrement of >10% considered abnormal (Oh et al 1992; Sande). RNS is a specific test for MG but is not sensitive, being frequently negative in ocular MG (Oh et al., 1992). Single fibre EMG is more sensitive and is especially useful in ocular MG although it is less specific. Jitter, the trial to trial variation in the latency from stimulus to response is increased and there may also be intermittent failure of excitation of muscle fibres – “block” (Howard et al., 1994).

1.9.8.5 Serological tests

Serological tests are very useful in the diagnosis of MG. Not every patient with MG will have demonstrable antibodies but in those that do, these tests are highly specific. Approximately 85% of patients with generalised MG and almost all patients with an associated thymoma will have detectable circulating antibodies to AChRs and a variable proportion of the remainder will have antibodies to MuSK (Hoch et al., 2001). A small proportion of the remainder will have antibodies to LRP-4 (Zhang et al. 2012; Higuchi et al 2011; Pevzner et al 2012 Zouvelou et al 2013) or agrin (Zhang et al 2014).

1.9.8.6 Diagnosis of MuSK MG

The diagnosis of MuSK positive MG can be difficult. The clinical neurophysiology in MuSK MG patients usually shows abnormal SFEMG in the facial muscles but can be normal in the limb muscles (Farrugia et al., 2006). Moreover, clinical improvement on erdrophonium testing is much less common than in seropositive MG. Thymic pathology in MuSK patients does not show lymphocytic infiltrates or complement deposition and muscle biopsy does not show reduced AChR density as it does in AChR antibody positive patients (Leite et al., 2005).

1.9.9 Role of the thymus in MG

The thymus has an important role in the pathogenesis of early onset MG associated with anti AChR antibodies. The thymus is essential for T-cell differentiation and for the establishment of tolerance. Interactions between thymic stromal cells expressing self antigens and developing thymocytes lead to the elimination of autoreactive T cells.

The thymus is abnormal in a large proportion of patients with MG. Approximately 10% of MG patients have a thymic epithelial tumour, a thymoma (see below). In 65-70% of MG patients without a thymoma, the thymus is enlarged (hyperplastic) with lymphocytic infiltrates and germinal centres that contain a large amount of B cells and myoid cells which express AChRs (Vincent, 2002; Tsinzerling et al., 2007) – follicular hyperplasia which is particularly common in early onset MG. The thymus in patients with MG who develop the disease later in life is usually atrophic. The immunogenic role here is less clear, although atrophic thymic tissue has been shown to produce AChR antibodies in vitro (Katzberg et al., 2001).

1.9.10 Thymomatous MG

Approximately 10-15% of MG patients will have an underlying thymoma (Tsinzerling et al., 2007). Thymomas arise from tissue elements of the thymus and develop in the anterior mediastinum (Cowen et al., 1995). Myasthenia gravis is present in 30-40% of patients (Wright and Mathisen, 2001) but thymomas can also be associated with a variety of other systemic and autoimmune disorders including pure red cell aplasia, pancytopenia, hypogammaglobulinemia and collagen vascular disease (Hon et al., 2006; Miyakis et al., 2006; Murakawa et al., 2002). Thymomas are the most frequently encountered tumour of the anterior mediastinum but remain rare with an annual incidence of 0.15 cases per 100,000 (Engels and Pfeiffer, 2003).

Thymomatous MG is usually associated with AChR antibodies, and in younger patients, with anti titin antibodies (Voltz et al., 1997).

1.9.10.1 Classification system for thymoma

Several classification systems for thymomas exist. The WHO classification is one of the most frequently used and is based on the traditional descriptive classification and the corticomedullary classification. The Masoka staging system, commonly used in tandem with the WHO classification system is based on the presence of invasion and the anatomical extent of tissue involvement.

WHO classification	
A	Bland spindle /oval epithelial tumour cells with few or no lymphocytes
AB	Mixture of a lymphocyte poor A type thymoma with a more lymphocytic component
B1	Histological appearance of normal thymus composed of areas resembling cortex with epithelial cells scattered in a predominant population of immature lymphocytes and areas of medullary differentiation
B2	Large polygonal tumour cells arranged in a loose network , large nuclei and prominent nucleoli , background population of immature T cells
B3	Medium round or polygonal cells with slight atypical. Epithelial cells mixed with minor component of intraepithelial lymphocytes
C	Heterogeneous group of thymic carcinomas
Masoka stage	
I	Macroscopically completely encapsulated and microscopically no capsular invasion
II	Microscopic invasion into capsule (IIa) or macroscopic into surrounding fatty tissue or mediastinal pleura (IIb)
III	Macroscopic invasion into neighboring organs (i.e. pericardium, great vessels or lung)
IV	a-Pleural or pericardial dissemination b – lymphogenous or haematogenous metastases

Table 1.6 WHO grading and Masoka staging of thymoma (Tomaszek et al., 2009.)

1.9.10.2 Screening for thymoma

There is controversy about the best screening method for evaluation of possible thymoma in myasthenic patients. Computed tomography (CT) is often regarded as the first choice technique to characterize a mediastinal mass (Tomaszek et al., 2010) and can help in distinguishing thymomas from other mediastinal tumours (Maher and Shephard, 2009). However radiological differentiation is not always straightforward and histological characterization is necessary for definitive characterisation (Maher and Shephard, 2009). CT and MRI are thought to be equivalent for the evaluation of anterior mediastinal tumours with the exception of thymic carcinomas and thymic cysts, when CT is more reliable (Tomiyama et al., 2009).

1.9.10.3 Treatment of thymoma

Surgery, as first described by Blalock in 1941 (Blalock et al., 1941) remains the treatment of choice for thymoma unless the patient is not fit for surgery (Lanska et al., 1990). There is controversy about the surgical approach with increasing reports regarding the efficacy of minimally invasive techniques. However, most surgeons continue to opt for a transsternal surgical approach with completeness of resection the most important prognostic factor for survival (Kondo and Monden, 2003). Masaoka stage and WHO classification are also important prognostic factors (Tomaszek et al., 2009).

No additional survival benefit has been demonstrated for post-operative radiotherapy in the case of completely resected Masaoka Stage 1 thymomas (Nakagawa et al., 2003). However, radiotherapy is frequently used in the setting of an incompletely resected thymoma or if the thymoma is invasive (Zhu et al., 2004). It is controversial whether patients with completely resected Masaoka stage II thymomas should undergo radiotherapy (Tomaszek et al., 2009).

Chemotherapy is reserved for patients with inoperable disease or patients with gross residual disease after surgical treatment (Wright et al., 2005). No standardised regime exists but cisplatin based protocols are frequently used consisting of doxorubicin, cyclophosphamide, cisplatin vincristin or cisplatin with etoposide and ifosfamfide (Kim et al., 2005).

Myasthenic symptoms often remain after removal of a thymoma with over 80% of patients requiring continued immunosuppression (Evoli et al., 2002).

1.9.10.4 Thymoma recurrence

Thymomas are traditionally thought of as slow growing, rather indolent tumours but recurrence of a thymoma after surgery is not uncommon and has been reported in up to 30% of patients. Even after complete resection recurrence rates between 11% and 19% have been reported (Wright et al., 2005; Haniuda et al., 2001). Prognosis following thymoma recurrence is poor and successful treatment is more likely if recurrence is detected early. Currently there are no published guidelines as to the frequency, duration or mode of surveillance for thymoma recurrence.

We noted that there is a small cohort of patients that develop recurrence of thymoma many years after their initial surgery. We decided to describe the clinical characteristics of patients with late thymoma recurrence.

1.9.11 Medical treatment of MG

The treatment of MG is both symptomatic and immunomodulatory although there is a paucity of controlled randomised trials to provide definitive guidance. Treatment often relies on institutional preferences and the experience of the prescribing clinician.

1.9.11.1 Acetylcholinesterase inhibitors

Acetylcholinesterase inhibitors are usually first line therapy, providing symptomatic relief in ocular and generalised myasthenia. There are no randomised controlled trials but clinical experience and case reports support their efficacy. Side effects of acetylcholinesterase inhibitors are both muscarinic (abdominal cramps, salivation and lacrimation) and nicotinic (muscle cramps and fasciculations). Cholinergic crisis, which can mimic myasthenic crisis, can occur in high doses. AChE inhibitors have a short half-life and so require regular dosing although a controlled release preparation is available in some countries. Most patients will require immunosuppressive treatment as well but given the adverse effects associated with these drugs, it may be reasonable to treat with an AChE inhibitor alone initially. Patients with MuSK positive MG tend not to respond so well to acetylcholinesterase inhibitors and are more sensitive to their side effects (Kawakami et al., 2011). The basis of this cholinergic hypersensitivity in MuSK positive MG is thought to relate to the interference of MuSK binding to Col-Q which leads to a reduction of acetylcholinesterase at the neuromuscular junction (Kawakami et al., 2011).

1.9.11.2 Immunosuppression

1.9.11.2.1 Corticosteroids

The majority of patients with generalised MG will require immunosuppression and patients with MuSK positive MG may require early and aggressive immunosuppression due to symptom severity. Corticosteroids are generally the first line immunosuppressive treatment. A Cochrane review found only limited evidence from randomised controlled trials, nonetheless several observational trials support their

efficacy (Pascuzzi et al., 1984; Mann et al., 1976). It is thought that early use of steroids in ocular MG may prevent generalisation (Monsul et al., 2004). High initial doses of prednisolone can transiently worsen myasthenic weakness and trigger crisis and patients may require admission to hospital for escalation of treatment (Miller et al., 1986). Bone protection strategies and alternate day dosing are often used to counteract side effects.

If longer-term immunosuppression is required, steroid sparing agents are typically used. Azathioprine, methotrexate and mycophenolate are among the most frequently used oral immunosuppressant agents.

1.9.11.2.2 Azathioprine

Azathioprine is one of the most widely used second line immunosuppressant agents. It has been shown in two randomised controlled trials to have a steroid sparing effect (Myasthenia Gravis Clinical Study Group 1993; Palace et al., 1998). The maximal efficacy of azathioprine treatment may only be achieved after two years of treatment (Palace et al., 1998). Thiopurine methyl transferase levels are routinely measured before commencement of treatment as one in 300 people are homozygous for a variant of the TPMT gene that puts them at risk of developing azathioprine toxicity and bone marrow suppression (Relling et al., 2011).

1.9.11.2.3 Mycophenolate Mofetil

On the basis of small case series and case reports, mycophenolate mofetil has been used widely in MG (Hauser et al., 1998; Chaudhry et al., 2001; Meriggioli et al., 2003). Two randomised controlled trials suggested that it was not effective in MG or sparing prednisolone dose when used for 90 days or 36 weeks (Muscle Study Group. 2008; Sanders et al., 2008). However, these studies were of short duration and have been contradicted by retrospective studies that have shown that mycophenolate use improves symptoms and reduces steroid dose in a large proportion of MG patients after six months of therapy (Hehir et al., 2010).

1.9.11.2.4 Methotrexate

Methotrexate has also been recommended in MG by the European Federation of Neurological Sciences (EFNS) as a second line agent. It has been found to have a similar steroid sparing effect to azathioprine in a randomised controlled trial (Heckmann et al., 2011) and another phase II trial is currently underway (Pasnoor et al., 2012).

1.9.11.2.5 Cyclosporine

Cyclosporine has been found to be effective as monotherapy in MG in a small randomised trial and it has a fast onset of action compared to other steroid sparing agents in MG (Tindall et al., 1987). However, concerns about nephrotoxicity have limited widespread use.

1.9.11.2.6 Tacrolimus

Tacrolimus was shown to be effective in the treatment of MG in a pilot study (Nagane et al., 2005). However, as with mycophenolate, the results of a double blind placebo controlled trial were disappointing (Yoshikawa et al., 2011). The duration of the trial was 28 weeks, likely too short to demonstrate a steroid sparing effect, highlighting the importance of trial design in MG (Benatar and Sanders, 2011).

1.9.11.2.7 Cyclophosphamide

Cyclophosphamide is an alkylating agent that is rarely used due to its adverse effect profile and is generally reserved for truly refractory cases of MG. It has been shown to be of benefit, showing marked improvement in myasthenic weakness in a small group of myasthenic patients (Drachman et al., 2003).

1.9.11.2.8 Rituximab

More recently, the monocloncal antibody to the B cell antigen CD 20, rituximab has been used in MG. A growing number of retrospective studies have demonstrated its efficacy in MG (Maddison et al 2011; Collongues et al., 2012) in particular it is effective in MuSK MG - causing a sustained reduction in MuSK antibody tires (Diaz – Manera et al., 2012; Yi et al., 2013).

1.9.11.2.9 Eculizumab

Interest in other monoclonal antibodies has increased in recent years. Eculizumab is a humanised monoclonal antibody that cleaves the complement component C5 and hence

blocks the formation of the terminal complement complex. A recent small RCT has suggested that eculizumab may have a role in treating severe refractory MG (Howard et al., 2013).

1.9.11.2.10 Plasma Exchange and IVIG

Plasma Exchange (PLEX) and Intravenous immunoglobulin (IVIG) are treatments with a rapid but transient effect and are used in certain situations such as myasthenic crisis, rapidly worsening myasthenia and pre operatively. PLEX directly removes AChR antibodies from the circulation – adverse effects include hypotension, infection, thrombotic complications due to venous access and coagulopathy due to removal of circulating clotting factors. IVIG has been demonstrated in a placebo controlled randomoised controlled trial (RCT) to be effective for the treatment of worsening myasthenic weakness (Zinman et al., 2007), its mechanism of action is complex and involves inhibition of cytokine competition with autoantibodies, inhibition of complement deposition, interference with the binding of Fc receptor and interference with antigen recognition by sensitized T cells (Jayam Trouth et al., 2012). IVIG and PLEX have recently been compared in a randomised controlled trial for the treatment of moderate to severe MG. IVIG was found to have comparable efficacy to PLEX with the duration of effect of both treatments comparable (Barth et al., 2011).

1.9.12 Thymectomy in the management of MG

Removal of the thymus gland, thymectomy, is a frequently used treatment for MG. The first use of thymectomy for the treatment of MG was described by Blalock in 1939 for a patient with thymoma (Blalock et al., 1939). As discussed above, approximately 20%

of cases of MG are thymomatous and thymectomy is always indicated in these cases unless the patient is unfit for surgery.

Subsequently, Blalock performed thymectomy on MG patients without thymoma, found hyperplasia in the thymus glands and reported improvement in at least half the patients (Blalock et al., 1944). There is a rationale for the use of thymectomy in non-thymomatous AChR antibody positive MG as the B cell follicles and the germinal centres of the thymic tissue are the likely site of antibody production. Since these early reports, thymectomy has become an accepted treatment for early onset MG. However, there is a lack of evidence surrounding its efficacy. Non-randomised studies suggest that thymectomy is associated with an improvement in myasthenic symptoms and an increased likelihood of remission (Gronseth and Barohn, 2002; O'Riordan et al., 1998).

There is currently a randomised trial underway that will seek to answer whether thymectomy combined with prednisolone, compared to prednisolone alone will result in an improvement of myasthenic weakness, allow a lower dose of prednisolone and enhance quality of life by reducing symptoms and adverse events (Newsom-Davis et al., 2008). The data from this trial will not be available for some years however and so we took advantage of the large cohort of myasthenic patients attending our institution to examine the long-term outcome of MG after thymectomy.

1.9.13 Myasthenic Crisis

Myasthenic Crisis is the most serious complication of MG and is traditionally defined as acute respiratory failure due to worsening MG requiring admission to an intensive care unit (ICU) for ventilatory support (Jani-Acsadi and Lisak, 2007). It is potentially life threatening and is said to occur in 15-20% of patients with MG (Ahmed et al., 2005). The risk of developing crisis is greater in patients with oropharyngeal weakness, patients with a thymoma and patients who have MuSK MG (Oostehuis, 1981, Thomas et al., 1997; Vincent and Leite, 2005). The mortality of myasthenic crisis has declined considerably in recent years from between 50-80% in the 1960s (Thomas et al., 1997) to less than 5% nowadays (Thomas et al., 1997; Cohen and Younger 1981; Alshekhlee et al., 2009), due largely to improvements in the respiratory management of these patients in the ICU. Aside from the respiratory care, the treatment of MC rests on quick acting immunomodulation- namely plasma exchange and intravenous immunoglobulin (IVIG) (Newsom-Davis et al 1978; Gajdos et al., 2005; Qureshi et al and Suri, 2000; Vedeler et al., 2006). A randomised controlled trial of 84 patients with moderate to severe MG recently has shown that IVIG and PLEX have comparable efficacy (Barth et al., 2011). As the recognition of MG in the elderly has increased (Vincent and Drachman, 2002) so too has the recognition of MC in the older population. Some studies suggest that MC is actually more common in older myasthenics whereas others report it across the whole age spectrum of MG (Lacomis, 2005). There is little evidence regarding the long-term prognosis of MC, particularly in the older age group. We wished to describe the current management of patients admitted to a neurological intensive care unit (N-ICU) with severe exacerbations of MG and to examine their long-term outcome.

1.10 Questions to answer in this thesis

In summary, although there has been extensive research as detailed in both LEMS and MG, unanswered questions remain. I decided to focus on a number of important areas in these disorders.

Firstly in relation to LEMS I wished to gain a more in-depth understanding of the pathomechanisms of the disease. In particular, I had the following aims:

1) To visualise the binding of LEMS IgG to surface antigens in neuronal cultures in an attempt to clarify the antigenic target of LEMS antibodies and with a view to developing a cell-based assay for LEMS.
2) To define the pathomechanism of LEMS IgG by measuring the effect of LEMS IgG on synaptic vesicle release
3) To test the causal role of antibodies to P/Q-type VGCCs, and ascertain the role of antibodies to N-type VGCCs, by probing the effect of LEMS IgG on synaptic vesicle release in synapses lacking P/Q-type VGCCs from a *Cacna1a*$^{-/-}$ knockout mouse.

With regard to MG, I decided to take advantage of the large cohort of patients attending the NHNN for management of their MG. I was particularly interested in the long term outcomes of MG. The aims were as follows:

1) To assess the current policy toward thymectomy for MG patients and to examine the long term outcome of patients with thymomatous and non thymomatous MG after thymectomy
2) To describe the clinical characteristics of patients with late recurrent thymoma and to discuss post-operative surveillance for detection of recurrence.
3) To describe the current management of patients with severe exacerbations of MG and myasthenic crisis in the ITU and to assess their long term outcome.

2 Methods

This chapter describes the methods that were used for the laboratory based experiments in this thesis. It is subdivided into the following sections:

- Animal husbandry
- Preparation and maintenance of neuronal cultures
- Preparation of LEMS IgG
- Immunohistochemistry
- Synaptic vesicle imaging with FM dyes
- Electrophysiological recordings of excitatory post synaptic currents

2.1 Animal husbandry

All animal procedures were carried out in accordance with Home Office regulations under the UK Animal (Scientific Procedures) Act 1986. Animals were housed under controlled environmental conditions (24-25°C, 50-60% humidity, 12 h light/dark cycles and free access to food and water). For cortical glial cultures and rat hippocampal cultures, new-born (P0) Sprague Dawley rats were obtained from the Central Biological Unit, UCL. *Cacna1a* knockout mice were a kind gift of Arn van den Maagdenberg (Leiden University). The *Cacna1a*$^{+/-}$ breeding pairs were housed in the Biological Services Unit, Institute of Neurology. Transgenic mice were bred on a C57BL background for at least eight generations before being used in experiments and the strain was propagated by breeding heterozygous *Cacna1a* $^{+/-}$ animals with C57 mice. One pair was kept per cage. Breeding pairs were replaced if a litter was not produced

for 3 months. Litters were weaned at 21 days and were ear-tagged to ascertain genotype. The PCR based genotyping was carried out by Dr Stuart Martin, Huxley Building, UCL.

Pairs of heterozygous *Cacna1a* $^{+/-}$ animals were set up for the experiments where homozygous *Cacna1a*$^{-/-}$ and *Cacna1a*$^{+/+}$ pups were required. New-born pups were used for preparation of cultures at P0-P1. Pups that were not used for culture were culled before weaning to ensure that homozygous knockout pups did not reach maturity. Tail clips for PCR based genotyping were taken from pups used for neuronal cultures immediately after sacrifice.

2.2 Preparation and maintenance of neuronal cultures

This is the protocol that was used for the preparation of hippocampal neuronal cultures from rats and mice used in all our experiments. The method had been optimised previously in our laboratory as described in the thesis of F. Alder (2012) and was originally based on a modified version if the protocol described by Kaech and Banker,2007).

2.2.1 Materials consumables and timeline for hippocampal cell culture preparation.

Table 2.1 describes the equipment used for cell culture. Table 2.2 logs the laboratory consumables used. Table 2.3 describes the cell culture media and reagents. Table 2.4 describes the timetable for preparation of dissociated hippocampal cultures.

Cell culture equipment	Type	Source
Laminar flow hood	Gelaire BSB4	Labcaire
Tissue culture incubator 37 °C 5% CO_2 atmosphere	Galaxy S, Galaxy S +	Wolf laboratories
Water bath	JB Series	Grant
Dissection microscope		Zeiss
Dissection tools	2 x Dumont #5 2 x Dumont #55 Scissors	WPI WPI WPI
Centrifuge	Universal 32R	Hettich
Haemocytometer	Bright-line	Sigma
Ceramic coverslip racks		Thomas Scientific
Pipetters	Powerpette and Labmate pipetters	Jencons Scientific
Oven	Autoclave oven	Binder

Table 2.1 Cell culture equipment

Cell culture materials	Type	Source
Plasticware	Centrifuge tubes (15 ml and 50 ml)	Sigma
	Culture dishes (30, 60, 90 mm)	Sigma
	Pasteurpipettes (5, 10 and 25 ml)	Sigma
	Freezing vials	Sigma
	T25 and T75 flasks	Sigma
	12 well culture plates	Sigma
	Sterile eppendorf tubes	Sigma
Glass pipettes	Pasteur pipettes (plugged and unplugged)	Sigma
Filters	70 µm , 0,2µM	vWR
Glass coverslips	18 mm diameter	Fisher Scientific

Table 2.2 Cell culture materials

Media and other reagents	Component	Source	Amount
Nitric Acid 70%	Nitric Acid	Sigma	
	Water	Sigma	
PDL-Laminin	Laminin	Sigma	9 ml
	Poly-D-Lysine	Sigma	1ml
CMF-HBSS	Tissue culture grade H20	Sigma	900 ml
	10x Hanks BSS	Invitrogen	100 ml
	1M HEPES buffer pH7.3	Invitrogen	10 ml
Trypsin	Trypsin 2.5%	Sigma	
Glial Medium (GM)	MEM	Sigma	450 ml
	D-Glucose	Sigma 45% solution	6.7 ml
	Penicillin-streptomycin	Invitrogen 100x	5 ml
	Horse Serum	Invitrogen	50 ml
	L-Glutamine 200 mM	Sigma	5 ml
2 x cell freezing medium	FBS	Invitrogen	40 ml
	DMSO	Sigma	10 ml
Digestion solution (filtered and frozen in 10 ml aliquots)	137 mM NaCl (MW 58.44)	Sigma	1.6 g
	5 mM KCL (2M stock)	Sigma	0.5 ml
	10 M NaOH to pH to 7.2	Sigma	
	7 mM Na_2HPO4 (MW 141.96)	Sigma	0.2 g
	25 mM HEPES (MW 238.31)	Sigma	1.19 g
Wash solution stock (store at 4 °C)	HBSS	Sigma	500 ml
	5 mM HEPES	Invitrogen	2.5 ml
Dissociation solution (filtered and frozen in 10 ml aliquots)	Wash solution	Stock (as above)	200 ml
	12 MM Mg $S0_4$ (MW 120.37_	Sigma	0.288 g
Dissection solution	Wash solution	Stock (as above)	40 ml
	FBS 20%	Invitrogen	10 ml
DNAse stock solution (frozen in 25µl aliquots)	DNAse 75u/µl	Sigma	150 KU
	Tissue culture grade H20	Sigma	2ml
Digestion mix	Trypsin	Sigma	25 mg
	Digestion solution	Stock (as above)	2.5 ml
	DNAse	Stock (as above)	10 µl
Dissociation mix (filter)	Dissociation solution	Stock (as above)	2.5 ml
	DNse	Stock	12.5 µl
Complete Neurobasal A	Neurobasal A medium	Invitrogen	500 ml
	B27 serum free supplement	Invitrogen	10 ml
	Glutamax (25 µM glutamine)	Invitrogen	1.25 ml
AraC		Sigma	1 µM (1 ml stock)
Trypan Blue (0.4%)		Sigma	

Table 2.3 Cell culture media and reagents

Day	Glial Cells	Coverslips	Neurons
Prior	Prepare and freeze glial cells		
-14	Defrost and plate glial cells into T75 flasks		
-13	Feed with GM		
-10	Feed with GM	Soak coverslips in 70% nitric acid	
-7	Feed with GM	Rinse and bake coverslips	
-6		Coat coverslips with PLL	
-5	Dislodge glial cells and plate on coverslips		
-4	Feed with GM		
0	Feed with Complete Neurobasal A		Prepare and plate neurons onto glial cells
1			Add araC
7			Feed with Complete Neurobasal A
14-21			Use neurons for experiments

Table 2.4 Cell culture time -table

2.2.2 Preparation of cortical astroglial cells

Postnatal (P0) Sprague Dawley rats were sacrificed and decapitated. The heads were rinsed with 70% ethanol and were washed four times in 30 ml of wash solution. The heads were then placed in a 10 cm dissection plate that was filled with wash solution. The skin of the head was removed using a Dumont #5 forceps. The skull was pierced with the tip of a scissors and the parietal bones of the skull were peeled back to expose the brain. The brain was scooped out and placed onto a 60 mm dissection dish filled with dissection solution, ensuring that the brain remained submerged at all times. The dish was placed under the dissection microscope. The cerebral hemispheres were separated from the brain stem and all the meninges were removed using the fine forceps. Each hemisphere was then finely chopped using a small dissection scissors.

All steps up to trypsinisation were performed using ice cold solutions. The pieces of chopped cerebral hemisphere were transferred to a 50 ml tube containing 9.5ml of CMFH-HBSS, 1.5 ml of 2.5% trypsin and 1.5 ml of DNAse (10mg/ml). This was incubated for 5 minutes at 37°C in a water bath and swirled occasionally. The tissue was triturated through a 10 ml pipette ten times until large clumps were broken down before being returned to the water bath for another 10 minutes. A 5 ml pipette was then used to triturate the tissue again until remaining clumps of tissue were broken down. The suspension of cells was passed through a 70 µm cell filter and was collected in a 50 ml tube containing 15 ml of Glial Medium (GM). The suspension of cells in GM was centrifuged for 10 minutes at 2000 RPM to remove enzymes and lysed cells. The supernatant was discarded and the pellet was broken and re-suspended in 20 ml of GM. The cells were counted using the haemocytometer and cell viability was assessed using 0.4% trypan blue. A typical yield was around 10 million cells per pup.

The cells were plated at a density of 7-10 x 10^6 per T75 flask and topped up to a final volume of 12 ml with GM. The flasks were transferred to an incubator at 37°C, 5% $C0_2$. The following day, the media was exchanged. The flask was first tapped to remove loosely attached cells, the medium was then aspirated off and was replaced with fresh (warmed to 37 °C) medium. Media were exchanged every 3 days until the cells reached 90-95% confluence.

When the glial cells had reached confluence, they were split. Each T75 flask was divided into eight in order to amplify the colony. The cells were rinsed with 10 ml of warm CMF-HBSS and were then treated with 2 ml of trypsin/EDTA until the cells had

dislodged. The side of the flask was gently tapped to aid dislodgment of the cells. Once the majority of the cells were seen to dislodge, 8 ml of warmed glial medium was added to each flask to inactivate the trypsin/EDTA. The cells were transferred using a 10 ml pipette to a 15 ml tube. The cells were then centrifuged for 10 minutes at 2000 RPM. The supernatant was discarded and the pellet was re-suspended in GM and divided between eight flasks. This process was repeated in order to obtain a large batch of cells (approximately 150 flasks).

Glial cells were frozen in liquid nitrogen in order to create a stock of cells that could be defrosted at weekly intervals for neuronal cell preparation. The cells were dislodged from the T75 flasks with trypsin/EDTA as described above but were re-suspended in 0.5 ml GM and 0.5 ml 2 x freezing medium. The cells were then transferred to individual 1.8 ml cryotubes with the cells from two T75 flasks being transferred to one cryotube. They were frozen for 2 hours at -20 °C, and at -80°C before being placed in liquid nitrogen for long term storage.

The glial cells were defrosted two weeks before planned neuronal culture preparation. A cryotube was defrosted and the cells were re-suspended in ice cold GM. The cells were then centrifuged for 10 minutes at 2000 RPM to remove DMSO. The supernatant was discarded and the pellet was re-suspended in warmed GM. Cells from one cryotube were plated into nine T75 flasks. As described above, the media was changed the following day and cells were grown to 90% confluence with the medium changed every 2-3 days.

2.2.3 Preparation of coverslips

Using forceps, 19 mm glass coverslips were placed into ceramic racks and submerged in 70% nitric acid for 3-4 days. The coverslips in the racks were then rinsed four times for 2 hours each time in ddH20 before being baked overnight at 225°C. Once cooled, the coverslips were placed in 12 well plates (6 coverslips per plate) and were coated with 150 μL PLL. The coverslips were incubated with PLL overnight and were then rinsed twice for two hours with 3ml tissue culture grade H_20 per well. Coverslips were allowed to dry in the tissue culture hood.

Glial cells in T75 flasks were dislodged with trypsin/EDTA as described above and were re-suspended in GM. The cells were plated onto the treated coverslips at a density of 60K per cm^2. The GM was replaced the following day and the cells were left to form a monolayer.

2.2.4 Preparation of rat neuronal cultures

The rats were culled, decapitated and the heads were rinsed in ethanol and washed in wash buffer as described above. The skin and skull bones were removed and the brain was scooped out and placed in a 60 mm dish containing ice cold dissection buffer. The hippocampus was dissected out in the following fashion. First, the brain was divided in the midline by gently cutting the midline sagittal section. The hemispheres were then flipped outwards and the meninges were removed. Care was taken to completely remove all the meninges. The hippocampus became visible on the inner surface of the cerebral hemisphere and was removed carefully using a forceps. The same was done

for the other cerebral hemisphere and the process was repeated for all the brains that were to be used.

The dissected hippocampi were transferred to a new dish with dissection buffer and then to a 15 ml tube were washed 5 times in 5 mlof wash buffer. The pieces of hippocampus were transferred to a tube containing the digestion mix and were incubated in the water bath for ten minutes at 37°C. After ten minutes the trypsin was inhibited with 5 ml of dissection buffer. The cells were rinsed twice with 5 ml of dissection buffer and twice with 5 ml of wash buffer.

The tissue was triturated 5 times in 2 ml dissociation mix using a half diameter fire polished cotton-plugged Pasteur pipette. The triturated pieces were allowed to settle and the suspended cells were transferred to a new 15 ml tube. The remainder of the cells were triturated with a 1/3 diameter fire polished Pasteur pipette. Remaining clumps were allowed to settle before the solution was transferred to a new 15 ml tube. 10 ml dissection solution was added and the cells were centrifuged for 5 minutes at 2000 RPM.

The supernatant was discarded; the pellet was broken and was re-suspended in Complete Neurobasal A. The cells were counted using the haemocytometer and cell viability was assessed using trypan blue. The GM was removed from the glial cell coated coverslips and 1 ml warmed complete Neurobasal A was added to each well. The cell solution was diluted to a concentration of 30,000 cells per well with complete Neurobasal A and 1 ml was added to each well. The cells were returned to the

incubator and the following day were fed with 1ml per well of warmed complete Neurobasal A containing 3 μM araC, giving a final concentration of 1 μM. Seven days later, the cells were fed; 1 ml of medium was removed from each well and was replaced with 1 ml warmed Neurobasal A.

Cells were used for experiments from day 14 after plating to day 20.

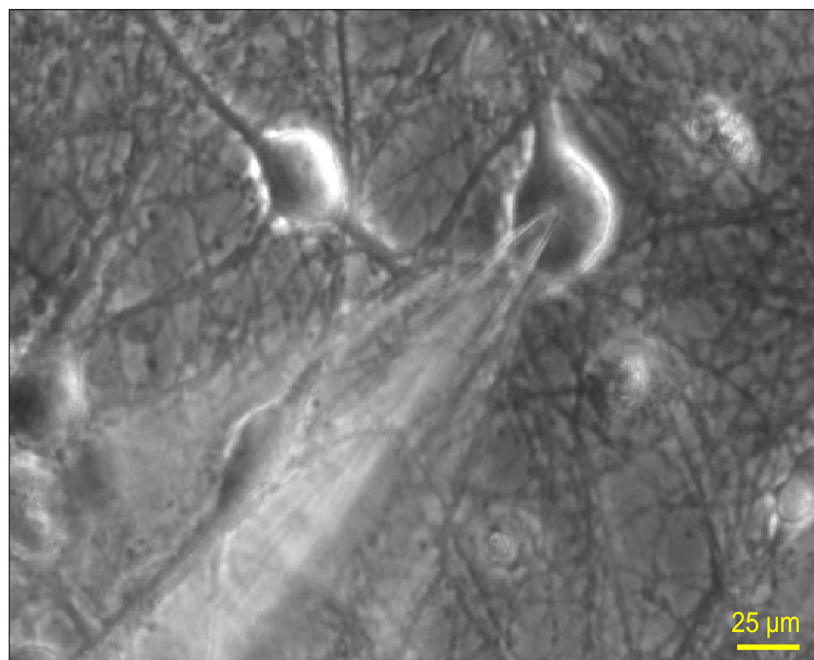


Figure 2.1 Neuronal cultures 14 days in vitro (63x)

2.2.5 Modifications for Cacna1a knockout cultures

A number of modifications were made when culturing cells from mouse pups. The genotype of each pup was determined after the culture preparation by PCR based genotyping carried out on tail clippings that were taken immediately after sacrifice. As mouse litters could be large (6-12 pups per litter), typically 3-4 pups would be dissected

at a time. The whole culture process was completed before the next 3-4 pups were culled and dissected. Surgical instruments were cleaned with 70% ethanol between each pup. The volumes of media and reagents were reduced according to the physical size of the tissue. Cells from each mouse were plated onto 3-5 coverslips depending on the cell count on the haemocytometer. Coverslips from heterozygous mice were subsequently discarded after genotyping. All other steps were carried out as for hippocampal cultures.

2.3 Preparation of LEMS IgG

IgG samples from patients diagnosed with LEMS were provided by Dr Bethan Lang, Weatherall Institute of Molecular Medicine, Oxford. Samples generally came from plasma exchange procedures which form part of the treatment of LEMS (Newsom-Davis et al 1984). Control samples were pooled from healthy controls. Samples from patients with clinically and electrophysiologically confirmed LEMS were chosen for the functional studies that were carried out. Samples from four of these patients precipitated high levels of anti-voltage gated calcium channel antibodies in the diagnostic ^{125}I MVIIC radioimmunoprecipitation assay. A fifth patient did not have detectable anti VGCCs antibodies and was used as a “seronegative LEMS” sample. LEMS sample collection was approved by the Oxfordshire Regional Ethical Committee A (07/Q1604/28). Each patient provided written informed consent

Sample number	Age at disease onset	Gender	Tumor status	Electrophysiology		P/Q ab titer (pM)	N- ab titer (pM)
				CMAP amplitude (mV)	% Increment		
1	48	M	Nil	6.1 mV	146	261	Neg
2	68	M	Anaplastic large cell lung carcinoma	2.2 mV	145	575	60
3	44	F	SCLC	1.2 mV	1,233	10,755	127
4	58	M	Nil	4.2 mV	281	362	Neg

Table 2.5 VGCCs positive LEMS samples used in experiments

Plasma samples were routinely stored at -20 °C in the host laboratory.

The IgG was extracted from plasma samples using the ethacridine lactate-ammonium sulfate method. This was performed by Dr B. Lang. Samples were transported from Oxford to London on dry ice. The samples were allowed to defrost and were aliquoted into 0.5ml eppendorfs before being refrozen at -20 °C. The number of freeze-thaw cycles was kept to a minimum

Samples to be examined for antibody binding to cells by means of immunofluorrescence were not dialysed before use. Samples to be examined for effects on cultures cells were dialysed before use. Two days before the planned experiment, the IgG samples were defrosted. The samples were diluted with complete Neurobasal A to a ratio of 1mg IgG /0.5 ml Neurobasal. The IgG/NeurobasalA samples were dialysed overnight against complete Neurobasal at 4° C using Slide-A-Lyzer dialysis cassettes (Fisher). The diluted IgG sample was added to dialysis cassette that had been

prehydrated with Neurobasal A. The dialysis cassette fixed into a buoy was submerged in a 1000ml plastic beaker containing Neurobasal A. A magnetic stirrer was used to continuously agitate the sample which was kept in cold room storage at 4°C overnight. The next day, the IgG sample was removed from the dialysis cassette and was warmed to 37°C. The sample was then filtered using a 0.2 µm SFCA (surfactant free cellular acetate) filter (Fisher) to ensure low protein binding. Warm filtered IgG was added to each well for a final concentration of 1mg/1ml IgG. An equivalent amount of Neurobasal A was removed from each well before the IgG was added to ensure that the volume remained constant. The cells were returned to the incubator and the samples were left overnight. Control IgG samples were treated in tandem with LEMS IgG samples in exactly the same way.

2.4 Immunohistochemistry using LEMS IgG

Immunofluorescence techniques were used to visualise binding of LEMS antibodies to hippocampal neuronal cultures.

Equipment	Source	Usage
LSM 500 confocal microscope	Zeiss	Used for confocal microscopy imaging including various objectives, condenser and photmultiplier tube
Luckham Rotatest R100 shaker	vWR	To agitate coverslip during antibody incubation

Table 2.6. Equipment used for immunohistochemistry experiments

Material	Type	Source
Corning 6 well culture dish	6 well plate	SigmaAldrich
Plastic Pasteur pipette (for washing cells)		vWR
Bakefoil (to exclude light from fluorescent secondary antibodies)		
Slides	76 x 26 mm	Thermoscientific
Coverslips	19 mm glass coverslips	ThermoScientific
Nailpolish		

Table 2.7 Labware used in immunohistochemistry experiments

Reagent/Antibody	Source	Use
Phosphate Buffered Saline	Sigma	Wash cells
4% PFA	Sigma	Fix cells
BSA (5% BSA in PBS)	Sigma	Block cells (to reduce non specific binding)
Anti Human IgA 594	Invitrogen (A11014)	Secondary antibody – to human IgG primary
Permeabalization solution 5% BSA and 1% triton in PBS)	Sigma	
Anti GFP antibody	Abcam	Antibody to GFP (Transfected voltage gated calcium channels were tagged with GFP)
Anti Rabbit 488	Invitrogen	Secondary antibody to anti GFP primary
Anti-Synapsin antibody	Invitrogen	Antibody to synapsin
Zenon antibody detection kit	Invitrogen	Secondary antibody detection system for human IgG
Vectashield mounting medium	Vector Labs	Mount cells

Table 2.8 Antibodies and reagents used in immunohistochemistry experiments

Various methods were used to attempt to visualise the binding of LEMS IgG to neuronal cells using immunofluorescence techniques. The methods will be explained in more detail in Chapter 3. The method is described here in brief; the medium from the tissue culture well containing mature hippocampal neurons (14-21 days *in vitro*) was

aspirated. Cells were washed four times in PBS for five minutes each time. The cells were then fixed in 4% paraformaldehyde (PFA) in PBS for 30 minutes at room temperature before being washed in PBS (four times for two minutes each time). Coverslips were incubated in 5% BSA in PBS as a blocking agent for 30 minutes. They were washed in PBS (3 times) and incubated with the primary antibody –LEMS IgG in 1% BSA (1.500 or 1.1000) in PBS for 2 hours at room temperature or overnight at 4°C. The coverslips were washed with PBS (five times- 1-2 minutes per wash) and were incubated with the secondary antibody, anti human IgG A594 in PBS (1:1000) for one hour at room temperature. Following incubation with secondary antibodies, the coverslips were washed again in PBS (5 times, 2 minutes per wash) and were finally washed in water to remove excess salts. Excess fluid was removed and the coverslips were mounted onto slides cell side down using Vectashield Hardset Mounting Media. Once the mounting medium had set the coverslips were sealed with nail varnish. The cells were imaged the following day using a confocal fluorescent microscope. Alexa594 was excited using the 543 nm line of the He-Ne laser and its emission was recorded using a band pass 560-615 nm filter. Alexa 488 was excited by the 488 nm line of an argon laser and its emission was recorded using a band pass 505-530 filter.

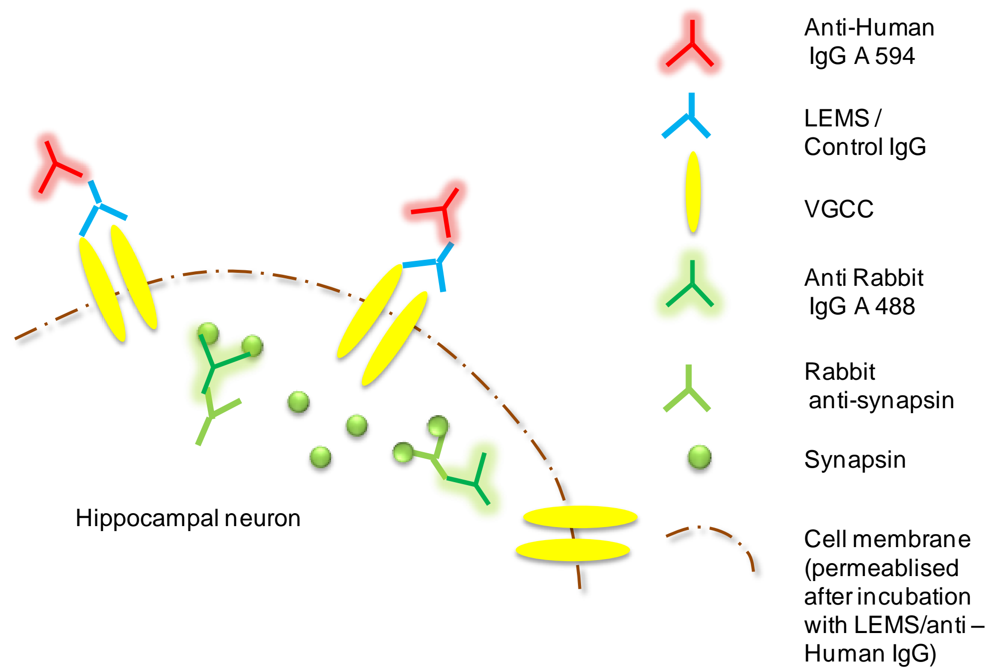


Figure 2.2 Schematic diagram of immunostaining for LEMS IgG with anti human IgG (Alexa 594) and co-staining for synapsin with anti rabbit synapsin and anti rabbit Alexa 488 as the secondary antibody.

2.5 Synaptic vesicle imaging with FM dyes

2.5.1 Equipment and apparatus used for synaptic vesicle imaging

Equipment	Source	Usage
Air Table	TMC	Isolates vibration for steady imaging. Microscope, recording chamber and, for electrophysiology experiments, micromanipulators were all placed on the air table
Faraday Cage	Custom made	Reduces electrical noise. Microscope and electrophysiology equipment within the Faraday cage are electrically grounded
Perfusion System	Custom Made	Gravity perfusion system. Cells were continually perfused with fresh extracellular buffer.
Perfusion pump	Masterflex: Easyload II	Pump used to drive perfusion
QuantEM 512 SC	Photmetrics	Live cell fluorescent imaging
Axiovert 135 microscope	Zeiss	
High Power LED Driver DC2100	ThorLabs	Excitation with 488nm LED 500nm dichoric filter Emission fluorescence collected with 510 Long Pass filter
Master8	A.M.P.I	Programmed to apply difficult electrical stimulation paradigms to the recording chamber. The Master8 was connected to the chamber via two constant voltage isolated stimulators
Constant voltage isolated simulators	Digitimer DS21-MKII	Two isolated constant voltage simulators were connected in series to apply voltage across the chamber.
Oscilloscope	Tektromix TDS1002	The oscilloscope was used to visualise the voltage of the stimulation applied to the chamber
Recording Chamber	Custom made	The recording chamber held the coverslip in place during the experiments. It had an inflow and outflow for perfusion and parallel platinum stimulation wires that were connected to the voltage stimulators

Table 2.9 Lab equipment for synaptic vesicle imaging

2.5.2. Extracellular solutions used for synaptic vesicle imaging

The following extracellular solution was used in all experiments

Extracellular solution	Composition (mM)	Source
pH 7.4 adjusted with 1M NaOH (Sigma) Osm 305-310 Made on day of experiments	NaCl 125mM	Sigma
	KCl 2.5mM	Sigma
	$CaCl_2$ 2 mM	Sigma
	$MgCl_2$ 2mM	Sigma
	Glucose 30 mM	Sigma
	HEPES 25 mM	Sigma

Table 2.10 Composition of experimental extracellular buffer

Several software programmes were used for the acquisition and analysis of data from FM experiments

2.5.3 Software used for data acquisition and analysis

Software	Source	Usage
Capture Pro		Viewing images
Image J	Wayne Rasband, National Institute of Health USA	Viewing and analysing images off line
SigmaPlot	Systat Software Inc	Data analysis and manipulation
Mathcad	PTC	Data fitting and modelling
SigmaPlot 11.0	Systat software	Data analysis
Excel 2007	Microsoft Office	Data analysis

Table 2.11 Software used in acquisition and analysis of data for FM experiments

2.5.4 Protocol for imaging of vesicular recycling with FM dyes

All experiments were performed at room temperature. For the FM experiments the following neurotransmitter antagonists were added to the extracellular solution in order to inhibit recurrent activity.

Drug	Target	Stock Concentration	Final Concentration	Source
NBQX (2,3 dihydroxy-6-nitro-7-sulfamoyl-benxol[f]quiniozaline-2-3,-dione)	AMPA receptor antagonist	25mM (stored at -20 °C)	10µM	Ascent Scientific
DL-AP5 (DL-2-Amino-5-phosphonopentalnoic acid)	NMDA receptor antagonist	100mM (stored at -20 °C)	50µM	Ascent Scientific

Table 2.12 Neurotransmitter blockers used in FM experiments

The styryl FM dye used to label recycling vesicles was SynpatoRedC1 (SRC1), which was obtained from Biotium USA. The excitation and emission wave lengths for SRC1 are 558 and 734 nm respectively. The dye was made up with MilliQ water and stored at 20 °C at a concentration of 20 mM. The final concentration used was 200 µM.

All imaging experiments were carried out using a 63X oil immersion microscope. Cultures were imaged at a resolution of 512 x 512 pixels, an area which typically contained several hundred boutons. Each pixel was 0.254 µM.

The following stimulation protocols were programmed into the Master 8. The Master 8 was connected to the stimulators to allow loading of synaptic vesicles, low frequency stimulation and complete destaining.

Paradigm	Frequency (Hz)	Number of pulses	Number of rounds	Delay between rounds (seconds)
Loading	30	120	4	20
Low frequency stimulation	0.5	450	1	-
Complete destaining	10	300	3	120

Table 2.13 Master 8 stimulation protocols

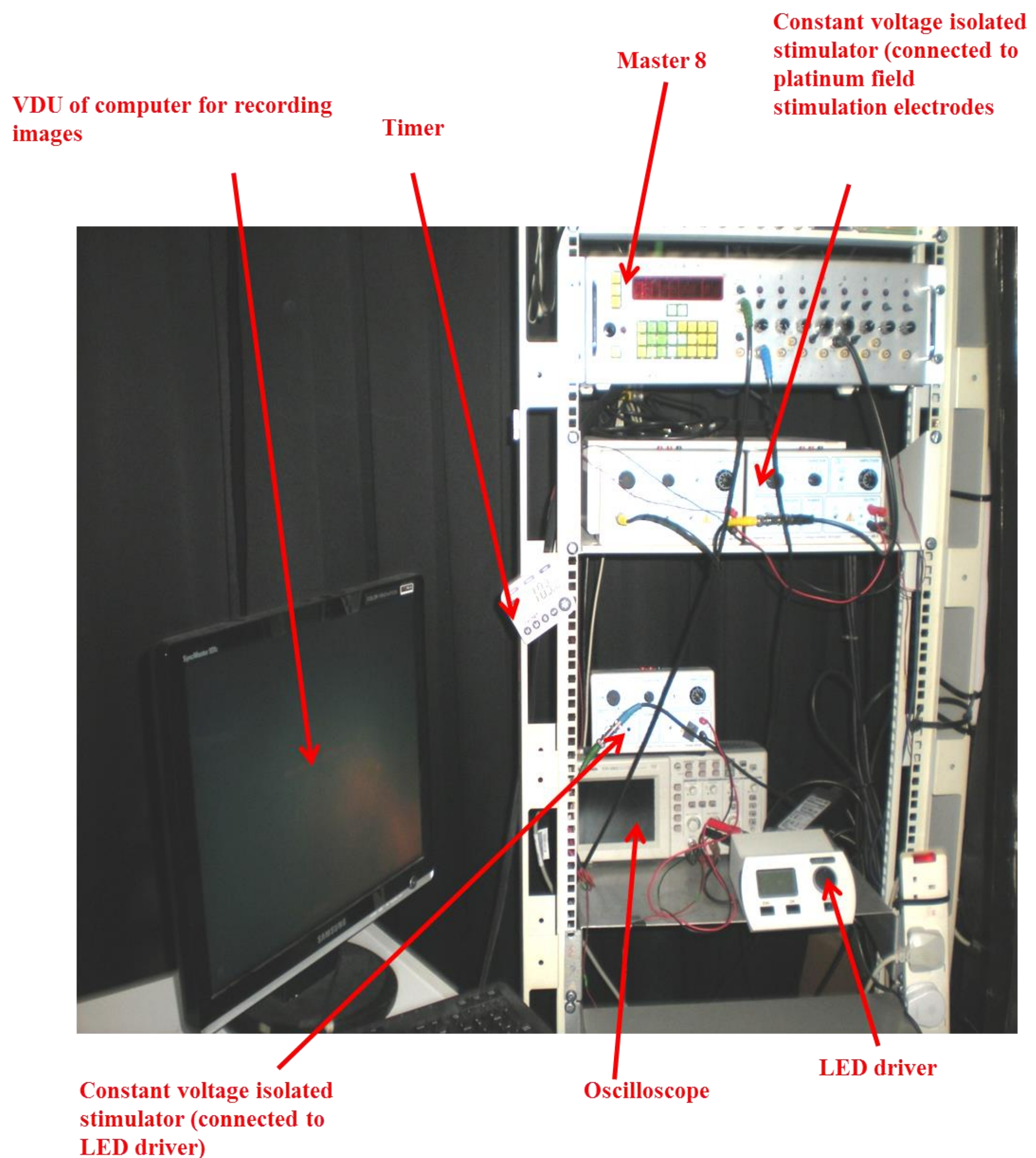


Figure 2.3 Rig for FM experiments

Before the experiment began, the perfusion system was washed with MilliQ water and was then perfused with extracellular buffer at room temperature. A coverslip was placed in the chamber, which was connected to the gravity perfusion system and the stimulation electrodes. The flow rate of the perfusion system was adjusted to 1 ml/minute. The perfusion was then stopped and buffer was allowed to drain until approximately 400 µl of buffer remained in the chamber. Action potentials were

evoked by field stimulation via platinum electrode separated by 1 cm.   The stimulation intensity was adjusted to 15V by adjusting the amplitude on the stimulators. This stimulus intensity was chosen because it was previously shown to trigger all the action potentials on an invidual coverslip (Ermolyuk et al., 2012; F Alder PhD thesis 2012). The SRC1 dye was added to the extracellular solution and was gently mixed using expelled air from a 1ml pipette. The loading paradigm (see above) was applied. Approximately 30 seconds after the last pulse, the dye was removed from the bath with fresh extracellular solution (5 x 1 ml washes) and the perfusion system was restarted for 15 minutes. During this washing time, the cells were focused on using the transmitted light function on the camera. An appropriate region of interest (ROI) was chosen by viewing the area using transmitted light. The focus was then adjusted using epifluorescence imaging and a reference image was taken. When it was confirmed that the ROI was in focus, a corresponding image was taken with transmitted light. This provided the reference image for refocusing of the microscope during the experiment. The microscope was focused using transmitted light before a snapshot image with a fast exposure time was taken with epifluorescence.

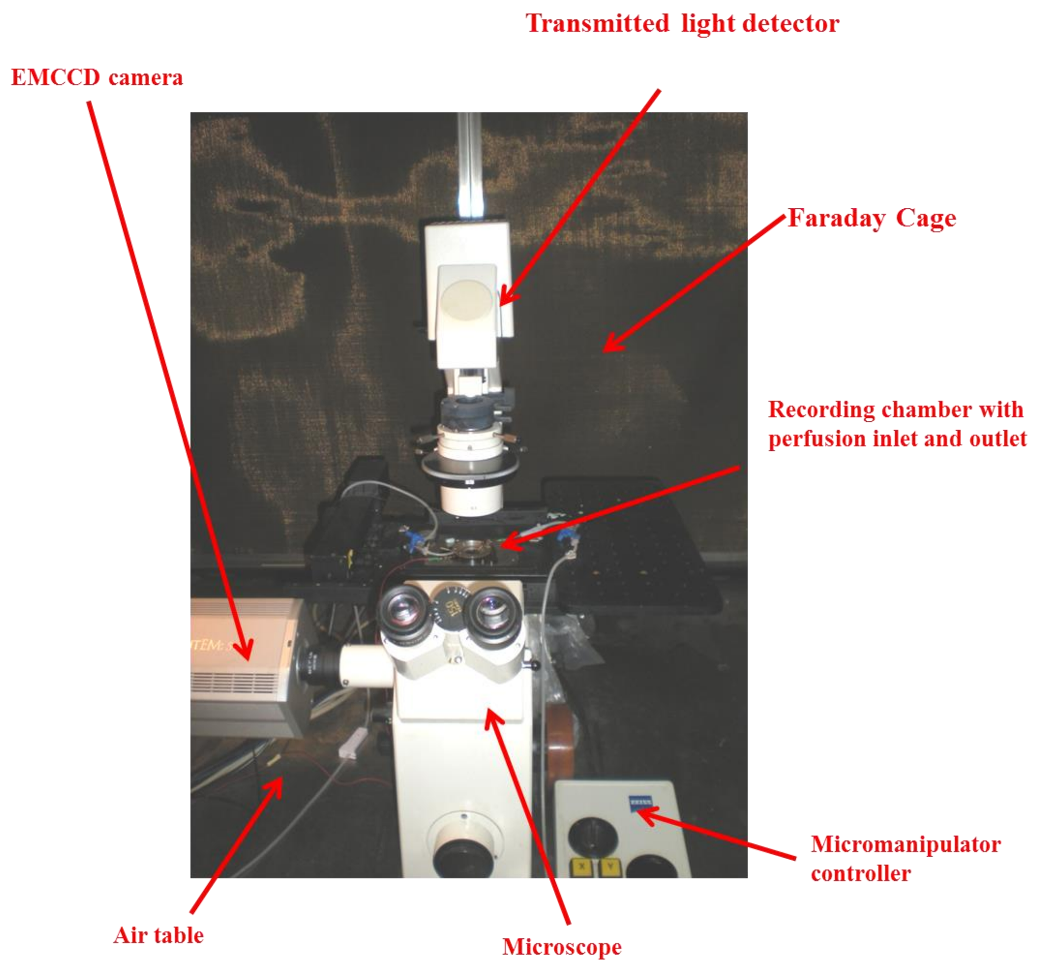


Figure 2.4 Imaging equipment for FM

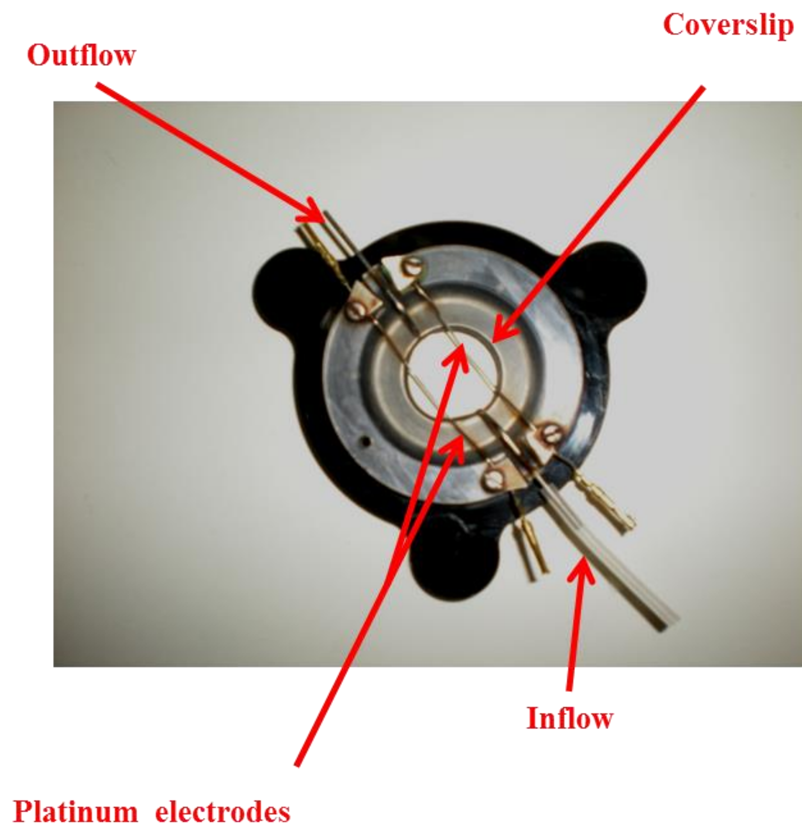


Figure 2.5 The recording chamber for FM experiments. The cells were plated onto glass coverslips and secured in a custom-made recording chamber for the duration of the experiment. A rubber O-ring was used to prevent leakage of the extracellular solution. Two parallel platinum electrodes were used to evoke field stimulation across the coverslip. The coverslip was perfused with fresh extracellular solution during experiments via the in- and outflow.

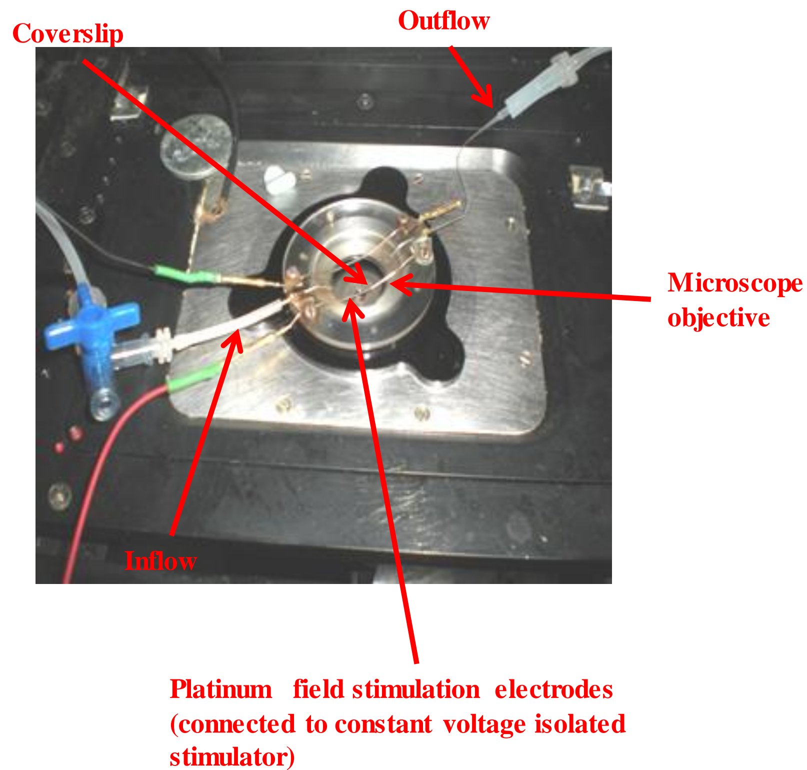


Figure 2.6 Custom made chamber for FM destaining experiments in place on rig

Each fluorescent snap shot took a z stack of ten images which were then combined using a prewritten macros to give one high quality combined image. After 15 minutes of washing the imaging was started. Images were first taken in the absence of any stimulation to obtain measurements for spontaneous destaining (k_{SP}) for ten minutes. The region was imaged every 40 seconds approximately with refocusing using transmitted light between each image. After ten minutes, the low frequency stimulation paradigm was applied for 15 minutes while images were taken every 40 s to measure evoked synaptic vesicle release (k_{EV}). The cells were then subjected to the complete destaining paradigm after which the region was imaged five times to determine the background fluorescence. The perfusion system was rinsed with Milli-Q water between each experiment and 30% ethanol at the end of each day.

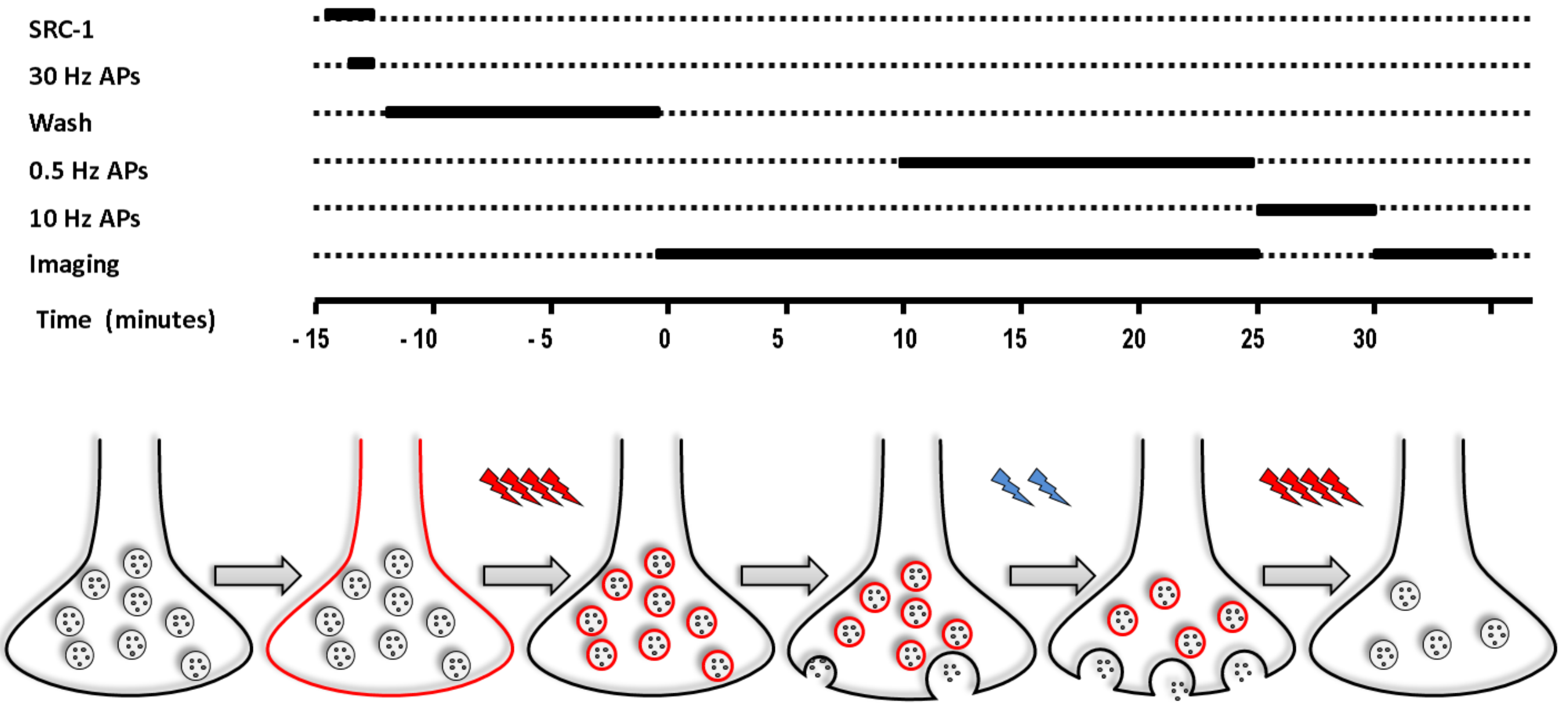


Figure 2.7 FM experiments

2.5.5 Analysis

The images from these experiments were analysed in the following way. Time stacks of fluorescence images were run through macros in Image J to time sort, z project and x-y align the images. A mask file was created by subtracting the average of the last five images (which accounted for background fluorescence) from the average for the first five images to allow identification of active boutons. Boutons were selected using the look up tables (LUT) red hot" assay for easy visualisation This showed dim areas as red, brighter areas as yellow and the brightest areas as white in a graded fashion so areas of high intensity corresponding to boutons could be readily identified. The drawing tool was used to draw around and so to select areas corresponding to individual boutons. Boutons in areas of high background fluorescence, overlapping boutons and boutons that were obscured by a large amount of movement artefact were discarded. Once all potential boutons were identified, the selected regions of interest (ROIs) were applied to the time stack image. The area and mean grey value at each time point for each individual bouton were extracted to a Sigma plot file.

The data were split into three stages: spontaneous destaining before starting low-frequency stimulation, during 0.5 Hz stimulation, and after full destaining by 10 Hz stimulation. The start and end times and corresponding frame numbers were specified for each stage. The data were imported to a Mathcad program, and the fluorescence intensity profiles for each putative bouton were fitted with mono-exponential fits after subtracting the final residual fluorescence. For the initial stage of the experiment, before stimulation, the exponential fit gave an estimate for the rate of spontaneous destaining, k_{SP}. During low frequency stimulation, the monoexponential fit gave an

estimate for evoked destaining k_{EV}. Boutons with high spontaneous destaining rates (k_{SP} >0.001 s^{-1}) were discarded, as were boutons with low signal to noise ratios as defined by a goodness of fit criterion χ^2 larger than 0.1. The values for k_{SP} and k_{EV} for the remaining boutons were transferred back to SigmaPlot. The specific action-potential evoked destaining rate was then calculated by subtracting the spontaneous destaining rate from the evoked destaining rate ($k_{AP} = k_{EV} - k_{SP}$). All selected boutons from a particular set of experiments were combined and were plotted in SigmaPlot.

2.6 Electrophysiological recording of excitatory post synaptic currents

The same extracellular buffer as described for the FM destaining experiments was also used for the electrophysiology experiments although without glutamate receptor blockers. A Caesium chloride based intracellular solution was used for whole cell patch clamp electrophysiology experiments.

Cesium Chloride intracellular solution pH was adjusted to 7.4 with cesium hydroxide Osmolality 290 Stored at -20 °C	**Compound (concentration)**	**Sources**
	CsCl 120 mM	Sigma
	HEPES 10mM	Sigma
	EGTA 2 mM	Sigma
	NaCl 8 mM	Sigma
	$MgCl_2$ 0.2 mM	Sigma
	MgATP 2mM	Sigma
	Na_2GTP 0.3 mM	Sigma
	Qx 314 Br 5 mM	Sigma
	Phosphocreatinine 10 mM	Sigma

Table 2.14 Intracellular recording solution used in electrophysiology experiments

Equipment	Source	Usage
Scientifica Slice-scope	Scientifica	Imaging cells
Air table	TMC	Isolated vibrations for steady recording
Faraday Cage	Custom Made	Reduced electrical noise. The Faraday cage confined the microscope and electrophysiological recording equipment
XCite Series 120 Q	Olympus	Used to visualise cells in transmitted light. Image acquired was observed using Patch Vision software run on computer 2.
Perfusion System	Custom Made	2 way pump perfusion system used to continually perfuse cells with fresh extracellular buffer
Perfusion pump	Masterflex	Used to drive perfusion
Constant voltage isolated stimulator	Digiterm DS2A-MkII	Connected to the field stimulating electrode to apply voltage adjacent to a cell
Oscilloscope	Tektronix TDS 1002	Visualising voltage stimulation applied to the chamber and visualising EPSC recorded
Micro manipulators	Scientifica	Used to direct recording pipettes during electrophysiological recording
Headstage	Axon instruments	Contains voltage clamp and voltage follower circuutis for voltage and current clamp recordings. Connected to the mircromanipulator
Pipette holder	Custom made	Connected to the headstage. Used to securely hold pipettes.
Multiclamp 700B amplifier	Axon instruments	Amplifier used for electrophysiological recordings. Run on computer 1
PCI-6221 and BNC-2090	National Instruments	Analogue to digital signal converter and interface. Used to communicate between the computer and amplifier and stimulator.
Recording chamber	Custom Made	Holds coverslip during an experiment. Chamber included an inflow and outflow for perfusion and a ground pellet
P97 Flaming/brown micropipette puller	Sutter Instruments company	Used to pull recording pipettes
Filamented borosilicate glass pipettes	Warner Instruments	Pipettes were pulled to a resistance of 3.5-5 MΩ.
Concentric Bipolar stimulating electrode (1.0mm, impedance 10nA	FHC	Used to simulate the presynaptic cell

Table 2.15 Equipment for electrophysiology experiments

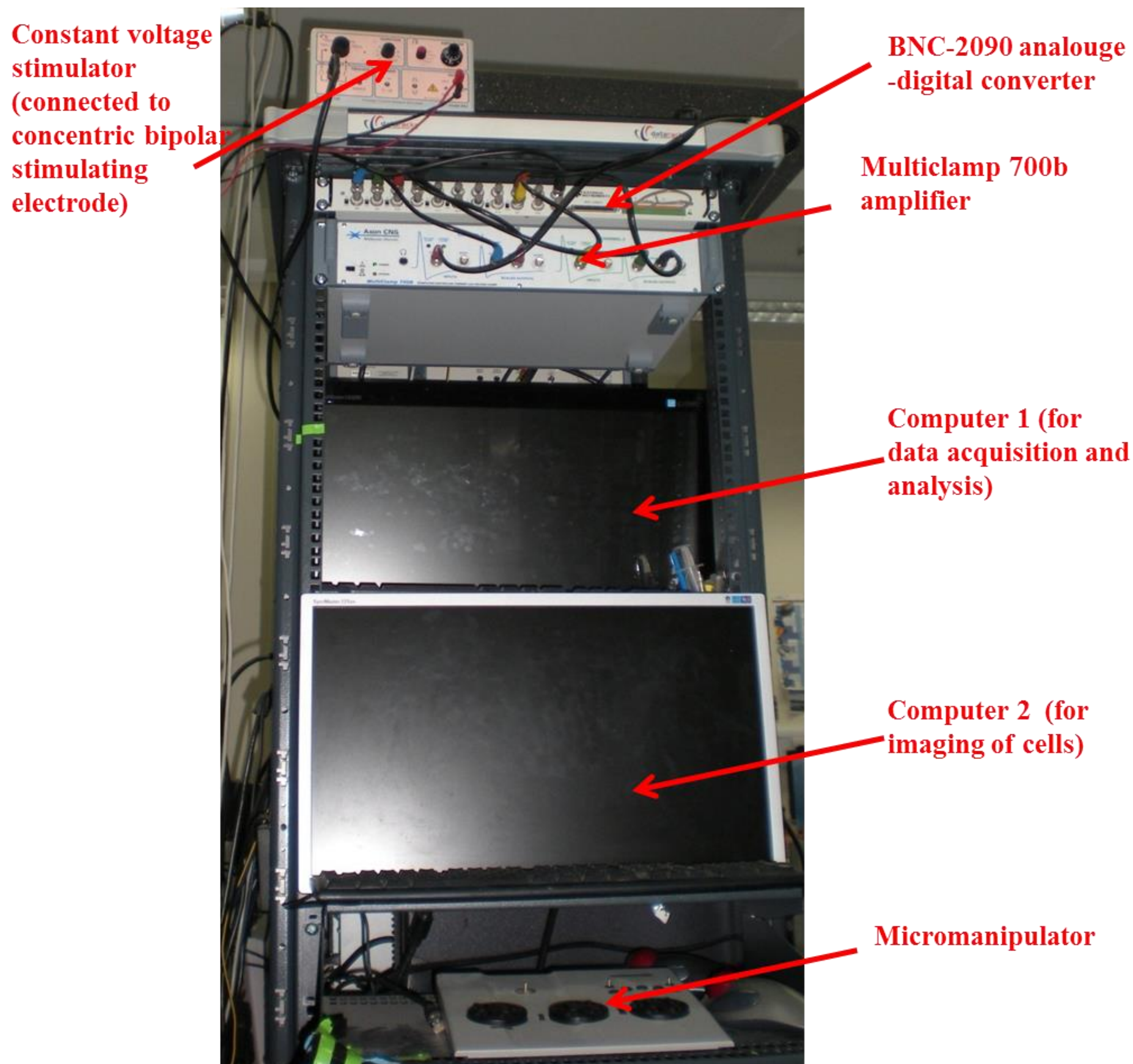


Figure 2.8 Electrophysiology rig I

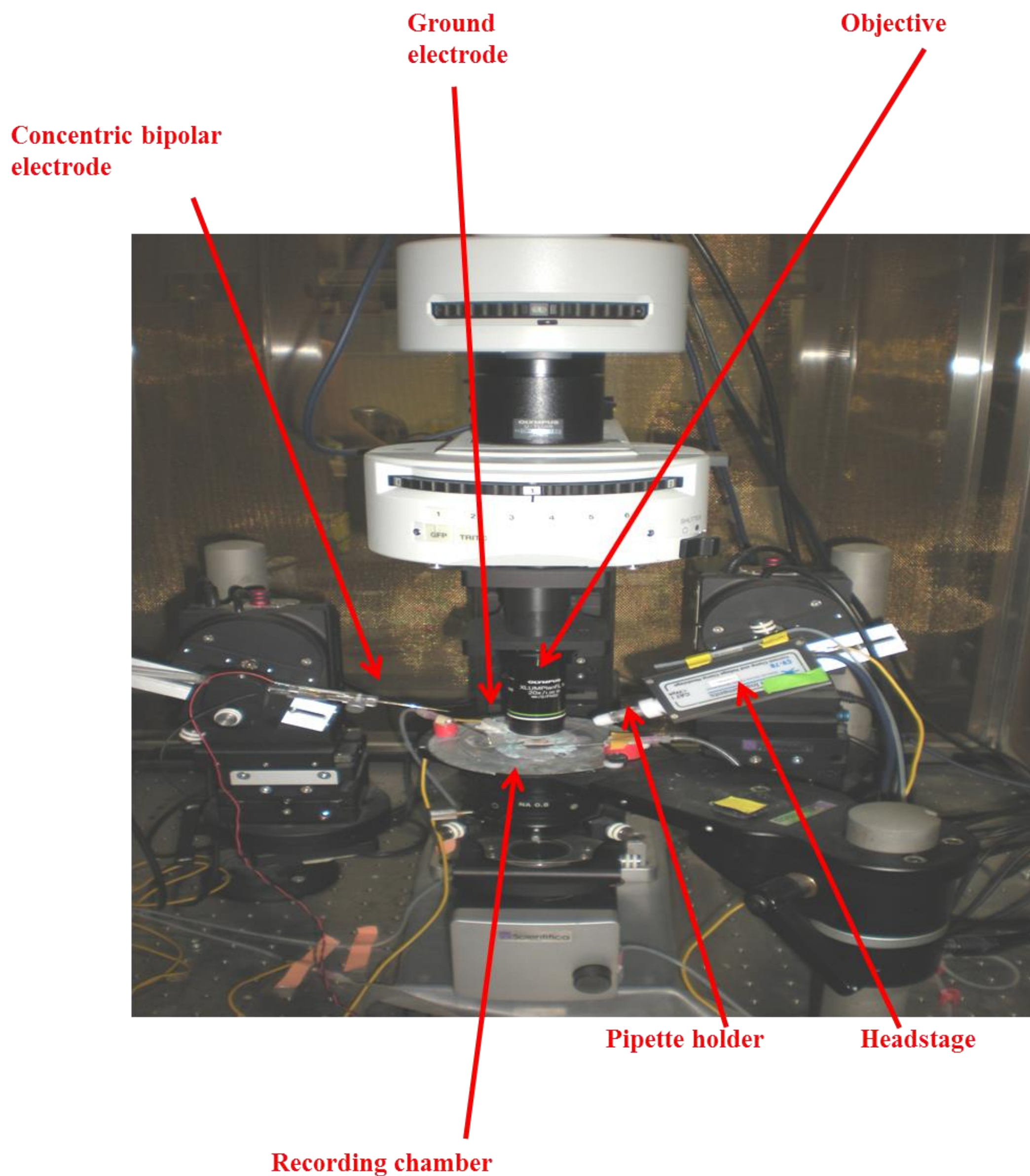


Figure 2.9 .Electrophysiology rig II micromanipulators and headstage

Software	Source	Use
Multiclamp		
Labview	National Instruments and custom written	Acquisition software for electrophysiology experiments. Used to record output from the amplifier. Software run on Computer 1
LinLab		Used to record position of chosen cell
Patch vision		Used to image cells
Excel		Used for analysis of raw data

Table 2.16 Software used for data acquisition and analysis in electrophysiology experiments

The contribution of VGCC subtypes to the amplitude of the excitatory post synaptic potential (EPSC) was assessed using the specific blockers ω-conotoxin GVIA (ω-ctx), ω agatoxin IVA (ω-aga) and SNX 482. These experiments were performed in Cacna1a knockout mice and their heterozygous and wildtype littermates. DL-APV and NBQX were used in the extracelluar solution to prevent recurrent excitability.

Drug	Target	Stock Concentration	Final Concentration	Storage	Provider
ω-ctx	N type Calcium channel antagonist	500 µM	5 µM	-20°C	Ascent Scientific
ω-aga	P/Q-type calcium channel antagonist	25 µM	250nM	-20°C	Ascent Scientific
SNX - 482	R type calcium channel antagonist	20 µM	200 nM	-20°C	Ascent Scientific

Table 2.17 VGCCS toxins used in electrophysiology experiments

2.6.1. Protocol for electrophysiological recording of EPSCs

Cells were patched in the somatic whole cell voltage clamp configuration at room temperature. Excitatory post synaptic currents (EPSCs) were recorded via a MultiClamp 700B amplifier, filtered at 4 Hz and digitized at 10 kHz. Filamented borosilicate glass pipettes were pulled to a resistance of 3.5-5 MΩ. The glass coverslip was placed in the recording chamber and was constantly perfused with extracellular buffer at a rate of 1ml/minute throughout the experiment.

The cells on the coverslip were visualised with the 10x objective and a suitable cell was located. The position of the cell was recorded using the LinLab software. The stimulating electrode was brought to the area of the coverslip adjacent to the cell of interest using the coarse micromanipulator. The recording pipettes were filled with intracellular solution and were placed in the pipette holder of the Ag/$AgCl_2$ electrode which was attached to the headstage. The chloride of the silver wire was regularly replaced by electroplating to avoid electrical drift. Positive pressure was applied to the glass pipette before it was lowered into the bath using the coarse manipulators. The pipette resistance was calculated using the seal test and the pipette offset was zeroed. The pipette was manoeuvred to just above the cell using the coarse manipulator. LinLab software was used to locate the chosen cell. The objective was changed from 10x to the 63x and the pipette was brought closer to the cell using the fine micromanipulator. Just before the cell was patched, the pipette offset was zeroed again and the pipette was gently moved in a diagonal fashion until it was brought in contact with the cell surface. This was observed as a slight dimpling of the cell surface. The pressure was released and a gentle suction was applied to achieve a gigaseal between the membrane and the pipette. If a gigaseal was not obtained the cell was discarded. The pipette potential was switched a holding potential of -70mV and the capacitance transients were neutralised using the amplifier control software.

Once the gigaseal was stable, a short sharp suction was applied to rupture the membrane and enter the whole cell configuration. The access was monitored and a small pressure was applied to the pipette in order to stabilise it below 25 MΩ if this was required. The access resistance was monitored throughout the experiment and the cell was discarded if the access resistance drifted by more than 25%. The patch was held for 5 minutes

before recording to allow for equilibration of the intracellular solution between the cell and the pipette. An extracellular bipolar electrode was positioned nearby to stimulate one or more axons at a frequency of 2 pulses 50 milliseconds apart every 5 seconds. The amplitude of the stimulating pulse was adusted for each experiment – the lowest amplitude that achevied a post synaptic response was chosen. The EPSCs evoked were recorded using LabView software.

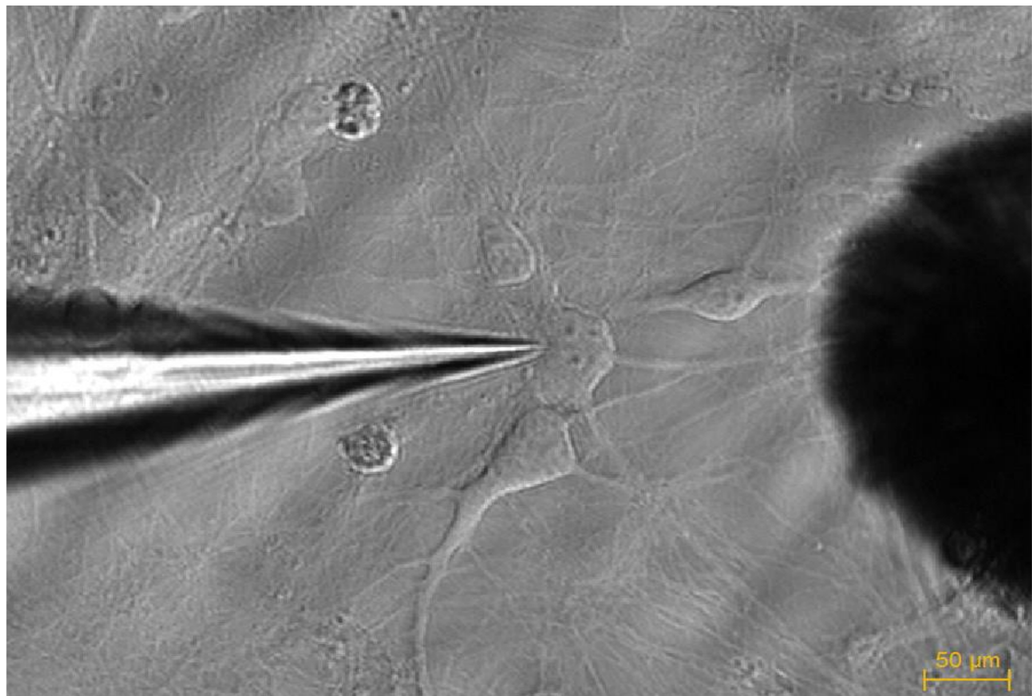


Fig 2.10 Hippocampal cell culture 14 days in vitro with concentric bipolar stimulation (right) and electrode and pipette for whole cell patch clamp (left)

In order to isolate the contribution of each type of presynaptic VGCC, the amplitude of the EPSCs were measured at baseline and after sequential application of toxins to block P/Q, N and R type VGCCs. After 15 minutes of baseline recording, ω –Aga (250 nm) was added to the perfusion solution. After 30 minutes, ω- ctx (5 μM) was added and after 45 minutes, SNX-482 (200nM) was added.

2.6.2 Analysis of EPSCS

The average EPSC peak for each experiment was calculated in LabView 8.0. As demonstrated in the figure 2.11, baseline start and end constraints (see cursors on figure 2.11) were placed roughly 20 ms apart 10 ms before the beginning of the EPSC. The start find (blue cursor) was placed at the start of the EPSC to initiate the search for the EPSC. The maximum of the peak was calculated to be the maximal point between the peak start and peak end (green cursors). The position of the cursors was applied to individual EPSC traces and the maximal amplitude of each EPSC was calculated. Once the peak of each EPSC trace was found, the data were extracted and exported to Excel.

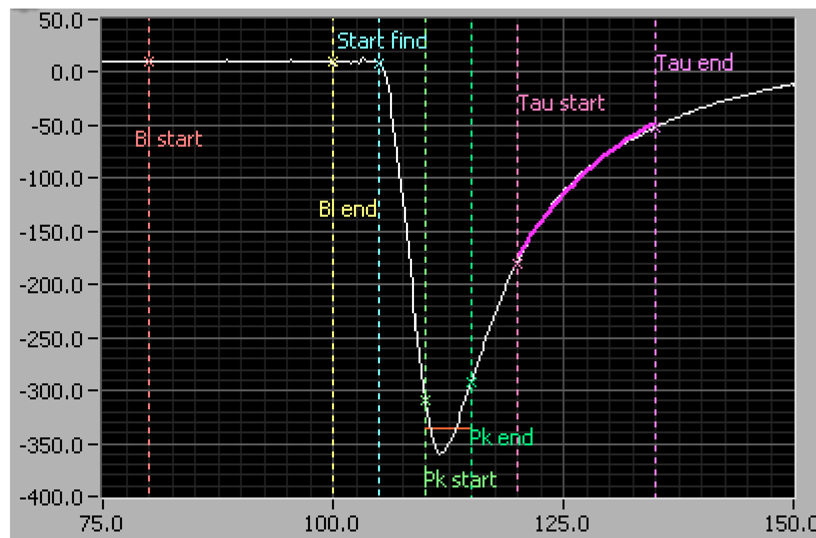


Figure 2.11 Analysis of EPSCs. The average EPSC peak for each experiment was calculated in LabView in order to calculate where to place the baseline (Bl) and peak (Pk) cursors. The "Bl start" (red) and "Bl end" (yellow) cursors were placed roughly 20 ms apart, 10 ms before the start of the EPSC. The "Start find" cursor (blue) was

placed at the start of the EPSC. The peak of the EPSC was calculated as the maximal point between the "Pk start" and "Pk end" cursors (green).

2.7 Statistical Methods

All electrophysiology and synaptic vesicle imaging data were described by the mean and the standard error of the mean. The 2 tailed unpaired t test was used for statistical analysis to assess significance.

3. Immunostaining for LEMS IgG

3.1 Background

As was discussed in Chapter 1, it has long been accepted that the pathology of LEMS is autoimmune. Initial clues about this came from clinical observations, including the association of LEMS with other autoimmune diseases and the improvement of LEMS symptoms after immunomodulatory treatment. The definitive evidence however, came from passive transfer studies (Lang et al., 1981; Lang et al., 1984, Fukunaga et al., 1983; Kim et al., 1986) whereby the electrophysiological and morphological features of the disease were transferred to rodents.

3.1.1 Antigenic target in LEMS

The presumed antigenic target in LEMS is the presynaptic voltage gated calcium channel (VGCC). Antibodies directed against P/Q-type VGCCs can be detected by radioimmunoprecipitation assay in the vast majority of LEMS patients (Motomura et al., 1997) and Ca^{2+} currents are reduced in a wide variety of cell types after incubation in LEMS IgG (Garcia and Beam, 1996; Hewett and Atchison,1991; Kim and Neher, 1988; Roberts et al., 1985; Grassi et al., 1994; Viglione et al., 1995; Meriney et al. 1996; Pinto et al., 1998; Johnston et al 1994)**.**

Ambiguity remains however,regarding the exact target of antibodies in LEMS patients; not least because 10-15% of patients have no detectable P/Q- or N-type antibodies (Motomura et al., 1997; Nakao et al., 2002). Moreover, Western blots and immunoprecipitation assays rely on the use of detergents to solubilize proteins from

cells which may result in the detection of antibodies that are not necessarily pathogenic. The results of immunoprecipitation assays can also be affected by the particular protocol used; for example, mouse anti-synaptotagmin monoclonal antibodies can detect radiolabelled VGCC from rat brain precipitated in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent but not from VGCC precipitated in digitonin, (Lennon et al., 1995).

There is previous literature regarding how immunoprecipitation assays can be misleading. Antibodies that react against a different ion channel; the voltage gated potassium channel (VGKC) have been detected in association with three different clinical syndromes- neuromyotonia, Morvan's Syndrome and limbic encephalitis (Irani et al., 2010). These antibodies are detected in a similar fashion to the immunoprecipitation assay described above for detection of antibodies in LEMS syndrome. VGKCs from homogenized mammalian brain, solubilized with digitonin, are labelled with iodinated α- dendrotoxin (^{125}I-α-DTX) and a radioimmunoprecipitation assay with serum from patients is performed. A recent study, however, revealed that IgG from most patients with these conditions fails to bind to VGKCs expressed on HEK 293 cells (Irani et al., 2010). This was a surprising finding and further investigation subsequently revealed that patient antibodies actually bind to various proteins that are complexed with ^{125}I-α-DTX labelled VGKC in brain extracts – namely contactin associated protein-2, leucine-rich glioma inactivated 1 protein and Tag-1/contactin-2 that associates with contactin associated protein- 2.

The aim of this study was to visualise the binding of antibodies from LEMS patients to cultured neuronal cells using high resolution microscopy. LEMS antibody binding to neuronal cells has not been visualized in this way before but if successful, immunohistochemistry techniques could be used to develop a cell-based assay which would help to clarify further the exact antigenic target in LEMS. This could have important implications for the diagnosis of LEMS and could help develop more specific therapies for patients with LEMS.

3.1.2 Previous attempts to visualise binding of LEMs IgG using immunohistochemistry

Previous studies have attempted to detect binding of IgG at the motor end plate in mice passively treated with LEMS IgG (Fukuoka et al., 1987b). Hitherto, this has been a difficult task due in part, to the relatively low density of active zone particles which are the morphological representation of VGCCs (Fukuoka et al., 1987). The density may be even lower in LEMS as LEMS antibodies are thought to cross link and internalize VGCCs (Nagel et al., 1988). In these experiments, there has also traditionally been a lot of non-specific background staining as passive transfer of IgG means that the end plates are exposed to high concentrations of IgG. Moreover, the reaction product can diffuse and be reabsorbed onto cell membranes making it difficult to distinguish between pre and post synaptic staining (Engel et al., 1987). Fukuoka et al. used the avidin-biotin detection system to increase sensitivity of immunolocalization and found significant immunostaining of presynaptic mouse active zones in mice treated with LEMS IgG (Fukuoaka et al., 1987). However this method resulted in a significant amount of non-specific staining and the resolution of the microscopy was limited.

In another experiment, Polizzi et al. tested sera from 20 patients with LEMS for binding to frozen sections of adult rat brain using standard immunohistochemistry techniques. However, only five of the 20 patients sera showed binding to neuronal antigens (Polizzi et al., 1998). Moreover, the immunostaining seen in this study was not specific and was not consistent with antibody binding to cell surface antigens.

3.2 Specific aims of this chapter

With improvements in imaging technology and increasing sophistication and quality of fluorescent secondary antibodies, I hoped that I would be able to specifically detect binding of LEMS IgG to cell surface antigenic targets using immunohistochemistry. I also wished to assess whether any immunostaining seen could be specifically co-localized to presynaptic sites.

3.3 Methods

The reagents and equipment used and methods are outlined in more detail in chapter 2. Briefly, non permeabilized hippocampal neuronal cultures were used to detect the binding of LEMS IgG with anti human IgG Alexa 594 as a fluorescent secondary antibody. I chose to use hippocampal neuronal cultures for our experiments as they have been well validated in other immunohistochemistry experiments in the past (Irani

et al., 2010b). Furthermore, the main aim was to detect binding of LEMs IgG to extracellular epitopes of VGCCs. As there is a high degree of sequence homology between mouse, rat and human CACNA1A gene products (figure 3.1),an antibody that binds to an extracellular epitope of the human P/Q-type VGCC is likely to bind to the corresponding region of the rat P/Q-type VGCC.

Species	Position	Sequence
Human	118-136	EQHLPDDDKTPMSERLDDT
Rat	120-138	EQHLPDDDKTPMSERLDDT
Mouse	120-138	EQHLPDDDKTPMSERLDDT
Human	183-190	ATVGTEFD
Rat	185-192	ATVGTEFD
Mouse	185-192	ATVGTEFD
Human	249-335	MGKFHTTCFEEGTDDIQGESPAPCGTEEPARTCPNGTKCQPYWEGPNNGITQFDNILFAVLTVFQCITMEGWTDLLYNSNDASGNTW
Rat	251-337	MGKFHTTCFEEGTDDIQGESPAPCGTEEPARTCPNGTKCQPYWEGPNNGITQFDNILFAVLTVFQCITMEGWTDLLYNSNDASGNTW
Mouse	251-337	MGKFHTTCFEEGTDDIQGESPAPCGTEEPARTCPNGTKCQPYWEGPNNGITQFDNILFAVLTVFQCITMEGWTDLLYNSNDASGNTW
Human	507-521	VHYNQPEWLSDFLYY
Rat	509-523	VHYNQPEWLSDFLYY
Mouse	509-523	VHYNQPEWLSDFLYY
Human	569-578	AVIKPGTSFG
Rat	571-580	AVIKPGTSFG
Mouse	571-580	AVIKPGTSFG
Human	637-689	GGQFNFDEGTPPTNFDTFPAAIMTVFQILTGEDWNEVMYDGIKSQGGVQGGMV
Rat	639-691	GGQFNFDEGTPPTNFDTFPAAIMTVFQILTGEDWNEVMYDEIKSQGGVQGGMV
Mouse	639-691	GGQFNFDEGTPPTNFDTFPAAIMTVFQILTGEDWNEVMYDGIKSQGGVQGGMV
Human	1262-1280	AAEDPVQPNAPRNNVLRYF
Rat	1213-1231	AAEDPVQPNAPRNNVLRYF
Mouse	1215-1231	AAEDPVQPNAPRNNVLRYF
Human	1329-1339	AFTGNSKGKDINTI
Rat	1283-1293	AFTGNSKGKDINTI
Mouse	1283-1293	AFTGNSKGKDINTI
Human	1398-1486	LFKGKFFHCTDESKEFEKDCRGKYLLYEKNEVKARDREWKKYEFHYDNVLWALLTLFTVSTGEGWPQVLKHSVDATFENQGPSPGYRME
Rat	1349-1437	LFKGKFFHCTDESKEFERDCRGKYLLYEKNEVKARDREWKKYDFHYDNVLWALLTLFTVSTGEGWPQVLKHSVDATFENQGPSPGYRME
Mouse	1349-1437	LFKGKFFHCTDESKEFERDCRGKYLLYEKNEVKARDREWKKYEFHYDNVLWALLTLFTVSTGEGWPQVLKHSVDATFENQGPSPGYRME
Human	1587-1598	FYGASVAYENALRV
Rat	1538-1552	FYGASVAYENALRV
Mouse	1538-1552	FYGASVAYENALRV
Human	1646-1656	LVTEFGNNFIN
Rat	1597-1607	LVTEFGNNFIN
Mouse	1597-1607	LVTEFGNNFIN
Human	1711-1786	MQVFGNIGIDVEDEDSDEDEFQITEHNNFRTFFQALMLLFRSATGEAWHNIMLSCLSGKPCDKNSGILTRECGNEF
Rat	1666-1737	MQVFGNIGIDGEDEDSDEDEFQITEHNNFRTFFQALMLLFRSATGEAWHNIMLSCLSGKPCDKNSGIQKPECGNEF
Mouse	1666-1737	MQVFGNIGIDGEDEDSDEDEFQITEHNNFRTFFQALMLLFRSATGEAWHNIMLSCLSGKPCDKNSGILTADCGNEF

Figure 3.1 Amino acid sequences of the extracellular elements of the alpha$_1$ subunit of the P/Q VGCCs demonstrating a high degree of amino acid sequence homology between the species. Sequences in grey highlight areas where there is some uncertainty

about the exact start and end of the extracellular regions. (Sequences taken from uniprot.org)

Both fixed and live neuronal cultures were used. Medium was aspirated from the neuronal cultures and the cells were washed thoroughly with PBS. If the cells were to be fixed, they were incubated with 4% PFA for 10 minutes. Following fixation, the cells were washed with PBS. When live cells were used, the cells were gently washed with PBS after removal of the culture medium. In both cases, non specific epitopes were then blocked in 5% BSA in PBS for 30-60 minutes after which they were washed in PBS. The cells were incubated with the primary antibody (either a LEMS or control sample, typically in a concentration of 1:1000 of 1% BSA in PBS) for 60 minutes at room temperature. The IgG samples were of varying concentrations ranging from 5mg/ml to 22 mg/ml and the concentration required for dilution was adjusted for each sample. The primary antibody was then removed and the cells were washed with PBS. When the primary antibody had been incubated with live cells, they were fixed with 4% PFA at this point. The cells were then incubated with the secondary antibody (typically anti human IgG Alexa 594 at a concentration of 1:1,000 for one hour at room temperature followed by washing with PBS.

Another antibody was used for co-localisation in some experiments, typically this was rabbit anti-synapsin but anti-Green Fluorescent Protein (GFP) was used to localise GFP tagged VGCCs and the synaptic vesicle FM dye, SRC-1 was also used in some experiments. In these experiments, the cells were fixed once again with 4% PFA and were then washed with PBS. The cells were permeabilized by incubating them with 5%

BSA in 0.1% Triton in PBS for 30 minutes. After permeabilization the cells were PBS washed and blocked in 5% BSA in PBS. The cells were incubated with the primary and secondary antibodies (for example, rabbit anti-synapsin and anti-rabbit Alexa 488) in the same fashion as was described for LEMS IgG outlined above.

At the end of the experiment, the cells were washed thoroughly and the coverslips were mounted on glass slides using Vectashield mounting medium. The cells were imaged using an LSM 500 confocal microsope. Alexa 594 was excited using the 543nm line of He-Ne laser and its emission was recorded using a band-pass 560-615nm filter. Alexa 488 was excited by the 488nm line of an argon laser and its emission was recorded using a band pass 505-530 filter. I am grateful to Dr Y. Ermolyuk for his help with the use of the confocal microscope. There were a number of changes made to this protocol depending on the exact aim of the experiment being carried out and these are outlined below. The figures shown are representative of 10 different images taken from each coverslip.

3.4. Results

3.4.1. Immunostaining of one LEMS sample in fixed unpermeabilized rat hippocampal cultures using anti-human IgG Alexa 594 as a secondary antibody

The first experiment was designed to test whether our secondary antibody – anti human IgG Alexa 594 – was specific for human IgG. Fixed, non-permeabilized rat hippocampal neuronal cultures were incubated with (a) LEMS IgG at a concentration of

1:100 in 1% BSA in PBS, or (b) no primary antibody in 1% BSA in PBS followed by incubation with anti-human IgG Alexa 594 as the secondary antibody (figure 3.2).

In the cells incubated with LEMS IgG from one patient,immunostaining of cell bodies and neurites was detected (Figure 3.3.a). There was no immunostaining of cells that were not incubated with a primary antibody (figure 3.3.b), suggesting that the LEMS IgG bound to some antigenic target on the surface of neuronal cells and that the secondary antibody was specific for human IgG. On high resolution images it was observed that LEMS stained both cell bodies and neurites of some but not all cells (figures 3.4 and 3.5). The cells were not permeabilized and so, only cell surface binding was observed. No significant glial cell staining was observed. The images shown are representative of experiments from two different cultures and ten images were taken from each coverslip.

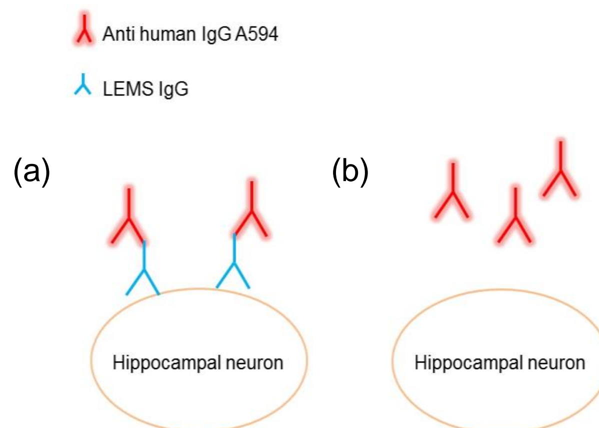


Figure 3.2 Schematic diagram showing binding of anti human IgG A594 to LEMS IgG in hippocampal neurons (a), compared to hippocampal neurons that were not incubated with a primary antibody (b).

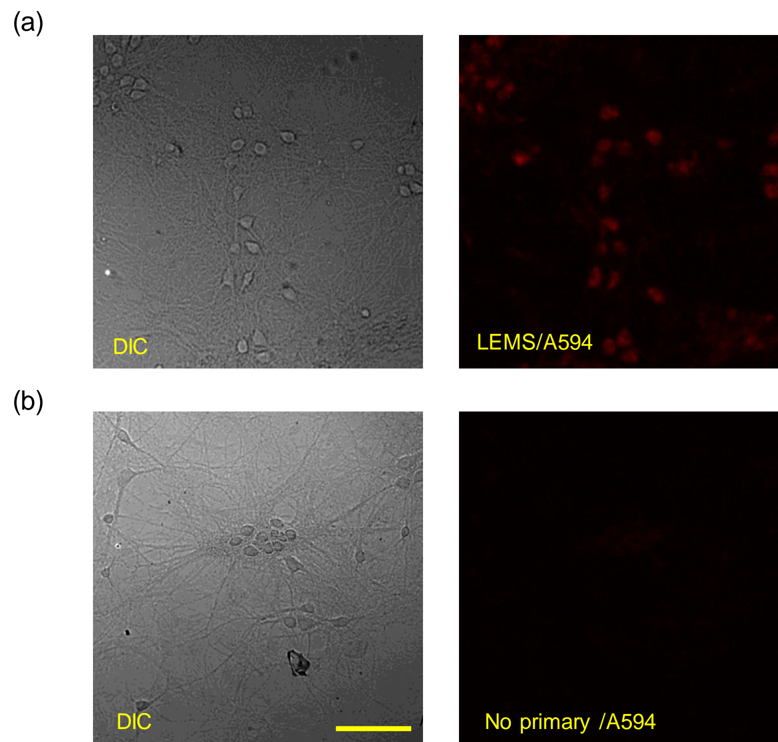


Figure 3.3 Fluorescence images (right panels) showing (a) immunostaining of LEMS IgG with anti human IgG 594 on cell bodies and neurites of hippocampal neurons. The cultures that were not incubated with a primary antibody did not exhibit any immunostaining (b) implying that the secondary antibody was specific for human IgG DIC images are shown for comparison (left panels). Scale bar =100 µm

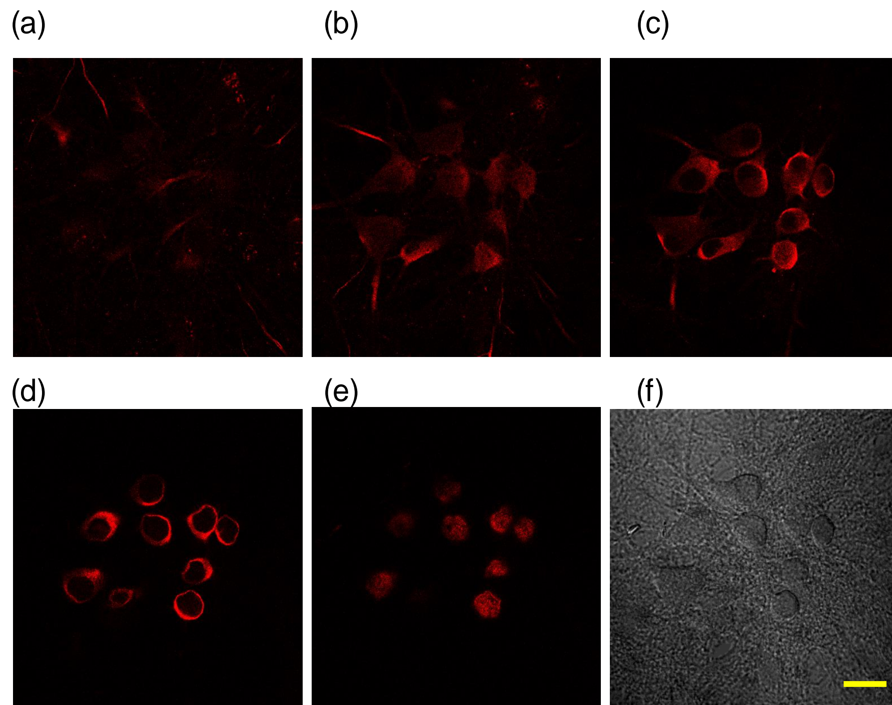


Figure 3.4 A section of hippocampal neurons showing that LEMS IgG immunostained neurites and cell bodies of hippocampal neurons (a-d). DIC image is shown for comparison (f). Scale bar = 10 µm. The thickness of each slice was 1 um and the distance between sections was 2.5 µm

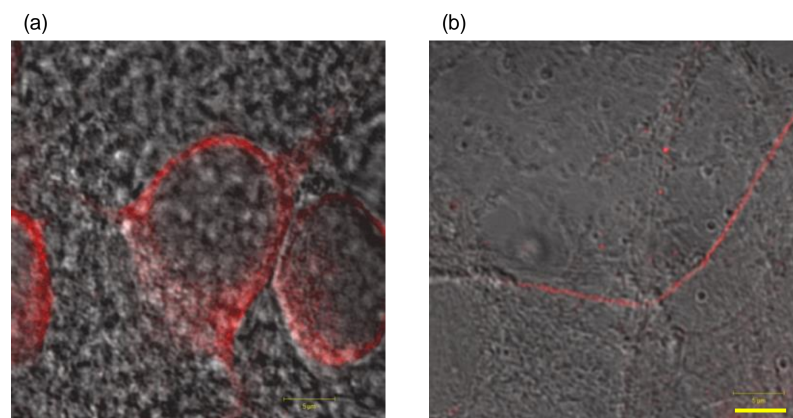
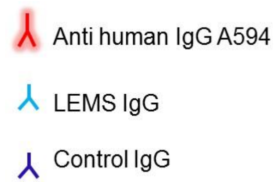


Figure 3.5 High resolution fluorescence image superimposed on DIC showing that LEMS IgG immunostains soma and neurites of hippocampal neurons in culture. Scale bar = 5 µm

3.4.2 Immunostaining of LEMS IgG compared to control IgG in fixed unpermeabilised rat hippocampal cultures using anti human IgG Alexa 594 as a secondary antibody

Having confirmed that the secondary antibody was specific and having detected LEMS IgG binding to hippocampal cell bodies and neurites as outlined above, the next step was to compare the immunostaining of LEMS IgG to control IgG.

The experiments were carried out in the same manner as described above. Binding of one LEMS sample was compared to a pooled sample of control IgG from healthy volunteers (figure 3.6). It was once again confirmed that LEMS IgG can be detected binding to fixed non permeabilised cells (figure 3.7a) but the degree of this staining and its specificity was difficult to quantify. Moreover, there was a high degree of non-specific staining with control IgG (figure 3.7b). The images shown are representative of experiments from three different cultures and ten images were taken from each coverslip.



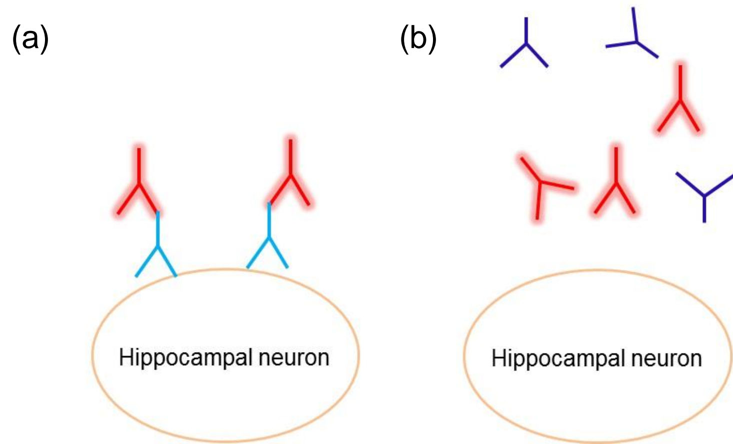


Figure 3.6 Schematic diagram showing (a) immunostaining of LEMS IgG in fixed non-permeabilised hippocampal neurons compared to (b) immunostaining with control IgG

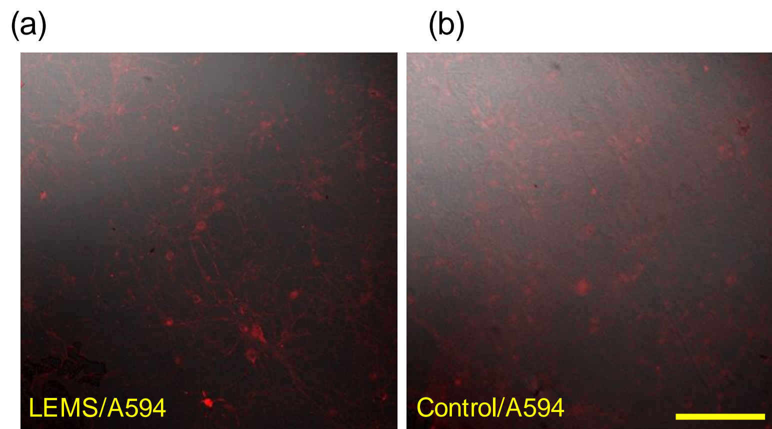


Figure 3.7 (a) Fluorescence images showing immunostaining of LEMS IgG (a) compared to control IgG (b). Scale bar = 100 µm.

3.4.3 Immunostaining of LEMS IgG compared to control IgG in neurons transfected with GFP tagged VGCCS

The previously described experiment was disappointing because there was a high degree of non-specific binding detected in cultures incubated with control IgG and the immunostaining detected with LEMs IgG was difficult to quantify. The next step was to attempt to specifically localise the binding of LEMS IgG to VGCCs using neurons that were transfected with green fluorescent protein (GFP) tagged human P/Q-type VGCCs. The enhanced GFP-Ca_v2.1 fusion plasmid was previously used by a group in our laboratory (Imbrici et al., 2004). As the cDNA used was human, it would also allay any concerns that difference in antigens between the species was the reason why binding was not detected.

3.4.3.1 Methods

The experiments were conducted in the same way as outlined above. After completion of staining for LEMS with primary and secondary antibodies the cells were washed with PBS, fixed with 4% PFA and then permeabilised with 5% BSA in 0.1% Triton in PBS for 30 minutes. The cells were then washed with PBS and incubated with rabbit anti-GFP primary antibody at a concentration of 1:1,000. The remainder of the experiment was carried out in the same way as outlined above for the incubation with LEMS antibodies except the secondary antibody used was anti rabbit-IgG Alexa 588 at a concentration of 1:1,000 (Figure 3.8.)

3.4.3.2 Results

Some LEMS IgG binding to surface antigens in neuronal culture was detected (3.9b) but the LEMS IgG binding was not co-localised to the GFP tagged VGCCs (figure 3.9a). The images shown are representative of experiments from two different cultures and ten images were taken from each coverslip.

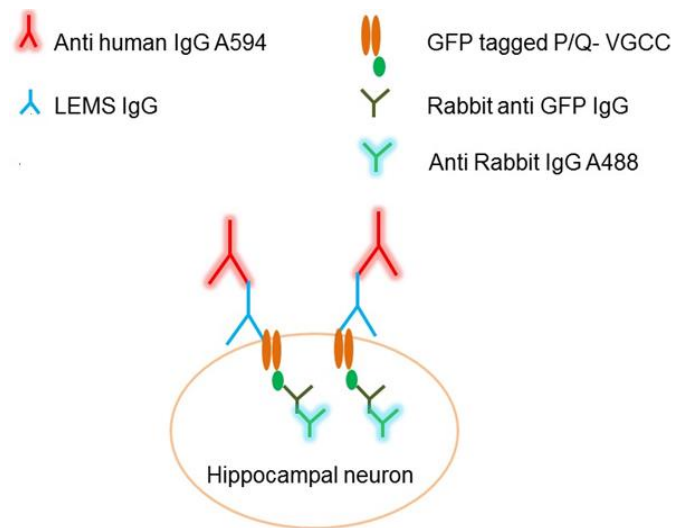


Figure 3.8 Schematic diagram showing the immunostatining of LEMS IgG with anti human IgG A594 in neurons transfected with GFP-tagged VGCCs.

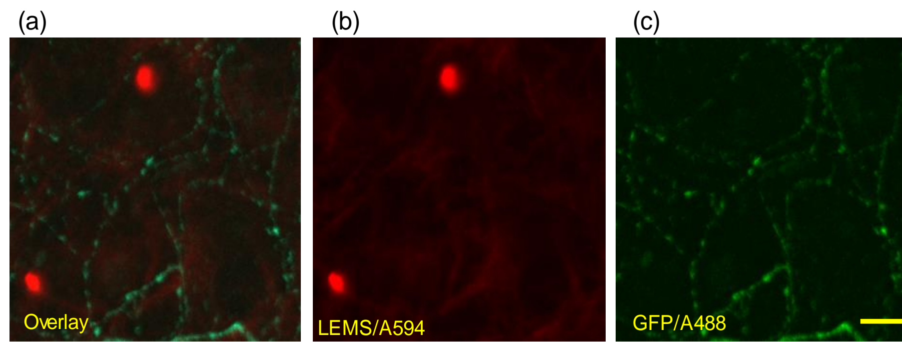


Figure 3.9 Fluorescence images showing immunostaining of LEMS IgG with anti-human IgG 594 (red) in neurons transfected with GFP- tagged VGCCs (green) (a) overlay of green and red channels (b) LEMS IgG (c) GF- tagged VGCCs. Scale bar = 5 μm.

3.4.4 Immunostaining of LEMS IgG compared to control IgG in neurons transfected with GFP tagged VGCCS in permeablized hippocampal neurons

The above experiment was repeated in neurons that had been permeabilised before incubation with LEMS antibodies. LEMS IgG could be immunolocalised to some cell bodies and neurites but was not definitively confirmed to bind to transfected VGCCs (Figure 3.10). The images shown are representative of experiments from two different cultures and ten images were taken from each coverslip.

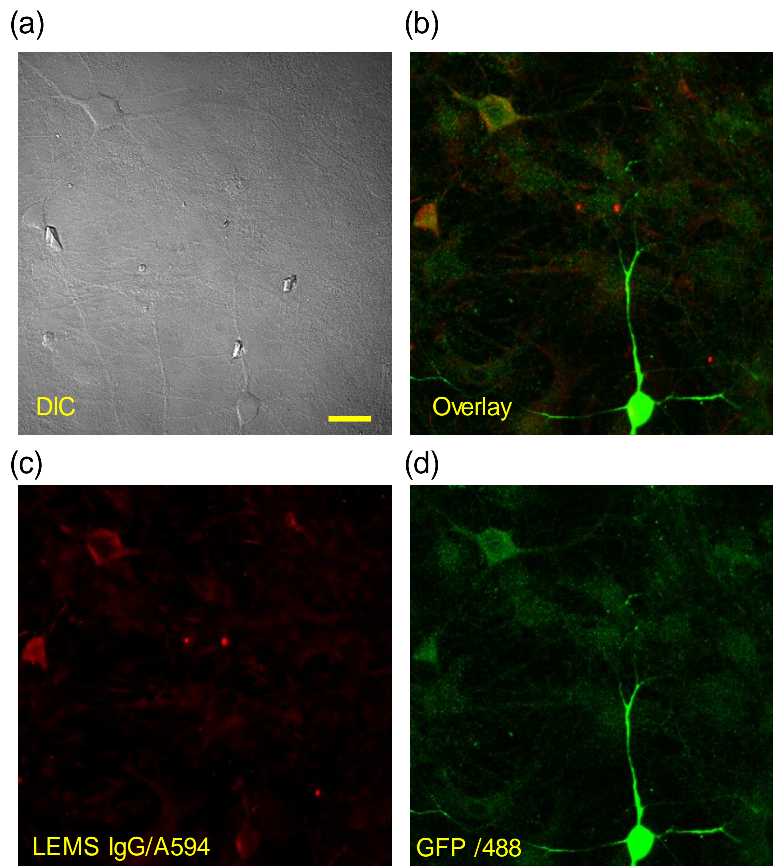


Figure 3.10 Immunostaining of LEMS IgG (red) in permeabilised neurons transfected with GFP tagged P/Q- VGCCS (green). (a) DIC image (b) Overlay of green and red channels (c) LEMS IgG immunostaining with anti-human IgG A594 (d) immunostaining of GFP tagged VGCCs with A488   Scale bar = 10 µm.

3.4.5 Immunostaining of live neurons with zenon labelled LEMS IgG after preblock with control IgG.

So far, the success with immunostaining for LEMS IgG was limited by a high degree of non–specific staining. There was a concern that fixation of neuronal cultures could cause a conformational change in the epitope which could affect antibody binding. It was also postulated that the LEMS IgG may have a low avidity and that if binding were unstable, it may impede the immunolocalisation experiments. Alternatively, it was

possible that LEMs IgG did not specially bind to VGCCs. Thus a different approach was taken for the next experiment.

3.4.5.1 Aim and background

The aim was to image the binding of LEMS IgG labelled with anti human IgG Zenon® Fab fragments in live neuronal cultures and to attempt to localise IgG binding to synapses using synaptic vesicle imaging with the FM dye, SRC-1.

The Zenon® labelling reagents used in this experiment contain a fluorophore, labelled, Fc specific anti Human IgG Fab fragment. This labelled Fab fragment binds to the Fc portion of an antibody (such as LEMS IgG) with high affinity and selectivity. Non specific IgG is used to complex unbound Fab fragments and to ensure that any fluorescence seen is due to specific binding of the Zenon® Fab fragments to human IgG. Using live cultures would ensure no conformational changes in epitopes occurred and could also allow a divalent IgG to bind to two epitopes through cross linking, thus increasing the affinity of antibody binding.

Synapses were stained using the synaptic vesicle dye SRC1 after incubation with Zenon® labelled LEMS IgG. Synaptic vesicle imaging with SRC-1 is discussed in detail in Chapter 2 and in Chapter 4. SRC-1 is an FM dye that is fluorescent when incorporated to the lipid bilayer of a cell membrane. It can be specifically used to label recycling synaptic vesicles and in this experiment was used to attempt to co-localise LEMS IgG binding to the site of synapses.

3.4.5.2 Methods

Live neuronal cultures were incubated in Neurobasal containing control IgG (at a concentration of 250µL per 1ml) to preblock non-specific binding of IgG. The solution also contained NBQX (10 µM) and DL APV (50 µM) to prevent recurrent electrical activity in the live cells. After 15 minutes the coverslip was incubated with LEMS IgG at a concentration of 1:100 that was labelled with human IgG Zenon fragments® (Invitrogen). (Figure 3.11). The coverslip was then transferred to the imaging rig where recycling synaptic vesicles were labelled with the fluorescent styryl dye, SRC-1 (See Chapter 2 and Chapter 4 for methods).

3.4.5.3 Results

Zenon ® labelled LEMS IgG stained neurites and some cell bodies but binding could not be reliably localised to the sites of recycling synaptic vesicles labelled with SRC-1 (figure 3.12). The images shown are representative of experiments from two different cultures and ten images were taken from each coverslip.

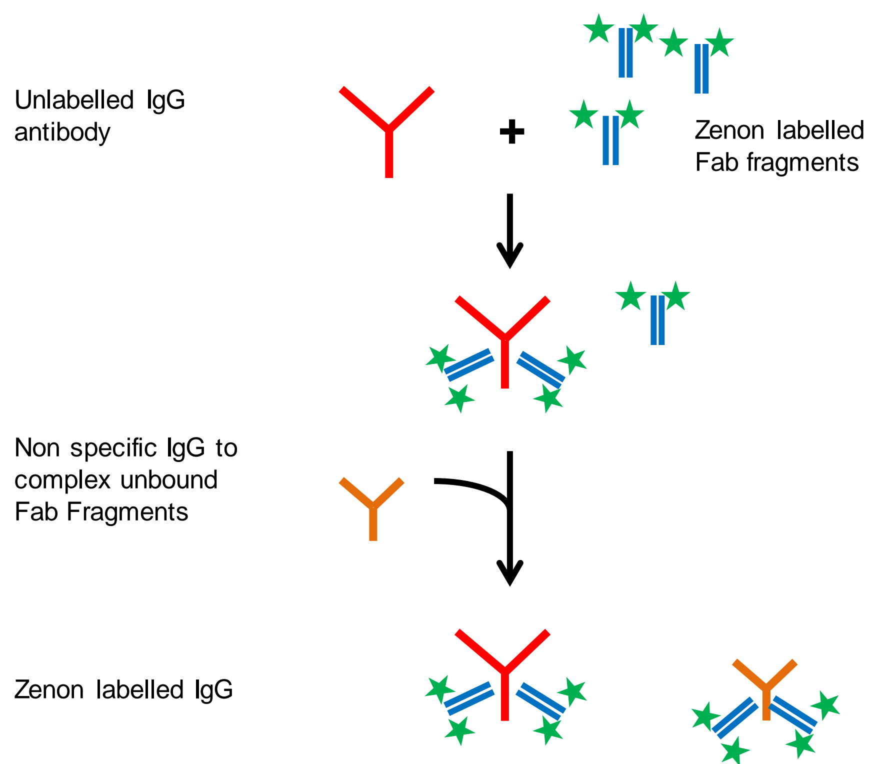


Figure 3.11 Schematic diagram demonstrating labelling of primary IgG with Zenon® Fab fragments

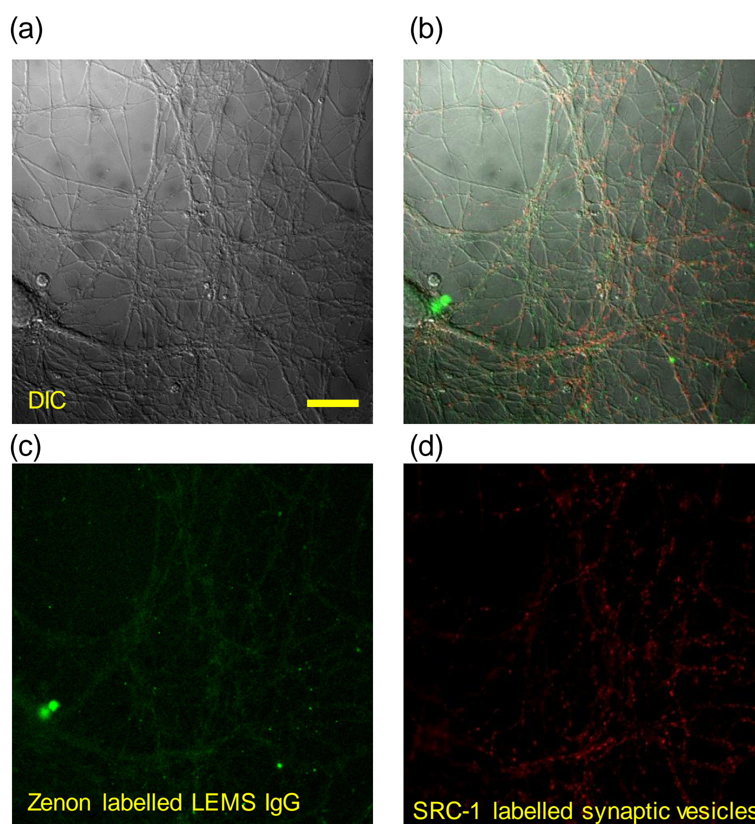


Figure 3.12 Fluorescence images demonstrating Zenon® antibody labelling (green channel) and labelling of synaptic vesicles with SRC-1 (red channel) in live neuronal cultures. (a) DIC image) (b) Fluorescence images superimposed on DIC (c) LEMS IgG lablelling with Zenon fragments (d) Labelling of synaptic vesicles with SRC-1. Scale bar = 10 µm.

3.4.6 Immunostaining of LEMS IgG in fixed neuronal cultures with co-localisation with synapsin

3.4.6.1 Aim

As there was limited success with the methods described thus far, the next step was to attempt to detect specific binding of LEMS IgG to neuronal cultures using co-localisation with the synaptic membrane protein synapsin. It was also decided to use

multiple different LEMS samples as hitherto, just one LEMS sample had been used. A uniform concentration of 1,1000 was used for each sample.

3.4.6.2 Methods

Four different LEMS samples were used – each of these samples had a different concentration and the amount of IgG used in each experiment was adjusted to achieve a concentration of 1:1,000. A pooled control sample was used for comparison and the concentration was also adjusted to achieve a final concentration of 1:1,000.

The experiments were conducted in a similar way as described for fixed neuronal cultures above. The cells were washed, fixed in PFA and blocked with 5% BSA in PBS. They were then incubated with LEMS or Control IgG and washed before application of the secondary antibody. After incubation with anti-human IgG Alexa 594, the cells were fixed, permeabilised, blocked and were then incubated with rabbit anti synapsin antibody (Invitrogen). The cells were washed in the usual way and then anti-rabbit IgG A 488 was used as the secondary antibody (figure 3.13).

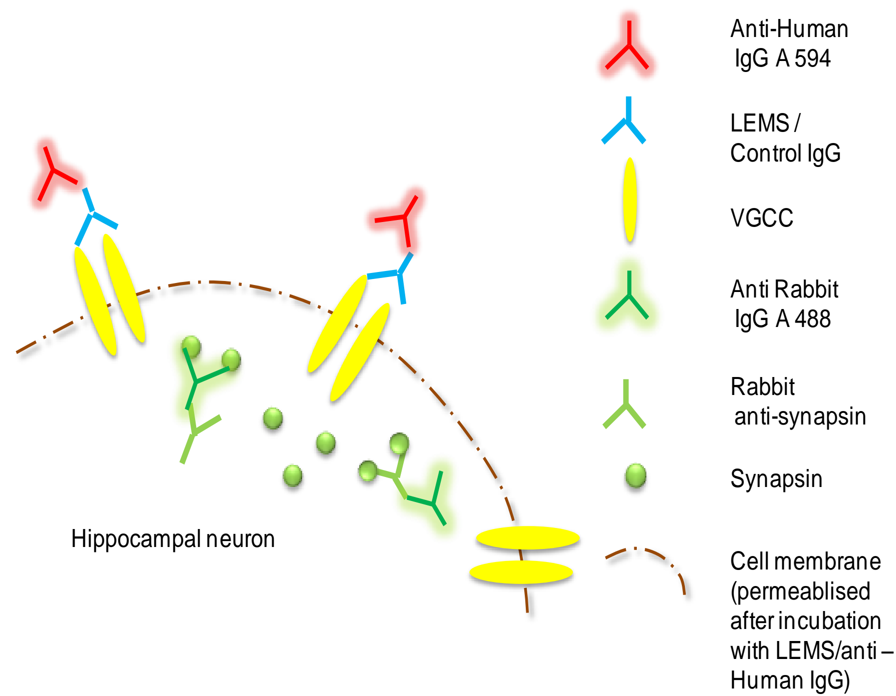
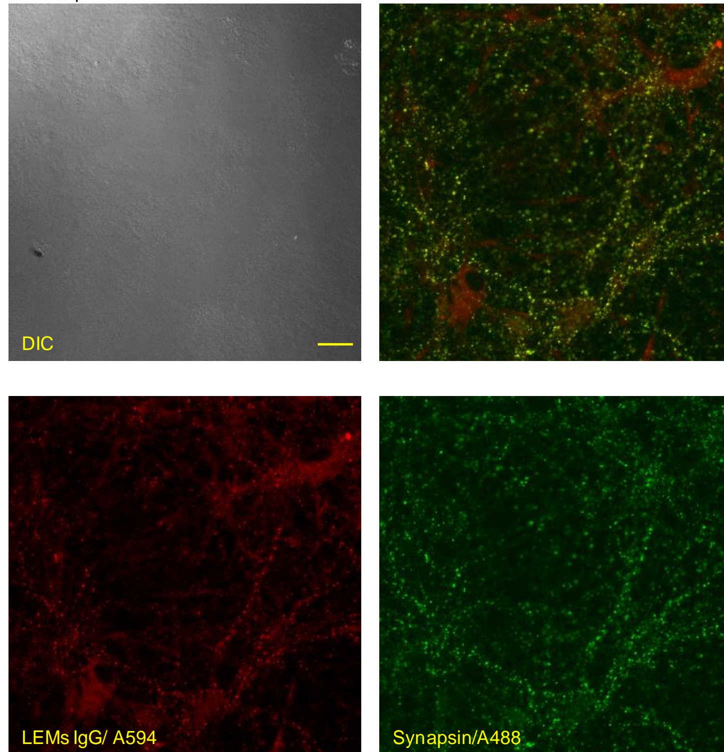


Figure 3.13 Schematic diagram showing immunolocalization of LEMSs IgG and co-staining with synapsin

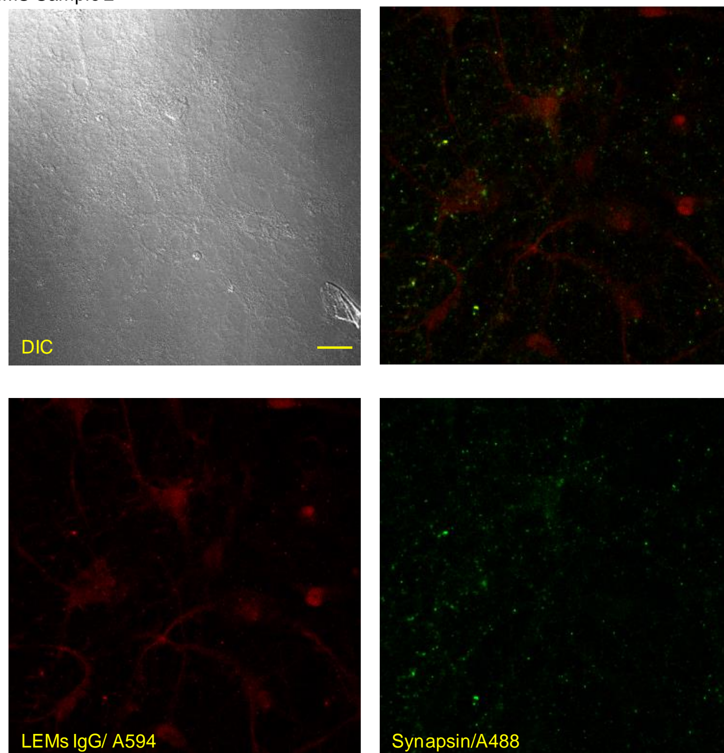
3.4.6.3 Results

Control IgG caused non-specific immunostaining (figure 3.14e). However, LEMS IgG from the four patients used did show variable patterns of immunostaining of cell surface antigens. As was seen before, LEMS IgG bound to both cell bodies and neurites (figure 3.14 a-d). However the pattern and degree of binding was not the same in each LEMs sample. Furthermore, the binding of LEMS IgG could not be specifically localised to synapses as identified by synapsin immunofluorescence (figure 3.14 (a-d)). The images shown are representative of experiments from four different cultures and ten images were taken from each coverslip.

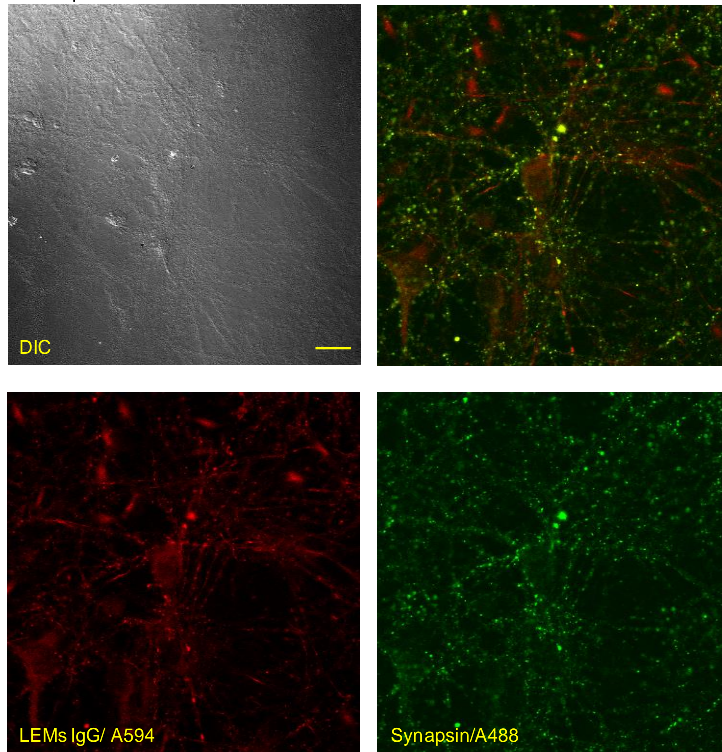
(a) LEMS Sample 1



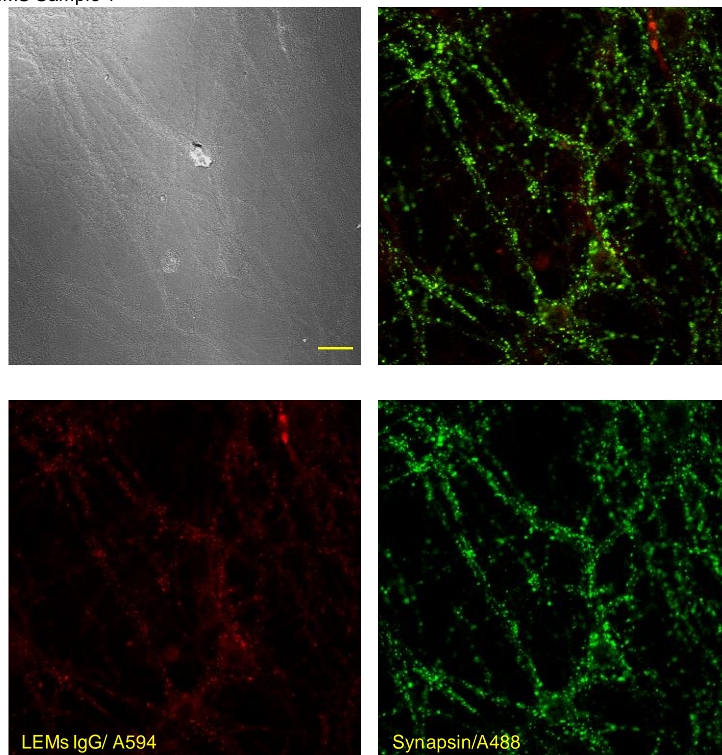
(b) LEMS Sample 2



(c) LEMS Sample 3



(d) LEMS Sample 4



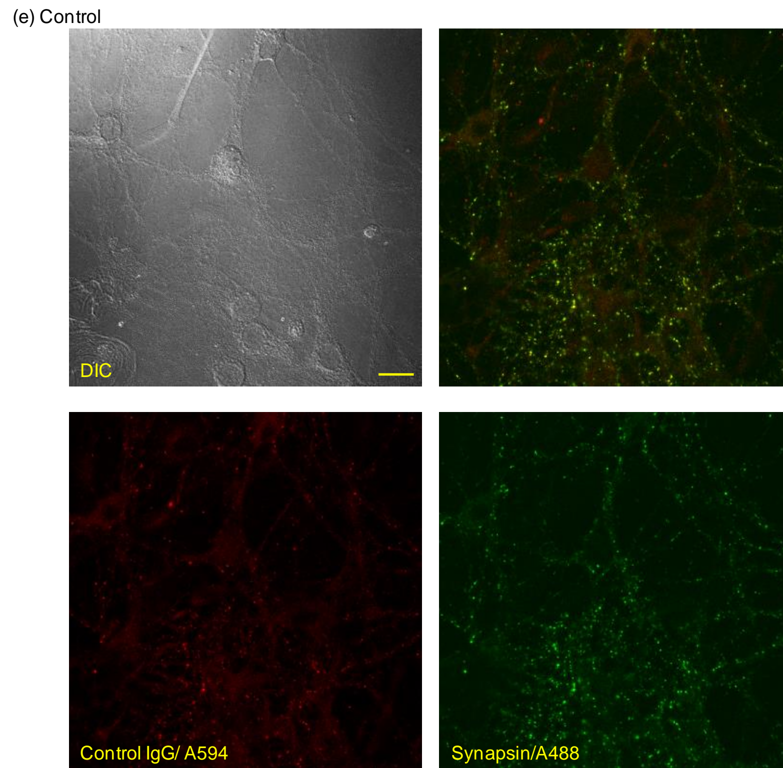


Figure 3.14 Immunostaining of fixed non permeabilized hippocampal cultures with LEMS IgG and co-staining with anti-synapsin (a-d) (a: LEMS Sample 1; b: LEMS Sample 2; c: LEMS Sample 3; d: LEMS Sample 4) and Control IgG (e). Scale bar = 20 μM

3.4.7 Immunostaining of LEMS IgG in live neuronal cultures with co-localisation with synapsin

3.4.7.1 Aim

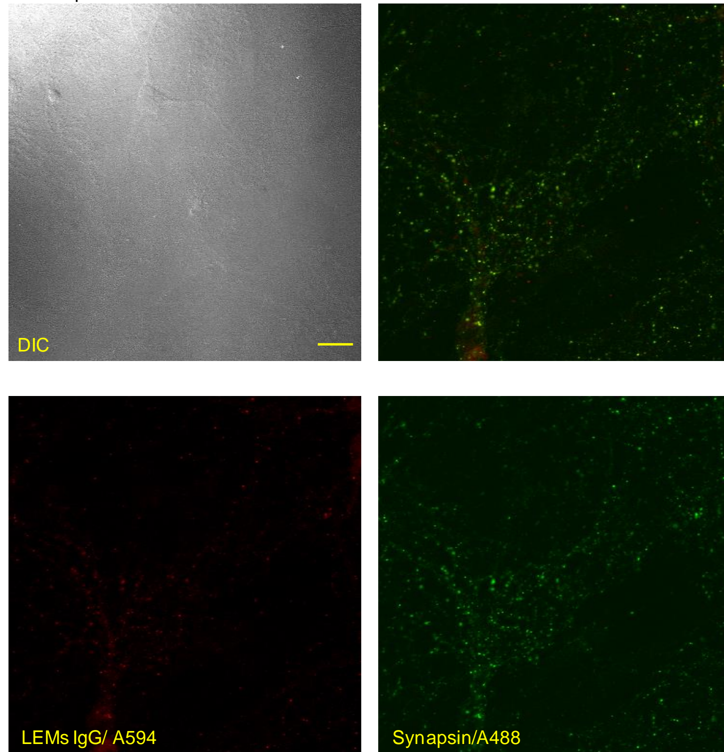
As described above, there was a concern that fixation could cause a conformational change in the epitope that could affect LEMS IgG binding. Therefore it was decided to repeat the above experiment (3.4.6) in live neuronal cultures.

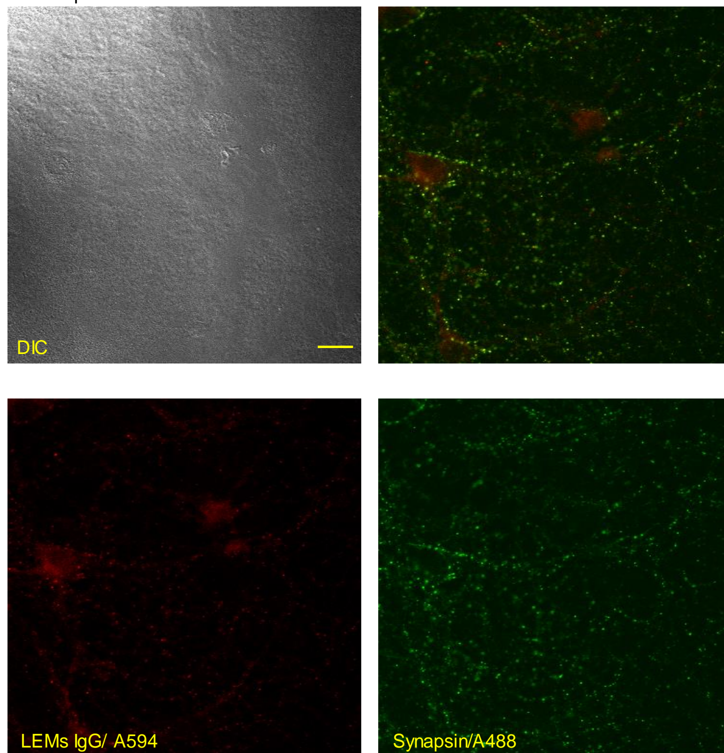
3.4.7.2 Methods

The experiments were carried out in the same way as described in 3.4.6 except that live neuronal cultures were gently washed in PBS before blocking in 5% BSA in PBS. The live cells were incubated with the primary antibody (LEMS or control IgG) The cells were then washed and fixed in 4% PFA and the rest of the steps were carried out in the same way as described above.

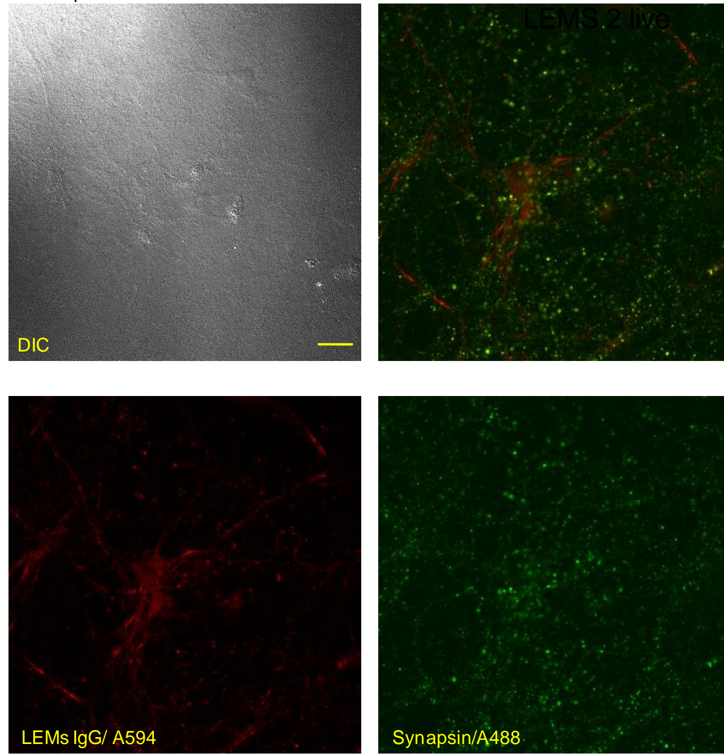
3.4.7.3 Results

A similar pattern of immunostaining as was observed for the fixed cells was seen in the live cells. LEMS samples showed a variable degree and pattern of immunostaining (figure 3.15 a-d). However it was difficult to quantify this or to determine its specificity and there was no reliable co-localisation with synapsin staining. Control IgG did not cause significant immunostaining (figure 3.15 e). The images shown are representative of experiments from three different cultures and ten images were taken from each coverslip.

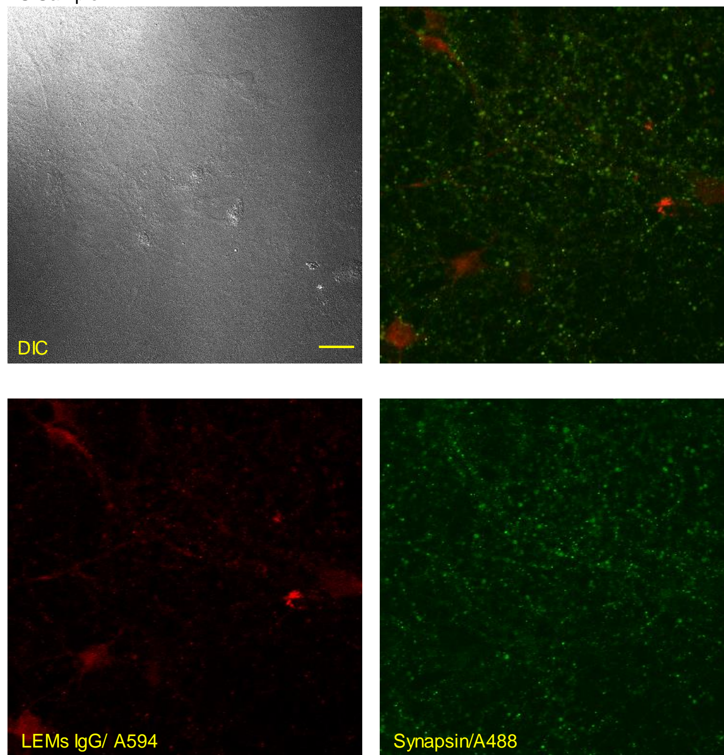

(a) LEMS Sample 1
DIC
LEMs IgG/ A594
Synapsin/A488


(b) LEMS Sample 2
DIC
LEMs IgG/ A594
Synapsin/A488

(c) LEMS Sample 3



(d) LEMS Sample 4



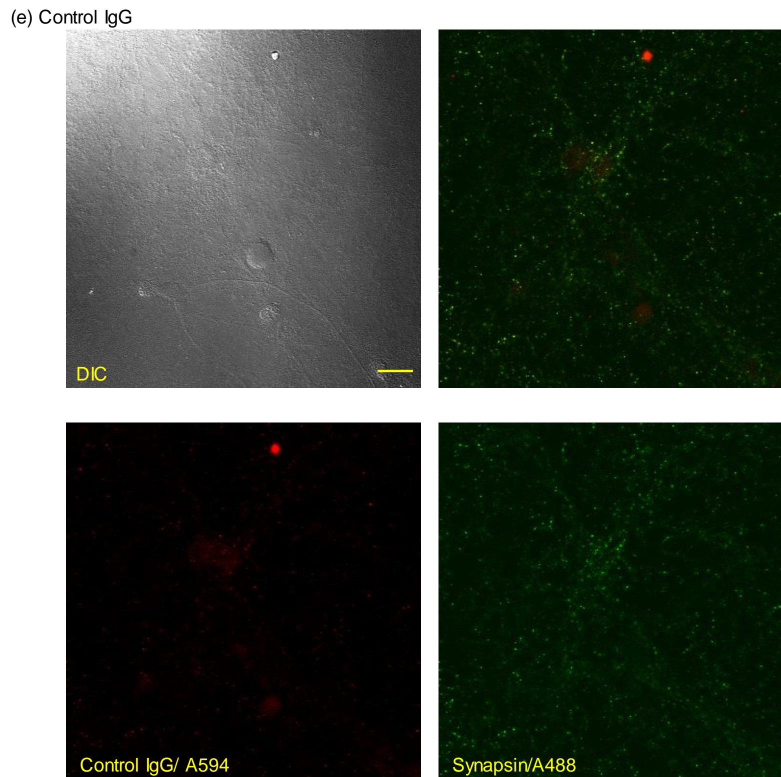


Figure 3.15 Immunostaining of live non permeablized hippocampal cultures with LEMS IgG (a-d) and co-staining with anti-synapsin (a: LEMS Sample 1; b: LEMS Sample 2; c: LEMS Sample 3; d: LEMS Sample 4) and Control IgG (e). Scale bar = 20 µM

3.4.8 Immunostaining of LEMS IgG in live Cacna1a WT and KO cultures

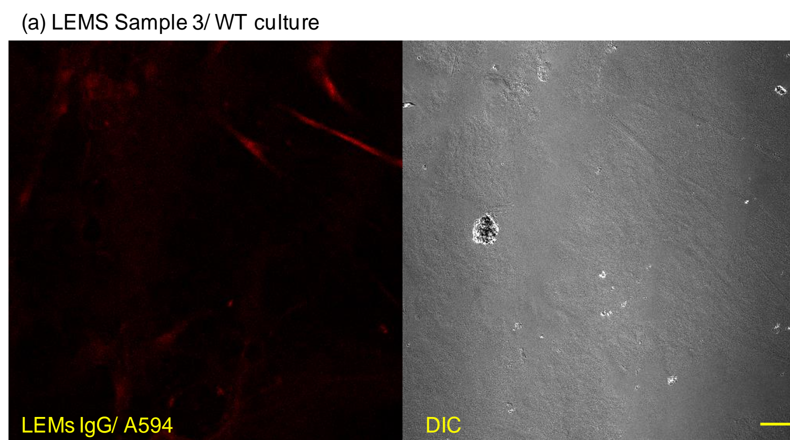
As described above, although, some immunostaining of LEMS IgG was detected in both fixed and live neuronal cultures, the pattern and the intensity of staining varied significantly amongst different LEMS samples and it was difficult to assess the specificity of this staining. Therefore it was decided to use hippocampal neurons that were lacking P/Q- type VGCCs from *Cacna1a* knockout mice. The aim was to compare immunostaining of LEMS IgG and Control IgG in WT and KO cultures to see if LEMS IgG immunostaining was specific for P/Q- type VGCCs.

3.4.8.1 Methods

The experiments were performed in the same way as outlined in the experiments described in section 3.4.7. WT and *Cacna1a* knockout cultures were used. LEMS Sample 3 was used and was compared to pooled control.

3.4.8.2 Results

LEMS immunostaining was seen in WT samples in a similar way to described above. However, LEMS immunostaining was also seen in the KO sample implying that the immunostaining that was observed was not specific for P/Q-type VGCCs in this particular sample (figure 3.16 a and b). There was minimal immunostaining seen with control IgG in the WT culture (figure 3.16c). There was large degree of non-specific staining, most likely due to technical issues seen in KO sample stained with control IgG (figure 3.16 d). The images shown are representative of experiments from two different cultures and ten images were taken from each coverslip.



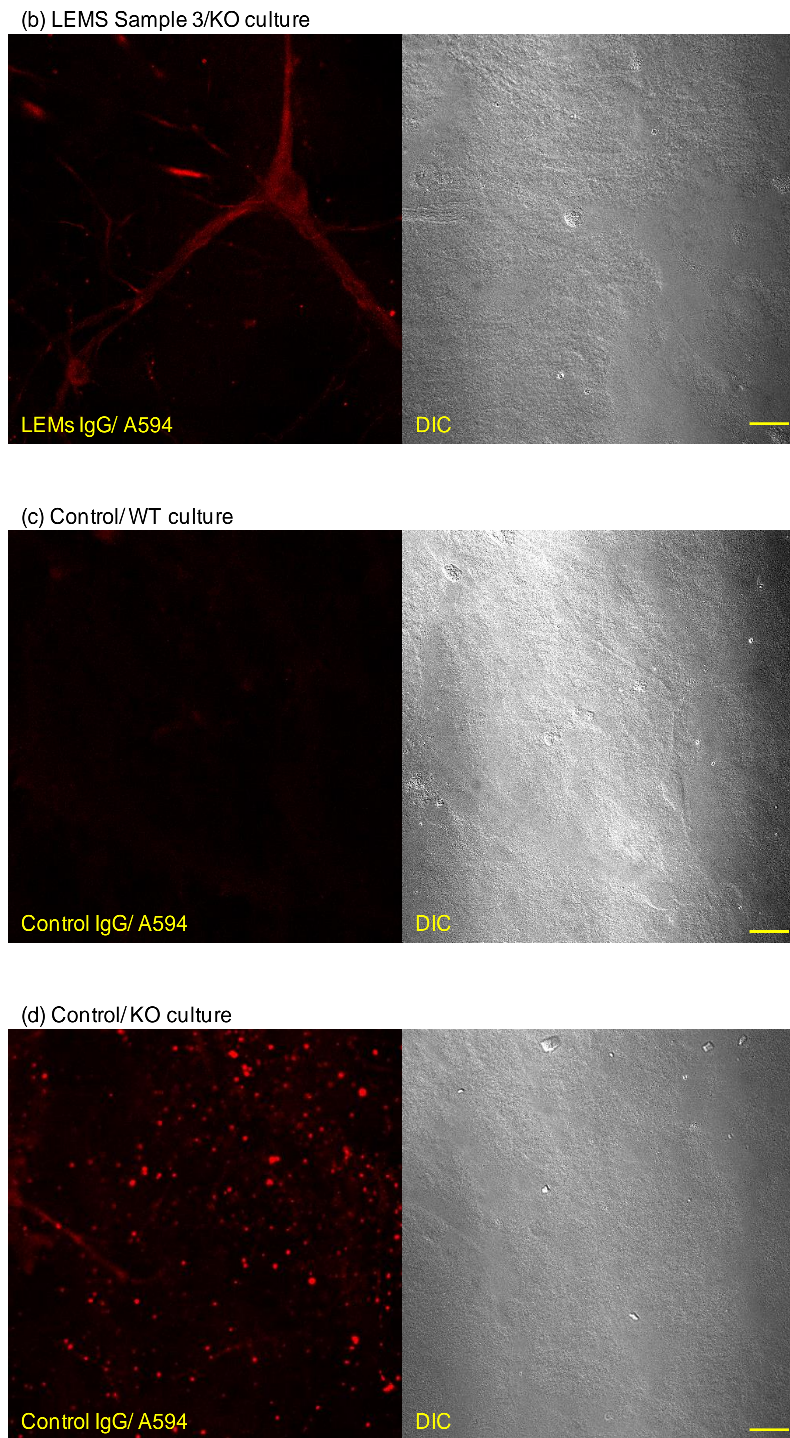


Figure 3.16 Immunostaining of LEMS IgG in WT (a) and Cacna1a KO cultures (b) compared to control IgG in WT (c) and KO (d) cultures. Scale bar = 20 µM

3.5 Discussion

These experiments showed LEMS IgG from five different patients binding to cell surface antigens in live and fixed hippocampal neurons. However, a high degree of non-specific immunostaining was seen with control IgG,although this improved slightly when experiments were repeated with an adjusted uniform concentration of IgG.

The immunostaining seen with LEMS IgG varied between samples and could not be localised to synaptic sites labelled with synapsin or the synaptic vesicle dye SRC-1. Moreover, the immunostaining was not specific for human VGCCs that were overexpressed and tagged with GFP. One LEMS sample tested in *Cacna1a* KO cultures showed a similar pattern of binding to that seen in WT cultures, meaning that the immunostaining observed was not specific for P/Q-type VGCCs in the sample tested.

There could be many reasons for the non-specificity of labelling observed in these experiments, including large volumes of IgG leading to non-specific binding, conformational changes in cell proteins that result in changes to cell surface antigens after fixation and a low density of the surface target antigen of LEMS IgG. Moreover, the K_d of the LEMS IgG may be high meaning that it was not possible to visualise binding of LEMS IgG. An alternative explanation in live cultures could be that the IgG cross links and internalizes LEMS IgG meaning that I was not able to visualise immunostaining in non-permeabilised cells.

A further alternative explanation is that the immunostaining that we were seeing was due to IgG binding to alternative antigens, unrelated to LEMS. Our collaborators in Oxford had performed similar experiments in cerebellar neuronal cultures. They had found that only a small proportion of LEMS samples reliably immunostained neuronal cultures (personal communication). Furthermore, many of the samples that did immunostain neurons were subsequently found to be positive for additional antibodies. In particular, LEMS patients who have an underlying malignancy may have antibodies to a wide variety of antigens other than VGCCs including GABA(B) receptors (Boronat et al., 2011) and antibodies against the VGKC complex (Kalra et al., 2014). It is possible that the immunostaining that was detected in these experiments could be due to IgG binding to antigenic targets other than the VGCC. Further experiments with additional secondary antibodies are required to investigate this.

Very recently, antibodies from three LEMS patients were found to bind to HEK293 cells expressing specific individual recombinant subunits of human neuronal VGCCs (Hajela et al., 2014). All three LEMS IgG samples were found to bind to HEK 293 cells expressing α_{1A}, α_{1B}, α_{1C}, or α_{1E}, subunits in combination with $\alpha_2\delta$ and β subunits – indicating that, in these experiments, LEMS IgG recognised multiple different types of HVA VGCCs - P/Q-, N-, L and R-type. LEMS IgG was also found to bind to HEK 293 cells expressing a single α_{1A}, α_{1B}, α_{1C}, or α_{1E} subunit without accessory subunits. Furthermore, LEMS IgG bound to HEK 293 cells expressing the β_3 subunit alone but not cells expressing $\alpha_2\delta$ alone (Hajela et al., 2014). These data suggest that LEMS IgG probably binds to a conserved region of the VGCC α and β subunits and that LEMS

antibodies recognise multiple types of VGCCs. These experiments were done in fixed permeabilised cells so it is difficult to be sure of the pathogenicity of the antibodies. In particular, as the β subunit is intracellular, it would seem likely that development of anti β antibodies is a secondary response rather than a primary pathogenic process. This group did not use a control IgG from a healthy patient but did use IgG from a patient with Myasthenia Gravis that did not demonstrate any immunoreactivity. This study is therefore open to alternative interpretations and further studies on nonpermeabilised cells are required to detect specific binding of pathogenic LEMS antibodies.

Having had disappointing results with immunohistochemistry experiments, the next step was to examine the functional effects of LEMS antibodies on synaptic vesicle release.

4. Effect of LEMS IgG on synaptic vesicle release

4.1 Background

As mentioned previously, the pathophysiology of LEMS is firmly established as autoimmune. This was long suspected by the clinical association of LEMS with autoimmune disease (Lennon et al., 1982; O'Neill et al., 1988) and the response of LEMS patients to immunomodulatory treatment (Lang et al., 1981; O'Neill et al., 1988). The definitive evidence came from passive transfer experiments where the functional effects of LEMS antibodies were first shown. Mice injected with IgG from LEMS patients had electrophysiological features resembling LEMS - reduced quantal release with a reduction in the end plate potential (EPP) amplitude in nerve muscle preparations, and facilitation in response to high frequency stimulation (Lang et al. 1981; Lang et al. 1983; Lang et al., 1987; Prior et al., 1985; Giovannini et al., 2002; Lambert et al., 1988). Facilitation is an increase in the amplitude of the compound muscle action potential/EPP after maximal exercise or high frequency stimulation. This phenomenon arises because action potential trains that occur during high frequency stimulation lead to a gradual temporary increase in presynaptic Ca^{2+} concentration and hence an increase in Ca^{2+} dependant exocytosis, thus suggesting that VGCCs may involved in the pathogenesis of LEMS. Acute exposure of muscle nerve preparations to LEMS IgG results in reduced quantal content *in vitro* (Flink and Atchison, 2002; Buchwald et al., 2005). These *in vivo* and *in vitro* experiments provide strong evidence that antibodies in patients with LEMS are directly pathogenic.

4.1.1 Antigenic targets in LEMS

As mentioned in the previous chapter, antibodies against P/Q-type VGCCs can be detected by immunoprecipitation of MVIIC-labelled VGCCs from solubilised cerebellar homogenates in 85-90% of patients with LEMS (Motomura et al,. 1995, Takamori et al., 2000; Nakao et al., 2002). Additional antibodies against N-type channels can be similarly detected by immunoprecipitation with conotoxin GV1A labelled VGCCS labelling in approximately one third of patients (Motomura et al., 1995). P/Q-type VGCCs and, to a lesser extent, N-type VGCCs are responsible for the majority of the Ca^{2+} influx that triggers release of ACh at the mammalian neuromuscular junction and at central synapses (Uchitel et al., 1992, Cao and Tsien, 2010). It has therefore been proposed that failure of neuromuscular transmission that occurs in LEMS results from a decrease in action potential dependent Ca^{2+} influx due to antibodies binding to presynaptic P/Q- and possibly N- type VGCCs. This hypothesis is consistent with the characteristic neurophysiological finding of facilitation both in LEMS patients and in synaptic preparations *in vitro* from animals treated with LEMS IgG.

4.1.2 Functional effects of LEMS antibodies

The finding that calcium currents are reduced following exposure to LEMS IgG in a wide variety of cell types, including SCLC cell lines (de Aizpurua et al., 1988; Johnston et al. 1994; Lang et al., 1989; Meriney et al., 1996; Viglione et al., 1995), cultured motor neurones (Garcia et al., 1996a; Garcia et al., 1996b), cortical synaptosomes (Hewett et al., 1991), bovine adrenal chromaffin cells (Kim et al., 1988), anterior pituitary cells (Login et al., 1987), insulinoma cell lines (Magnelli et al., 1996) and

neuroblastoma cells (Peers et al., 1990) is further supporting evidence for the hypothesis that LEMS IgG affects presynaptic VGCC function.

However, it remains unproven whether the effect of LEMS IgG on EPP amplitude is caused by a decrease in action potential dependent ACh exocytosis due to blocking of presynaptic voltage gated calcium channels. Studies of the effect of LEMS antibodies on synaptic transmission have focused almost exclusively on the postsynaptic effects of LEMS IgG. Postsynaptic responses are not always a direct read-out of presynaptic neurotransmitter release (Atwood and Wojtowicz, 1999) as synapses are subject to extensive plasticity meaning that alternative mechanisms could theoretically explain the impaired synaptic transmission in LEMS.

There have been some studies that have examined the effect of LEMS IgG on presynaptic neurotransmitter release. Studies on biopsied intercostal muscle from patients with LEMS showed that the rates of spontaneous and evoked release of acetylcholine are reduced compared to healthy human muscle (Molenaar et al., 1982). Decreased rates of resting and evoked release of acetylcholine were also found in diaphragmatic muscles of mice passively treated with LEMS IgG *in vitro* whereas the actual ACh, choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) content of homogenised gastrocnemius muscles was normal (Lang et al., 1984). Moreover, incubation of LEMS IgG with electric ray synaptosomes has been found to reduce depolarisation evoked ACh release (as measured using a chemiluminescence reaction) by 39-45% (Satoh et al., 1998).

Synaptic vesicle release has also been estimated in bovine adrenal chromaffin cells using increases in membrane capacitance (Cm) during perforated patch whole cell voltage clamp as a measure of membrane area, and therefore, exoctytosis. Treatment with LEMS IgG resulted in a smaller exocytotic response to a single depolarising pulse compared to control IgG. Furthermore, the magnitude of the reduction in exocytosis was proportional to the reduction in Ca^{2+} current caused by the individual LEMS samples (Engisch et al., 1999). LEMS IgG was not found to change the basal Ca^{2+} -exocytosis relationship observed during single step depolarisations (Engisch et al., 1999). A similar reduction in the exocytic response to stimulation was seen when membrane capacitance was measured in SCLC cells exposed to LEMS IgG (Viglione et al., 1995). Although these studies suggest that the abnormal synaptic transmission that occurs in LEMS is due to an effect on stimulus-evoked ACh release, a direct effect of LEMS IgG on synaptic vesicle release at the presynaptic membrane has not been shown.

4.1.3 Seronegative LEMS

Approximately 10-15% of patients with LEMS do not have antibodies against P/Q-type VGCCs demonstrable byradioimmunoprecipitation assay (Nakao et al. 2002). The clinical features of LEMS are similar in these “seronegative” patients to classic “seropositive” LEMS with two exceptions; the incidence of small cell lung cancer is lower and the electrophysiological abnormalities are less pronounced (Nako et al, 2002; Oh et al., 2007). Passive transfer of seronegative IgG transfers the electrophysiological features of LEMS suggesting that seronegative LEMS is also an antibody mediated disorder (Nako et al., 2002). It may be that so called “seronegative” LEMS is caused by

the same antibodies as seropositive LEMS but at a concentration that it not detectable by immunoprecipitation, or perhaps the antibodies are directed against an epitope that is not recognised by the current assay (Titulaer et al,. 2011). Alternatively, the antibodies in "seronegative" LEMS may be directed against a completely different protein, disruption of which causes a similar phenotype. Various alternative antigenic targets have been suggested. Synaptotagmin was found to cause an immune mediated model of LEMS in rats (Takamori et al., 1994) and antibodies to recombinant synaptotagmin were identified in a small number of LEMS patients using immunoblot assays (Takamori et al., 1995, 2007). Immunoblotting against the solubilized muscarinic ACh receptor has resulted in the detection of autoantibodies against the M1 receptor in seropositive and seronegative LEMS patients (Takamori et al., 2007). More recently autoantibodies to a neuromuscular junction protein ERC 1 (ELKS/RAB6-interacting/CAST family member 1) were found in one LEMS patient using a recombinant ELISA assay and a cell expression assay (Huijbers et al., 2013). However, it is not known whether these are disease-causing antibodies or how antibodies against these targets might result in a LEMS phenotype.

4.2 Fluorescent labelling techniques to investigate synaptic transmission

Fluorescent markers which accumulate at the synaptic terminal of neurons during endocytosis and are released during exocytosis of synaptic vesicles have proved useful tools in the investigation of synaptic transmission for many years. Horse radish peroxidase (HRP) was one of the first such markers and has been used since the 1960s.

It is endocytosed by nerve terminals and can mark recycled synaptic vesicles in fixed and frozen preparations (Graham and Karnovsky, 1966; Heuser and Reese, 1973; Ceccarelli et al., 1972). The subsequent discovery of hydrophilic fluorescent dyes such as 3,3' –diethyloxadicarbocyanine iodide ($DiOC_2$) that stain live nerve terminal preparations from mouse frog and *Drosophila* was an important development in this field (Yoshikami and Okun,. 1984). The first description of staining and subsequent destaining of live nerve terminals came from the work of Lichtman et al. (Lichtman et al., 1985; Lichtman and Wilkinson, 1987) who showed that certain fluorescent dyes (e.g. sulforhodamine,8-hydroxypyrene trisulfonic acid) were internalized by nerve terminals of snake neuromuscular preparations in an activity-dependent fashion. However, for unknown reasons, these dyes did not work in mammalian preparations (Betz et al., 1996).

The development of the fluorescent styryl FM dyes in the early 1990s (Betz et al. 1992), allowed the visualisation of synaptic vesicle recycling in living mammalian and reptile cells in real time (Cousin, 2008). FM dyes are a family of modified styryl dyes originally synthesized by Fei Mao (hence "FM"). All FM dyes have a similar structure (see figures 4.1 and 4.2) with a lipophilic tail linked to a charged head via a multiple double bond bridge (Betz et al., 1996). The lipophilic tail region allows the dye to reversibly partition into hydrophobic domains, including the lipid cell membrane (Cousin, 2008) whereas the positively charged hydrophilic head group stops the dye from passing through cell membrane. The speed at which the dye departitions from lipid membrane is determined by the tail region; the longer the hydrocarbon tail, the slower the departition rate (Cousin, 2008). The fluorescence spectrum of the dye is determined by the fluorophore nucleus formed by the aromatic rings and the double

bond bridge; the more double bonds present in the flurophore, the longer the excitation and emission wavelength (Betz et al. 1996; Gaffield and Betz, 2006).

The fluorescent properties of a styryl dye are dependent on whether the dye is in solution or partitioned into a lipid membrane. In polar solvents, such as aqueous solutions, styryl dyes do not fluoresce. However, the quantum yield increases by over two orders of magnitude when the dye is incorporated into a non-polar substance such as a lipid bilayer (Gaffield and Betz, 2006). Hence almost the entire fluorescent signal emitted from a styryl dye is from the proportion that is buried within cell membranes.

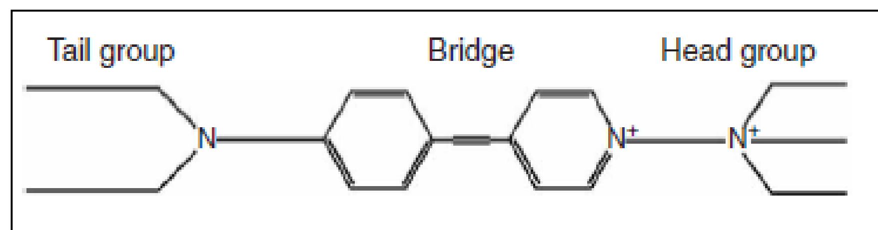


Figure 4.1 FM dye chemical structure (from Gaffield and Betz, 2007)

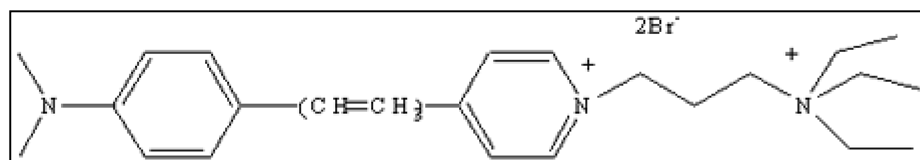


Figure 4.2 Structure of SRC-1 (from biotium.com)

FM dyes can be used to specifically label and monitor the release of synaptic vesicles (Fig 4.3). When an FM dye is incubated with neurons, the dye molecules initially

partition into the outer leaflet of the plasma membrane of the cells. If the neurones are stimulated, exocytosis of synaptic vesicles occurs, followed by endocytosis which internalizes the membrane bound dye. Excess dye that has not been internalized can be washed off, leaving fluorescently labelled synaptic vesicles inside the nerve terminals. Recycling synaptic vesicles are labelled specifically by this procedure (Cousin, 2008). A second stimulation of neurons can then unload the labelled synaptic vesicles. This is visualized as a decrease in fluorescence as the dye enters the extracellular medium; in theory the loss of fluorescence reflects of the extent of synaptic vesicle exocytosis.

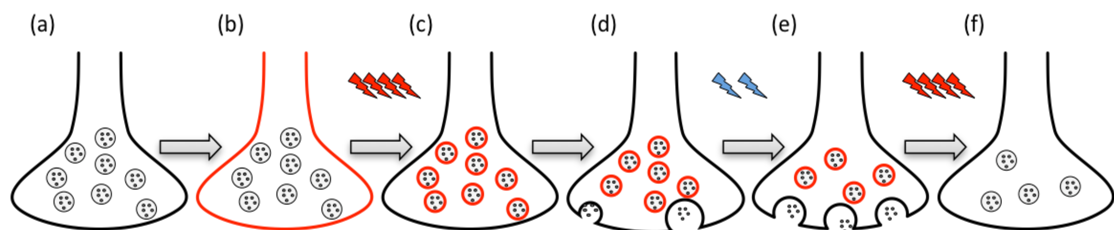


Figure 4.3 Schematic diagram for FM destaining experiments (a) Baseline (b) FM fluorescence of cell membrane (c) Specific labelling of synaptic vesicles after high frequency stimulation and wash out of excess dye (d) Spontaneous synaptic vesicle release (e) Action potential evoked synaptic vesicle release during low frequency stimulation (f) Complete destaining

FM dyes were first used in frog muscle nerve preparations (Betz et al., 1992; Richards et al., 2003). However, since then, they have been used in a wide variety of other synaptic preparations including the *Drosophila* larval neuromuscular junction (Ramaswami et al., 1994), crayfish neuromuscular junction (Wang and Zucker, 1998),

snake neuromuscular junction (Teng et al., 1999) and goldfish bipolar cells (Lagnado et al., 1996). FM dyes have also been used on studies in synaptic transmission in brain slices (Kay et al., 1999; Pyle et al., 1999; Winterer et al., 2006) and with relevance to this current project, in hippocampal neuronal cultures (Darcy et al., 2006; Harata et al., 2001; Hopf et al., 2002; Klingauf et al., 1998; Ermolyuk et al., 2013).

A number of different FM dyes exist, and are suited to different applications (Gaffield and Betz, 2006). Although FM-143 is the most commonly used dye (Cochilla et al., 1999; Harata et al 2001), FM dyes that have fast departition rates due to a shorter lipophilic tail, such as SynaptoRed C1 (SRC1), are particularly useful in experiments when fast recycling events are examined. SRC1 has a red shifted emission spectrum compared to FM-143 (the peak excitation and emission spectra are 734 and 558 nm respectively) and was shown in our laboratory to specifically label synaptic vesicles in hippocampal neuronal culture with a high signal-to-noise ratio (F. Alder 2012). As SRC-1 had been validated in our laboratory, has a known K_d and has a fast departition rate, this particular styryl dye was the most appropriate choice in our experiments.

4.3 Specific aims of this chapter

The aim of these experiments was to quantify the effects of IgG from LEMS patients on action potential evoked synaptic vesicle exocytosis in hippocampal neuronal cultures using the membrane specific fluorescent marker, SRC1. A protocol was used that would allow estimation of several key properties of neurotransmission at the level of

individual boutons in the presence of LEMS IgG or control IgG. These properties were: the size of the recycling pool of vesicles (RP) and the rates of spontaneous (k_{SP}), evoked, (k_{EV}) and action potential dependent (k_{AP}) synaptic vesicle release. A futher aim was to assess the functional effects of LEMS IgG from a LEMS patient without detectable anti-VGCC antibodies ("seronegative" LEMS).

4.4 Methods

4.4.1 Choice of hippocampal neuronal cultures

Hippocampal neuronal cultures from rodents were chosen as a model system for these experiments. These synapses have been extensively used to study the coupling of Ca^{2+} to synaptic transmission (Cao and Tsien, 2010) and show similar roles for N- and P/Q-type VGCCs as have been described at the neuromuscular junction. There is a high degree of conservation of the extracellular part of the P/Q-type channel between species, where epitopes for pathogenic antibodies are presumably located (see figure 3.1). Additionally there are many examples of functional effects of human LEMS IgG in passive transfer to rodents and in rodent *in vitro* experiments (Pinto et al., 1998; Garcia et al., 1996; Lang et al., 1981). Therefore it is highly likely that antibodies from LEMS patients would cross-react with P/Q-type VGCCs native to rat hippocampal cultures. Cells 14-19 days *in vitro* were used to ensure that the effect of LEMS IgG was being tested in relatively mature synapses (Grabrucker et al., 2009).

4.4.2 IgG samples used

IgG from four individual patients with confirmed LEMS was studied and was compared to IgG from healthy human controls. All patients had antibodies that immunoprecipitated ^{125}I-ω-MVIIC labeled VGCCs (See table 4.1). Samples were considered to be positive if the anti-VGCC antibody titre was greater than 50 pM. The titres ranged from 261-10,755pM. Experiments were performed and analysed blind to the disease status of each IgG sample. Two patients also had N-type antibodies.

Sample number	**Age at disease onset**	**Gender**	**Tumor status**	**Electrophysiology**		**P/Q ab titer (pM)**	**N- ab titer (pM)**
				CMAP amplitude (mV)	% Increment		
1	48	M	Nil	6.1 mV	146	261	Neg
2	68	M	Anaplastic large cell lung carcinoma	2.2 mV	145	575	60
3	44	F	SCLC	1.2 mV	1,233	10,755	127
4	58	M	Nil	4.2 mV	281	362	Neg

Table 4.1 LEMS IgG used in FM expermients

4.4.3 Concentration of IgG Samples

The neuronal cultures were incubated in Neurobasal A medium supplemented with IgG (control or LEMS) at a concentration of 1mg/ml overnight before each experiment. As IgG concentration of individual samples varied from 5-22 mg/ml, the dilutional volume for each sample was chosen individually. This concentration of 1mg/ml was chosen based on previous work in which LEMs IgG was incubated with a wide variety of cell types including cerebellar neuronal cultures, HEK cells, bovine adrenal chromaffin

cells, anterior pituitary cells small cell lung cancer rat cortical synaptosmes, atrial myocytes and neuroblastoma cell lines (Pinto et al., 1998; Pinto et al., 2002 ; Hewett and Atchison, 1991; Hewett and Atchison, 1992; Houzen et al., 1998; Kim et al., 1988; Roberts et al., 1985; Login et al., 1987; Grassi et al., 1994; Viglione et al., 1995; Meriney et al., 1996; Johnston et al., 1994). In these experiments a range of concentrations of LEMS IgG from 0.1 to 4 mg/ml was used. In the cell type most similar to ours, cerebellar neuronal cultures, 2 mg/ml LEMS IgG was used (Pinto et al., 1998), but concentrations as low as 0.4 mg/ml were shown to be effective in anterior pituitary cells (Login et al., 1987). It was decided to use a concentration of 1mg/ml IgG in Neurobasal A medium, since this concentration was adequate for examining functional effects in many previous studies and because only small samples of LEMS IgG were available, precluding the use of higher concentrations.

4.4.4 Duration of exposure to LEMS IgG

The duration of incubation of neurons with IgG was also considered. The dissociation constant (k_d) for antibodies depends on a number of variables, namely the concentration of the reactants, the temperature and pH of the solution. The affinity constants for antibodies usually lie in the range 10^{-6} -10^{-10} M. It was decided that with the concentrations present, it was likely that several hours would be needed for steady state equilibration. Furthermore, as LEMS antibodies may cause cross-linking and internalization of VGCCs, the effects may take some time to develop (Engel et al., 1988). In general, when functional effects of antibodies are examined in cultures, the IgG is allowed to incubate with the cells for 12-24 hours. In the system that was most similar to ours, Pinto et al., incubated cerebellar neurons with LEMS IgG for 15-22

hours (Pinto et al., 1998). However, there are examples of experimental protocols where the acute application of LEMS IgG was shown to have an effect. Buchwald et al., applied LEMS IgG directly to motor nerve terminals by macropatch clamp and an acute effect of LEMS IgG on evoked quantal release was demonstrated (Buchwald et al., 2005). Treatment of *in vitro* hemidiaphragm preparations with LEMS IgG for just 2 hours can reduce end plate potentials and cause facilitation at high frequency stimulation (Flink and Atchison, 2002). One hour incubation with LEMs IgG is sufficient to reduce calcium uptake in rat cortical synaptosomes (Hewett and Atchison, 1991). However, the vast majority of experiments in which IgG is incubated with a cultured cell system allow for chronic exposure of cell. As arguably this is also the most clinically relevant approach, our cultured neruons were incubated with IgG overnight. The range of exposure time of neurons to IgG in our experiments was 16-22 hours.

4.4.5 Experimental protocol

The full experimental protocol is described in Chapter 2 but briefly; experiments were performed at room temperature (23-26 °C) 15-19 days after plating. The imaging solution contained (in mM) 125 NaCl. 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 30 glucose and 25 HEPES (pH 7.4). The solution was supplemented with (in μM) 10 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) and 50 DL-2-amino-5-phosphonopentanoic acid (DL-AP5) to prevent recurrent activity from synaptically connected neurons.

Fluorescence imaging experiments were performed on an inverted epifluorescence microscope (Axiovert 135 Zeiss) using a 1.3 NA 63X oil immersion objective and a

QuantEM 512 SC EM-CCD camera. Recycling synaptic vesicles were fluorescently labelled using several rounds of saturating field stimulation (4 trains of 120 action potentials at 30 Hz) delivered via platinum bath electrodes in the presence of the amphiphilic dye SRC1 (SynaptoRed C1) at a bath concentration of 200 µM). These settings were previously calibrated to achieve complete labelling of recycling vesicles (Ermolyuk et al., 2012). Excess dye was subsequently washed out for 15 minutes leaving synaptic vesicles specifically labelled. The SRC1 fluorescence decay was monitored. Images were taken every 40 seconds, first in the absence of stimulation and subsequently during low frequency stimulation (0.5 Hz) (450 APs in total). SRC1 was excited with the 488 nm LED. The light passed through a 500 nm dichroic filter and emission fluorescence was collected with a long pass (LP) 510 nm filter. At the end of the experiment, the neurons were subjected to high frequency stimulation (four rounds of 300 AP at 10 Hz) to evoke exocytosis of all recycling vesicles at which point, five further images were taken.

The methods used in the analysis of these experiments are described in detail in Chapter 2. Briefly, images were analysed using Image J (NIH). Following X-Y alignment of consecutive images, active boutons were identified by subtracting the five-frame background average acquired after complete destaining from a five-frame average that was acquired immediately after SRC1 washout. Regions of interest (ROIs) corresponding to presynaptic boutons were outlined by hand in each frame to compensate for small degrees of drift. For each ROI, the mean pixel intensity integrated over the ROI was normalized by the initial maximal level, after subtraction of the image after full destaining.

The TRP (total size of the recycling pool) was calculated by measuring the fluorescence signal at the start of imaging (t=0), having subtracted the background fluorescence. The spontaneous (k_{SP}) and evoked (k_{EV}) destaining rates were calculated by fitting monoexponential functions to the fluorescence time course in each selected ROI, in unstimulated or stimulated conditions respectively. The specific AP-evoked SRC1 destaining rate was calculated as $k_{AP} = k_{EV} – k_{SP}$. Boutons with high spontaneous destaining rates ($k_{SP} > 10^{-3}$ s^{-1}) and boutons with low signal-to-noise ratios ($\chi^2 > 0.1$) were excluded from the analysis as it was not possible to fit the monoexponential functions to noisy signals accurately. Noisy signals were sometimes caused by movement of fluorescence during the experiment which could be due to mobile vesicles leaving or entering a synapse, movement of the synapse itself or particles of dirt in the perfusion system. Cells with high spontaneous destaining rates were generally not healthy and so were not included in the analysis.

4.5 Results: Effect of LEMS IgG on synaptic transmission

4.5.1 Estimation of k_{EV}, k_{SP}, k_{AP} and TRP

The average destaining trace for a single bouton normalised to the fluorescence at t= 0 is shown in figure 4.4. A slow fluorescence decrease in the absence of stimulation occurred (blue trace) corresponds to the spontaneous release of synaptic vesicles (k_{SP}). Applying low frequency stimulation (0.5 Hz) to the culture led to a faster decrease in fluorescence (red trace), giving an estimate of k_{EV}. The specific rate of of AP evoked

fluorescence, k_{AP}, which is proportional to the rate of AP-dependent synaptic vesicle exocytosis was estimated (Ermolyuk et al., 2012). The rate of action potential evoked synaptic vesicle exocytosis (k_{AP}) was calculated as $k_{AP} = k_{EV} - k_{SP}$. The background fluorescence was estimated after complete destaining (three rounds 300 APs at 10 Hz). This value was subtracted from the initial fluorescence to get an estimate for the Total Recycling Pool size (TRP) in each bouton (before normalisation).

The data were shown in two formats; bar chart and cumulative distributions. The means were best displayed as a bar chart. Cumulative figures were used as each data point represented one experiment, allowing every experiment to be shown. To acquire the cumulative graphs, the data were ranked in ascending order and the normalied cumulative distribution (Y axis) was then plotted against each value (X axis).

The time course and a typical destaining trace of a single bouton is illustrated in Figure 4.4.

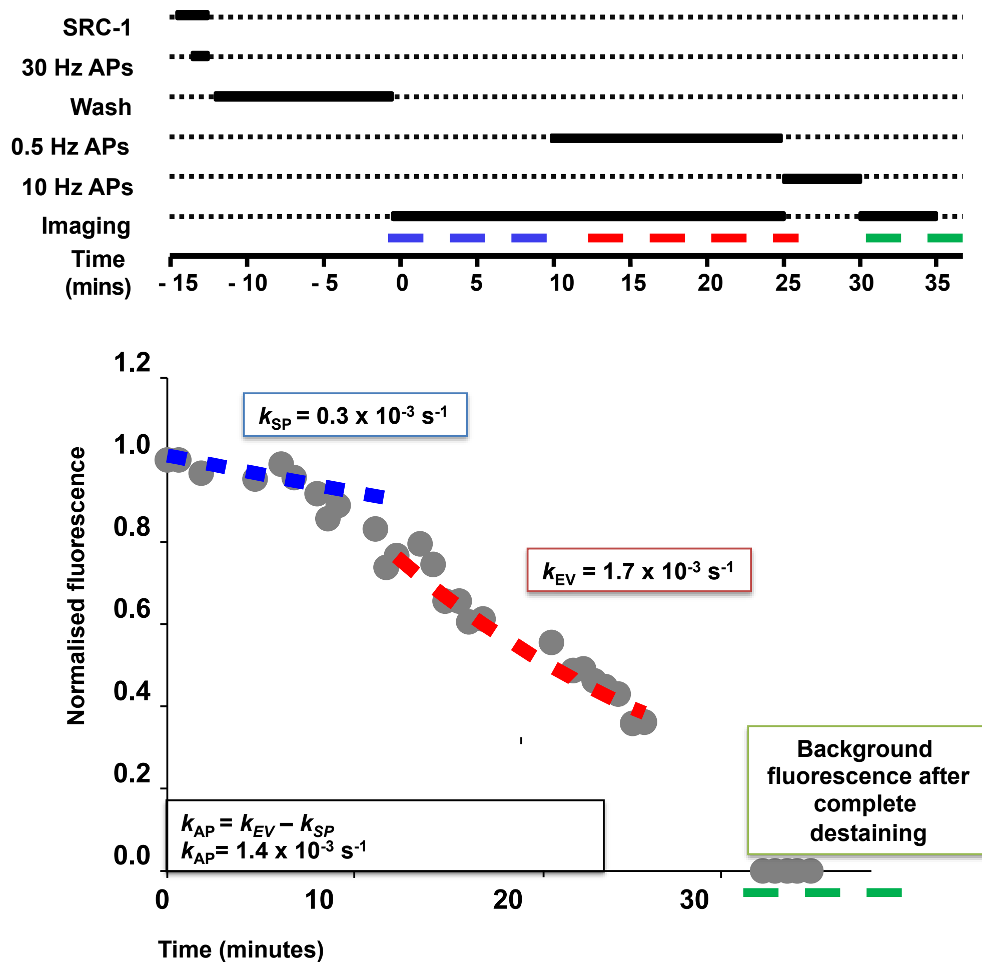


Figure 4.4 Time course and example trace from a typical experiment. The fluorescence trace was fitted with mono-exponential functions to determine k_{SP} *(blue trace) and* k_{EV} *(red trace). The background fluorescence was measured after high frequency stimulation at the end of the experiment (green). An average of five images taken at the end of the experiment was subtracted from an average of five images at the beginning of the experiment to estimate RP.*

Fluorescence images for a typical control and a typical LEMS treated experiment are shown in Figure 4.5. The destaining profiles for two representative boutons for each treatment (LEMS or control) are also shown. The profiles were fitted with

monoexponential functions to denote spontaneous (blue line) and evoked (red line) synaptic vesicle release.

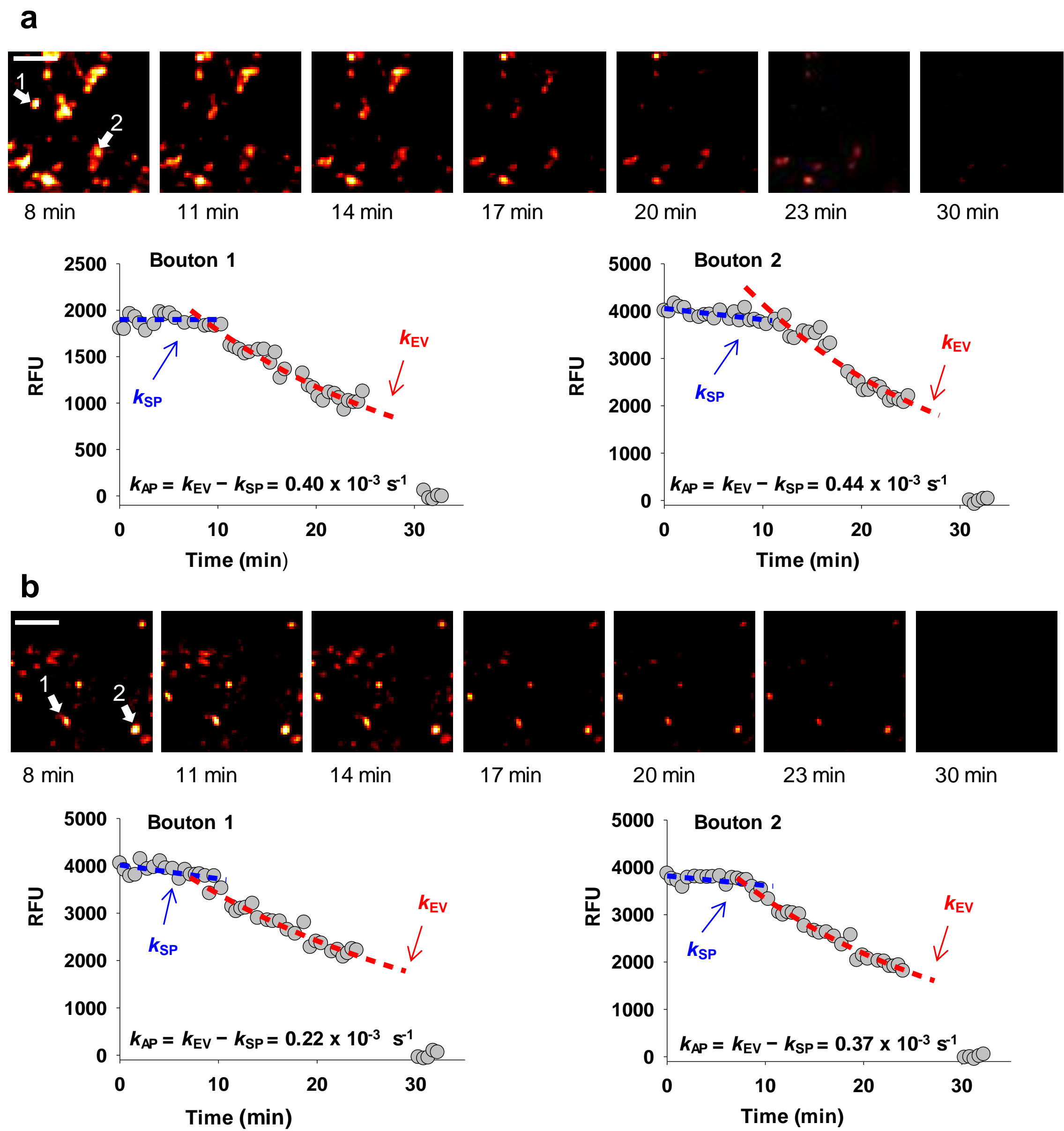


Figure 4.5 Fluorescence images and destaining profiles for a typical (a) control and (b) LEMS treated hippocampal culture. Scale bar = 5µm

4.5.2 Estimation of TRP size in LEMS treated neurons compared to controls

The Total Recyling Pool (TRP) was measured by taking the fluorescence signal at t=0 (the fluorescence signal at the start of imaging having taken the background fluorescence into account). There was no significant difference in the size of the TRP in neurons treated overnight with LEMS IgG compared to neurons treated with control IgG (p=0.49). (Table 4.2, Figure 4.6)

	Control	**LEMS ***	**LEMS 1**	**LEMS 2**	**LEMS 3**	**LEMS 4**
TRP (mean)	2.46	2.16	1.8	2.67	1.49	2.56
Standard deviation	1.63	1.26	0.34	1.68	1.34	0.94
SEM	0.36	0.25	0.2	0.75	0.47	0.31
Number of experiments	21	25	3	5	8	9
Number of boutons	927	791	47	222	205	317
Number of culture sets	9	9	1	2	3	3

*Table 4.2. Mean TRP size in LEMS treated neurons compared to control. *Pooled data for all LEMS samples*

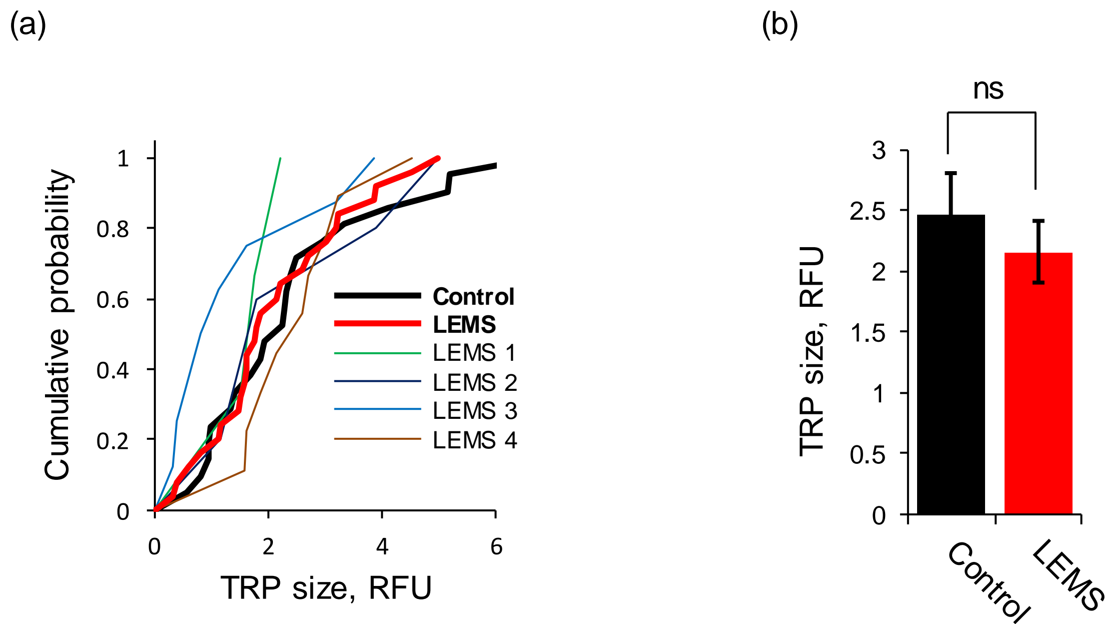


Figure 4.6 Effect of LEMS and Control IgG on relative TRP size. (a) Cumulative distributions of mean TRP size values obtained in individual experiments (average of 10-50 boutons in each experiment). Data corresponding to samples obtained from each individual LEMS patient are shown as thin coloured lines. LEMS samples 1-4 were used in 3, 9, 8 and 6 experiments respectively (b) Mean (± SEM) values for pooled data (LEMS IgG (red) n =26 experiments, Control IgG (black) n= 21 experiments) ns, non-significant)

4.5.3 Estimation of k_{SP} in LEMS treated neurons compared to controls

The rate of spontaneous neurotransmitter release was significantly reduced by LEMS IgG ($p < 0.05$). The overall effect was a reduction in the rate of spontaneous neurotransmitter release of 23% (range 18%-55% reduction for individual LEMS samples). The mean k_{SP} values for neurons treated with each LEMS sample and pooled control are shown in table 4.3 and figure 4.7.

	Control	LEMS*	LEMS 1	LEMS 2	LEMS 3	LEMS 4
k_{SP} x 10^3, s^{-1}(mean)	0.32	0.24	0.14	0.24	0.26	0.27
Standard deviation	0.07	0.12	0.14	0.11	0.02	0.10
SEM	0.02	0.02	0.08	0.05	0.01	0.03
Number of experiments	21	25	3	5	8	9
Number of boutons	927	791	47	222	205	317
Number of culture sets	9	9	1	2	3	3

*Table 4.3 Mean k_{SP} in LEMS treated neurons compared to controls. *Pooled data for all LEMS samples*

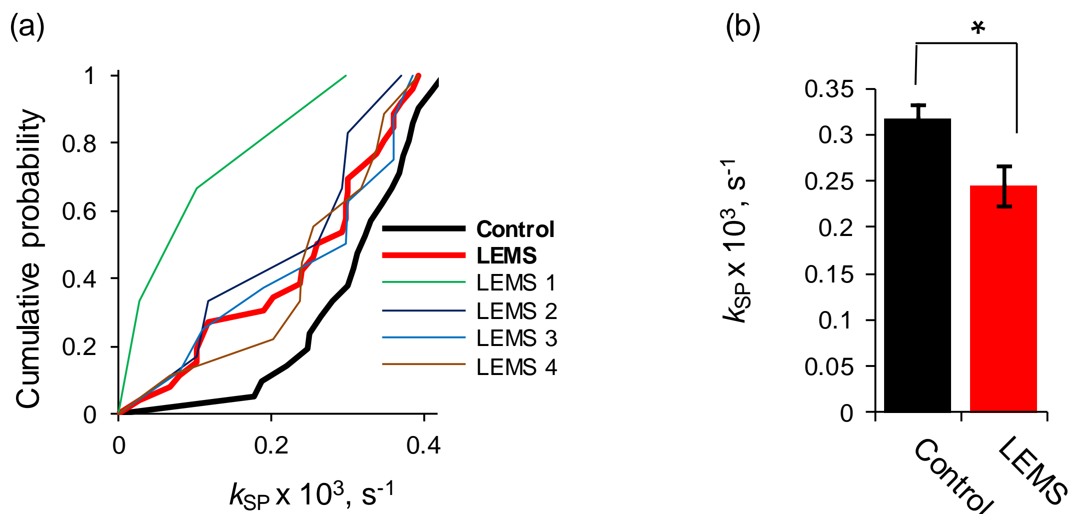


*Figure 4.7 Effect of LEMs and Control IgG on k_{SP}. (a) Cumulative distributions of mean k_{SP} values obtained in individual experiments (10-50 boutons in each experiment). Data corresponding to samples obtained from each individual LEMS patient are shown as thin coloured lines. LEMS samples 1-4 were used in 3, 9, 8 and 6 experiments respectively. Thick red line =pooled LEMS IgG data; thick black line = control IgG data. (b) Mean (± SEM) values for pooled data (LEMS IgG (red) n =26 experiments, control IgG (black) n= 21 experiments) *, $p < 0.05$*

4.5.4 Estimation of k_{EV} in LEMS treated neurons compared to controls

k_{EV} was calculated as the rate of fluorescence decay during low frequency stimulation (0.5 Hz) and includes both spontaneous and action potential dependant synaptic vesicle release. Overnight incubation with LEMS IgG caused a significant reduction in the rate of evoked synaptic vesicle release by an average of 24% ($p<0.01$). The range of reduction in k_{EV} was 20-47% (Table 4.4, Figure 4.8).

	Control	LEMS*	LEMS 1	LEMS 2	LEMS 3	LEMS 4
k_{EV} x 10^3, s^{-1} (mean)	0.96	0.73	0.5	0.75	0.77	0.75
Standard deviation	0.22	0.16	0.18	0.13	0.21	0.1
SEM	0.05	0.03	0.1	0.06	0.07	0.03
Number of experiments	21	25	3	5	8	9
Number of boutons	927	791	47	222	205	317
Number of culture sets	9	9	1	2	3	3

*Table 4.4 Mean k_{EV} in LEMS treated neurons compared to controls. *Pooled data for all LEMS samples*

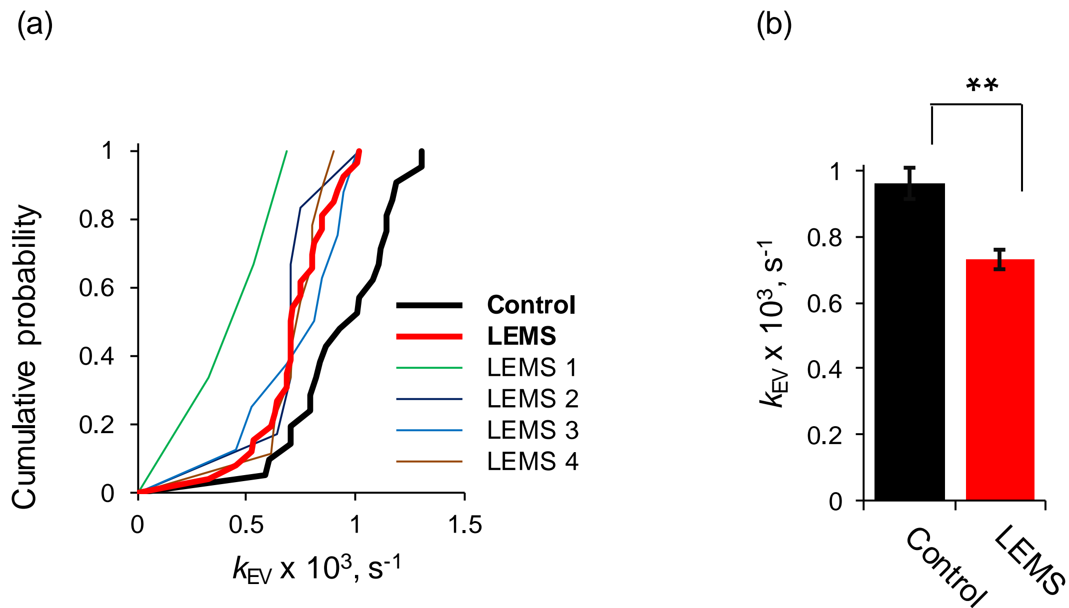


*Figure 4.8 Effect of LEMs and control IgG on k_{EV}. (a) Cumulative distributions of mean k_{EV} values obtained in individual experiments (average of 10-50 boutons in each experiment). Data corresponding to samples obtained from each individual LEMS patients are shown as thin coloured lines. LEMS samples 1-4 were used in 3, 9, 8 and 6 experiments respectively. (b) Mean (± SEM) values for pooled data (LEMS IgG (red) n =26 experiments, control IgG (black) n= 21 experiments) ** $p<0.01$*

4.5.5 Estimation of k_{AP} in LEMS treated neurons compared to controls

We next analysed the rate of action potential evoked synaptic vesicle release in neurons treated with LEMS IgG compared to those treated with control IgG. Overnight incubation with LEMS IgG (1mg/ml) reduced the rate of AP-dependent destaining by an average of 25% compared to neurons treated with control IgG ($p<0.05$). LEMS samples from all four patients resulted in a lower rate of exocytosis that the pooled

control sample with the size of the reduction ranging from 20% to 43% (Table 4.5; Figure 4.9).

	Control	LEMS *	LEMS 1	LEMS 2	LEMS 3	LEMS 4
k_{AP} x 10^3, s^{-1}(mean)	0.65	0.49	0.37	0.51	0.51	0.48
Standard deviation	0.21	0.20	0.30	0.2	0.23	0.17
SEM	0.05	0.04	0.17	0.09	0.08	0.06
Number of experiments	21	25	3	5	8	9
Number of boutons	927	791	47	222	205	317
Number of culture sets	9	9	1	2	3	3

*Table 4.5 Mean k_{AP} in LEMS treated neurons compared to control. *Pooled data for all LEMS samples*

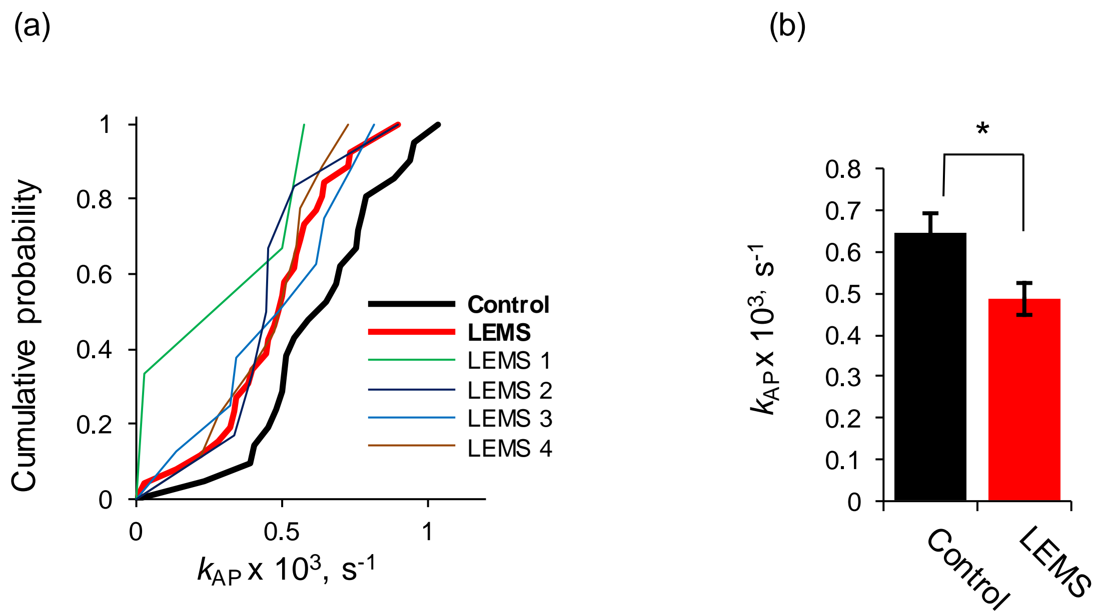


Figure 4.9 Effect of LEMS and Control IgG on k_{AP}. (a) Cumulative distributions of mean k_{AP} values obtained in individual experiments (average of 10-50 boutons in each experiment). Data corresponding to samples obtained from each individual LEMS patient are shown as thin coloured lines. LEMS samples 1-4 were used in 3, 9, 8 and 6

*experiments respectively (b) Mean (± SEM values for pooled data (LEMS IgG (red) n =26 experiments, control IgG (black) n= 21 experiments) * p<0.05*

4.6 Effect of seronegative LEMS IgG on synaptic transmission

Having confirmed that LEMS samples that contain antibodies directed against P/Q type channels reduce action potential evoked synaptic vesicle release; the next step was to test the effect of IgG from a patient with clinically confirmed LEMS but no demonstrable antibodies on radioimmunoprecipitation assay, either to either N or P/Q-type VGCCs.

The experiments were conducted in the same way as described above.

4.6.1 Effect of seronegative LEMS IgG on TRP size

Incubation of neuronal cultures with seronegative IgG overnight had no significant effect on the mean TRP size compared to incubation with control IgG (p=0.5) (Table 4.6, Figure 4.10).

	Control	Seronegative LEMS
Normalised TRP	3.98	2.2
Standard deviation	2.45	1.38
SEM	1.4	0.69
Number of experiments	3	4
Number of boutons	158	271
Number of cultures	1	1

Table 4.6 Mean TRP size in seronegative LEMS treated neurons compared to controls

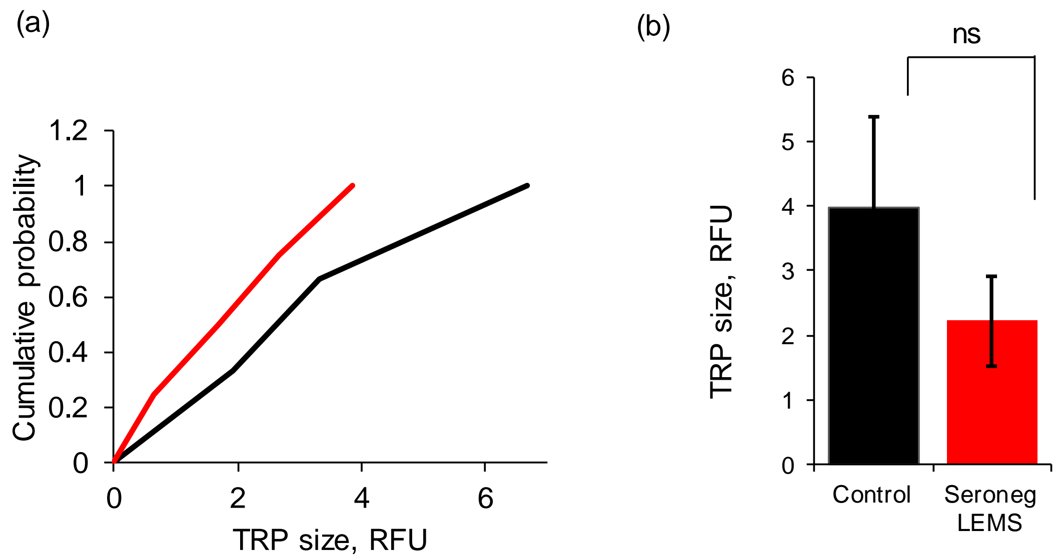


Figure 4.10 Effect of Seronegative LEMS and Control IgG on relative TRP size. (a) Cumulative distribution of TRP size in seronegative LEMS (red) compared to control IgG (black) (b) Mean (± SEM values) for pooled data (LEMS IgG n=4 experiments, Control IgG n= 3 experiments) ns, non-significant.

4.6.2 Effect of seronegative LEMS IgG on k_{SP}

Treatment with seronegative LEMS IgG did not cause a significant alteration in the rate of spontaneous synaptic vesicle release in neurons in culture (p=0.22) (Table 4.7, Figure 4.11)

	Control	Seronegative LEMS
k_{SP} x 10^3, s^{-1}	0.3	0.18
Standard deviation	0.11	0.1
SEM	0.06	0.05
Number of experiments	3	4
Number of boutons	158	271
Number of cultures	1	1

Table 4.7 Mean k_{SP} size in seronegative LEMS treated neurons compared to controls

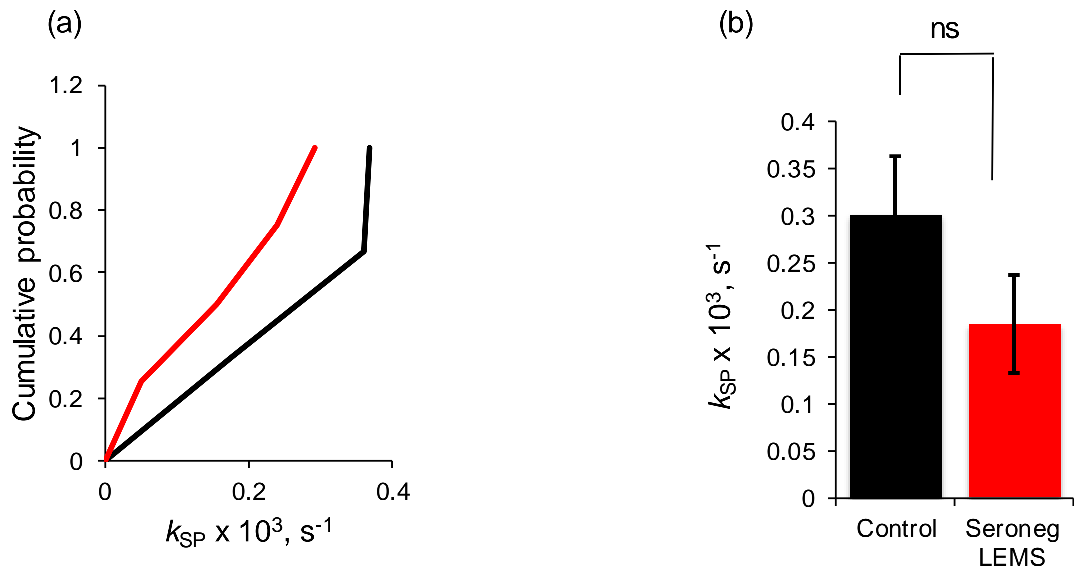


Figure 4.11 Effect of seronegative LEMS and control IgG on k_{SP}. (a) Cumulative distributions of mean k_{SP} values obtained in seronegative LEMS experiments (red) and control IgG experiments (black) (b) Mean (± SEM) values for pooled data (Seronegative LEMS IgG n =4 experiments, Control IgG n= 3 experiments) ns, non-significant

4.6.3 Effect of seronegative LEMS IgG on k_{EV}

The k_{EV} was not significantly different in neurons treated with seronegative LEMS IgG compared to those treated with control IgG (p=0.5) (Table 4.8, Figure 4.12).

	Control	**Seronegative LEMS**
k_{EV} x10^3, s^{-1}	0.82	0.7
Standard deviation	0.2	0.24
SEM	0.13	0.06
Number of experiments	3	4
Number of boutons	158	271
Number of cultures	1	1

Table 4.8 Mean k_{EV} in seronegative LEMS treated neurons compared to controls

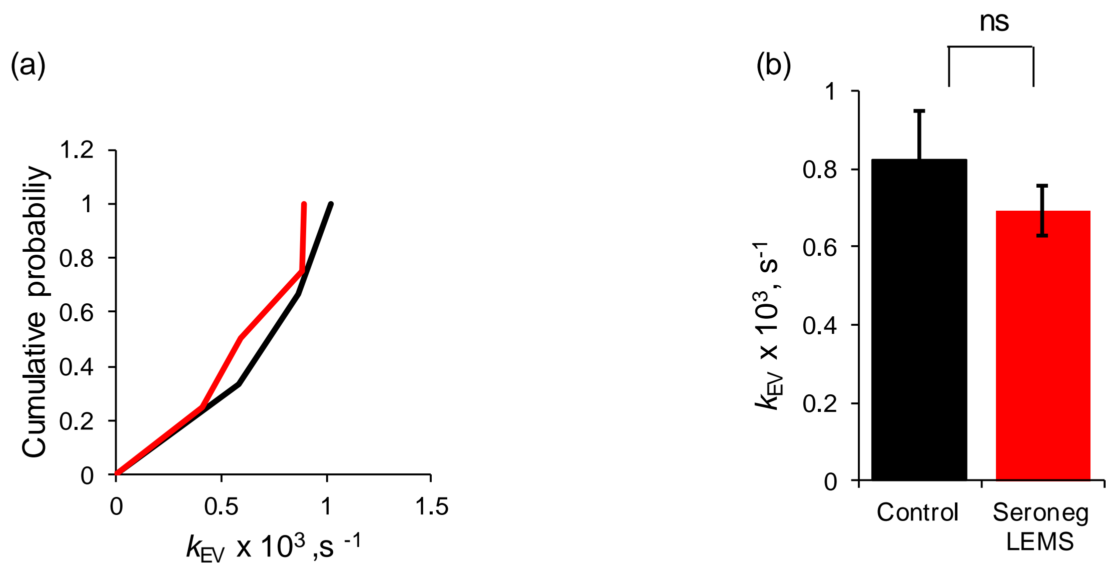


Fig. 4.12 Effect of seronegative LEMS and control IgG on k_{EV}. *(a) Cumulative distributions of mean* k_{EV} *values obtained in seronegative LEMs experiments (red) and control IgG experiments (black) (b) Mean (± SEM) values for pooled data (Seronegative LEMS IgG n =4 experiments, control IgG n= 3 experiments) ns, non-significant)*

4.6.4 Effect of seronegative LEMS IgG on k_{AP}

The results for action potential evoked synaptic vesicle release are displayed in Table 4.9 and Figure 4.14. There was no significant difference in the rate of action potential evoked synaptic vesicle release (p=0.95) (Table 4.9, Figure 4.13).

Treatment	Control	Seronegative LEMS
k_{AP} x 10^3, s^{-1} (mean)	0.52	0.51
Standard Deviation	0.12	0.28
SEM	0.07	0.14
Number of experiments	3	4
Number of boutons	158	271
Number of cultures	1	1

Table 4.9 Mean k_{AP} in seronegative LEMS compared to control IgG

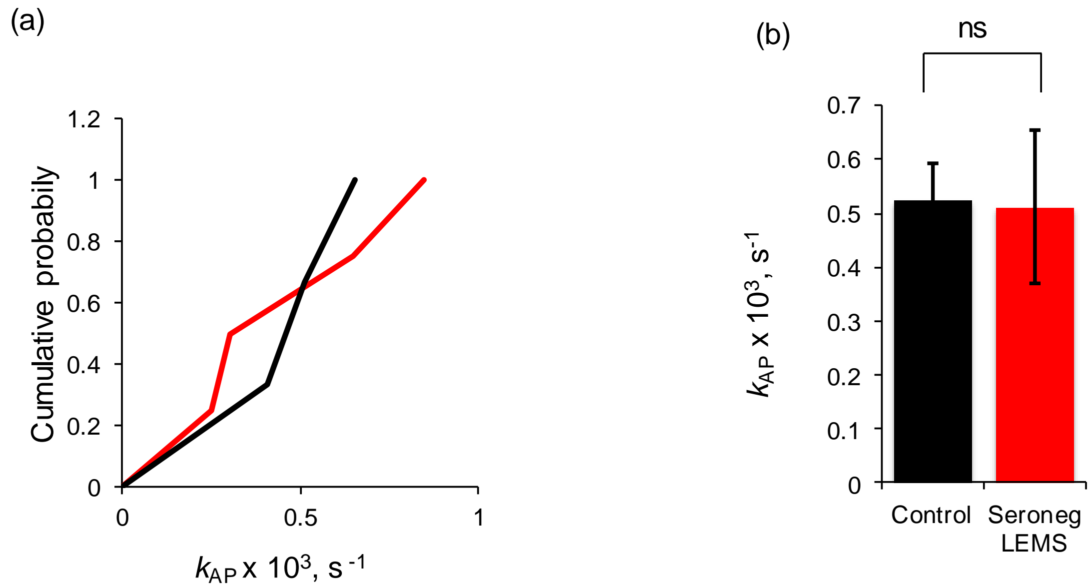


Fig 4.13 Effect of seronegative LEMS and control IgG on k_{AP}. (a) Cumulative distributions of mean k_{AP} values obtained in seronegative LEMS experiments (red) and control IgG experiments (black) (b) *Mean (± SEM) values for pooled data (Seronegative LEMS IgG n =4 experiments, control IgG n= 3 experiments) ns, non-significant*

4.7 Discussion

It has been accepted for many years that LEMS is an autoimmune disease and that LEMS antibodies affect synaptic transmission. Recordings from intercostal muscle biopsies of patients with LEMS have shown that the amplitudes of the EPP evoked by nerve stimulation are significantly reduced compared to controls (Lambert and Elmqvist, 1971). Similar findings have been described in passive transfer models of LEMS, where mice treated with LEMs IgG show a significant reduction in quantal content/ EPP amplitude (Lang et al., 1987; Giovannini et al., 2002; Flink et al., 2002). Curves of log EPP quantal content vs calcium concentration, are shifted to the right by LEMS IgG (Lang et al., 1987). As LEMS antibodies have been shown to reduce calcium current in a wide variety of cell types and IgG from LEMS patients can immunoprecipitate VGCCs *in vitro*, it has been proposed that LEMS antibodies affect synaptic transmission by binding to VGCCs and reducing the nerve action potential evoked Ca^{2+} influx that is necessary for the release of vesicles containing acetylcholine. This hypothesis is supported by the phenomenon of facilitation of the postsynaptic response in response to high frequency stimulation that occurs in LEMS.

Previously, evidence for the effect of LEMS antibodies on synaptic vesicle release has been indirect. The presynaptic imaging data described here directly show that LEMS IgG decreases AP-dependent synaptic vesicle release in rat hippocampal neuronal cultures. Although this finding was not unexpected, this work provides the missing link and conclusively shows that the rate of synaptic vesicle release is reduced at LEMS treated synapses.

VGCCs, mainly of the P/Q- and N- type are known to play a major role in action potential evoked synaptic transmission both at central synapses and at the neuromuscular junction and antibodies that affect these channels, as is thought to be the case in LEMs, would be expected to impede evoked neurotransmitter release. However, the role of VGCCs in spontaneous neurotransmission has up to recently been less clear and therefore the effect of LEMS IgG on spontaneous vesicle release could be considered surprising. In the past, mEPP amplitudes were shown not to be reduced in muscle biopsies from LEMS patients (Lambert and Elmqvist, 1972) or in passive transfer models of LEMS. (Lang et al., 1987; Giovannini et al., 2002; Flink et al., 2002.) However, mEPP frequency was found to be reduced when recorded in solutions containing high K^+ concentrations (Lang et al., 1987; Kim et al., 1986).

Spontaneous (action potential independent) neurotransmitter release occurs at the majority of synapses (Xu et al., 2009) and can be measured at the muscle fibre end plate as mEPPS and at neuronal synapses as "minis" or miniature excitatory or inhibitory post synaptic currents (mEPSC or mIPSCs). As outlined previously, it is now known that a large part of spontaneous neurotransmitter release is Ca^{2+} dependent and moreover, it was recently found in our laboratory that 50% of mEPSCs depend on the stochastic opening of VGCCs (Ermolyuk et al., 2013). The observation in the experiments described above, that spontaneous neurotransmitter release is reduced in the presence of LEMS IgG is in keeping with this. These data are also in keeping with the findings of Lang et al., who found that there was a reduced rate of spontaneous ACh release from nerve diaphragm preparations of mice passively treated with LEMS IgG (Lang et al,.

1984). However, my experiments were not specifically designed to measure spontaneous vesicle release. Action potentials were not blocked with tetrodotoxin and as many other factors such as cell health can affect the rate of spontaneous synaptic vesicle release, there are limitations to the conclusions that can be drawn about the effect of LEMS IgG on spontaneous exocytosis.

All four LEMS samples that had high titres of anti P/Q-type antibodies that were tested caused a reduction in the spontaneous and evoked synaptic vesicle release. However, the magnitude of the effect varied from sample to sample and this could not be correlated to the titre of antibodies in each sample. It was found, however, that there was substantial variability in k_{SP} values obtained from different boutons on the same coverslip and also between different experiments. The values for k_{AP} also varied substantially. Variability of neurotransmitter release at hippocampal synapses depends on a number of different factors, including the number of release ready vesicles (which is influenced by synaptic bouton size) and the fusion probability of individual vesicles (Ermolyuk et al., 2012). A strong correlation has been found between the fusion probability of synaptic vesicles and the magnitude of AP- evoked presynaptic Ca^{2+} influx amongst synaptic boutons located along the same axon (Ermolyuk et al., 2012). This could, at least in part, explain the variability in the k_{SP} and k_{AP} values in these experiments.

An effect on synaptic vesicle release from a "seronegative" LEMS sample could not be demonstrated in these experiments. Passive transfer of LEMS samples that lack any detectable antibodies have been shown to transfer the electrophysiological features of

LEMS to animals (Nakao et al., 2002) suggesting that the disease is antibody mediated and therefore these results are somewhat surprising. However, only a limited number of experiments with "seronegative" IgG were performed on only one culture set and therefore these results must be interpreted with caution; perhaps a larger sample number is required to detect a subtle effect, particularly considering the high degree of variability intrinsic to these experiments. Alternatively the mechanism of action of these antibodies may be different to that of classical "seropositive" LEMS. Further studies with more seronegative LEMS IgG samples would be required to resolve this question.

5 The effect of LEMS IgG in neurons lacking P/Q- calcium channels

5.1 Background

It was shown in the previous chapter that LEMS antibodies reduce the rate of synaptic vesicle release. The next step was to use this information to define the antigenic target of LEMS IgG. Although a compelling account of the pathophysiology of LEMS is that IgG binding to P/Q-type VGCCs lead to a decrease in neurotransmitter release, available data do not exclude the possibility that different antibodies to different antigens affect current through VGCCs and hence, exocytosis of synaptic vesicles.

5.1.1 Antigenic target in LEMS – radioimmunoprecipitation assays

The antigenic target of antibodies in LEMS is thought to be the presynaptic VGCC. Radioimmunoprecipitation assays using ^{125}-I-ω-contoxin MVIIC (^{125}I-ω-CnTx) labelled VGCCs precipitated from mammalian brain detect antibodies against P/Q- type channels in over 90% of LEMS patients (Motomura et al., 1997). A variable proportion of LEMS patients also have antibodies to N-type channels that are precipitated with ^{125}I-ω-conotoxin GVIA (^{125} I-ω-CgTx) (Sher et al., 1989; Lennon et al., 1989; Leys et al., 1991; Leys et al., 1989; Lennon et al., 1995; Suenaga et al., 1996). In the most specific assay for LEMS antibodies described by Motormura et al., 92% of LEMS patients have antibodies against P/Q type VGCCs and 33% have antibodies to N-type channels detected using ^{125}I-ω-Conotoxin-GVIA (Motomura et al., 1995; 1997). In this group, all patients with N-type antibodies also had antibodies directed against P/Q- type VGCCs. However the functional significance of N-type antibodies is unknown (Motomura et al., 1997).

5.1.2 Antigenic target in LEMS – functional studies

Functional studies have attempted to define the specific targets of antibodies in LEMS. As discussed in the Introduction, the effect of LEMS IgG on Ca^{2+} current was first shown in SCLC cells, which are known to express VGCCs (Roberts et al., 1985; Johnston et al., 1994; Lang et al., 1989; Viglione et al., 1995), suggesting this as the antigenic target. LEMS IgG has also been shown to reduce Ca^{2+} currents in a variety of other cell types including bovine adrenal chromaffin cells (Kim and Neher, 1988), motor neurons, (Garcia et al,. 1996), neuroblastoma cells, (Peers et al., 1990) insulinoma cells, (Magnelli et al., 1996) and IMR 32 cells (Grassi et al., 1994), all of which express VGCCs. Additionally passive transfer of LEMS IgG has been shown to reduce Ca^{2+} currents at the motor nerve terminals of mice by over 50% (Smith et al., 1995), where VGCCs are also expressed.

5.1.3 Specific calcium channel targeted by LEMS IgG

A number of studies have employed specific Ca^{2+} channel toxins to help determine which specific types of VGCCs are affected by LEMS IgG. Some of these studies have suggested that LEMS IgG can target multiple different VGCC subtypes. Engisch et al. studied the effect of LEMS antibodies on Ca^{2+} currents and exocytosis in bovine adrenal chromaffin cells (Engisch et al., 1999). By pharmacologically isolating specific calcium channels, they showed that IgG from all five of their LEMS patients significantly inhibited P/Q –type current and in addition, two of the five samples also significantly reduced N-type current. This implied that IgG from a single patient can act on at least two different VGCC subtypes (Engisch et al., 1999). There have been conflicting data regarding the effect of LEMS IgG on VGCCs in SCLC cells. In one study application

of LEMS IgG decreased P/Q type currents and to a lesser extent, L-type currents, whereas N-type currents were unaffected (Viglione et al., 1995). However another study of the effects of LEMS IgG on SCLC VGCC currents showed that multiple different types of High Voltage Activated (HVA) VGCCs were affected including both P/Q- type and N-type currents (Meriney et al., 1996). In motor neurons LEMS sera reduced both HVA and Low Voltage Activated (LVA) components of the Ca^{2+} current; but within the HVA component, L-type currents were spared (Garcia and Beam, 1996). In contrast, an earlier study found that LEMS IgG selectively reduced L-type currents in neuroblasotoma x glioma hybrid cell lines but did not affect T-type VGCCs (Peers et al., 1990). LEMS IgG was also found to reduce current thorough L-type VGCCs in IMR32 cells, but N-type currents were more severely affected by LEMS IgG in these experiments (Grassi et al., 1994). In the rat insulinoma RINm5F cell line, LEMS IgG reduced the dihyrdropyridine sensitive L-type current by 20-30% but current through Q-type channels was reduced by 31-36% (Magnelli et al., 1996).

There have been a number of studies that have suggested that the effect of LEMS IgG is specific for P/Q-type VGCCs. LEMS IgG has been found to affect Ca^{2+} currents from murine neonatal motor neurons to a greater extent than in murine neonatal sensory neurons (Garcia and Beam, 1996). As the predominant VGCC subtype in sensory neurons are N-type channels and P/Q-type are the predominant type in motor neurons (Mintz et al., 1992), these findings may also be taken as evidence supporting pathogenic effects of LEMS IgG through their actions on P/Q-type channels (and also may explain the lack of sensory findings in LEMS patients). Moreover, LEMS IgG has been shown to decrease neurotransmitter release from autonomic nerve terminals subserved by P/Q- but not N- type channels (Houzen et al., 1998).

The use of HEK 293 cells transfected with cDNA closes encoding human VGCCs proteins has provided strong evidence that P/Q type channels are specifically affected by LEMS IgG. Voltage dependent Ca^{2+} entry into cell lines expressing P/Q-type VGCCs was reduced by LEMS IgG whereas current through N- type channels was not affected (Pinto et al., 2002). These findings were particularly interesting as two of the six samples used in these experiments were shown to bind to ^{125}I-ω-ctx-GVIA-labelled (N-type) channels expressed in a cell line. Even LEMS samples that were shown to bind to extracellular antigens of N-type VGCCs did not reduce current through N-type VGCCs (Pinto et al. 2002). Incubation of cerebellar granule and Purkinje cells with LEMS IgG was also shown to lead to a significant reduction in the ω-aga-IVA sensitive Ca^{2+} current (P/Q-type) whereas the ω-ctx-GVIA sensitive (N-type) current was unaffected (Pinto et al. 1998). This suggested that LEMS IgG exerts its effect exclusively through an action on P/Q-type channels with no functional effect on N-type currents.

The above studies are conflicting and show that the effect of LEMS IgG on non P/Q VGCCs is far from certain. In particular, it is not clear whether N-type channels are affected by LEMS IgG. This is relevant as up to 33% of LEMS patients have antibodies against N-type channels detectable in their serum and the pathological significance of these antibodies is unknown (Motormura et al., 1997). There are a couple of possible reasons for the discrepancies in the literature. It is possible that the effect on certain VGCCs is specific to individual patients' sera with some sera reducing current through N-type channels and other sera having no effect. It is also possible that N-type current

is affected by LEMs IgG but that the smaller relative effect is more difficult to detect in certain cell types.

P/Q-type channels are the predominant VGCC required for Ca^{2+} entry during synaptic transmission both at central and peripheral synapses (Protti and Uchitel, 1993; Uchitel et al., 1992; Cao and Tsien, 2010; Luebke et al., 1993; Takahashi et al., 1993; Wheeler et al. 1994) and hence any disruption to the function of P/Q type VGCCs would be expected to impair neuromuscular transmission. However, N-type channels do play a role in synaptic transmission and it is important to define specifically which VGCCs are affected in LEMS.

5.2 Specific aims of this chapter

Using the SRC1 fluorescent imaging approach outlined in Chapter 4, the aim of the experiments in this chapter was to measure the rate of spontaneous and action potential evoked synaptic vesicle release in synapses lacking P/Q channels from *Cacna1a* knockout (KO) mice. I was particularly interested to ascertain whether LEMS IgG would reduce the rate of evoked release in these neurons compared to wild type (WT) cells. A colony of *Cacna1a* knockout mice was established in our laboratory, which were a kind gift of A Van den Maagdenberg. As outlined in the Introduction, these mice are normal *in utero* and at birth, but develop a rapidly progressive ataxic and dystonic phenotype from postnatal day 10 resulting in death after three weeks (Jun et al., 1999).

The specific aim of these experiments was firstly to define the electrophysiological characteristics of WT and *Cacna1a* KO neuronal cultures and then to compare the effect of LEMS IgG on synaptic transmission in cultures lacking P/Q-calcium channels to the effect of LEMS IgG in WT synapses.

5.3. Results

5.3.1. Electrophysiological characteristics of Cacna1a knockout neurons

The first step was to define the electrophysiological characteristics of *Cacna1a* knockout neurons. Specifically the aim was to define which VGCCs contributed to synaptic transmission in synapses lacking P/Q-type channels. Whole cell patch clamp experiments were performed and excitatory postsynaptic currents (EPSCs) were recorded from postsynaptic cells after stimulation with an extracellular electrode. EPSCs were recorded at baseline and after sequential application of specific VGCCs antagonists to block P/Q-type, N-type and R-type VGCCS (agatoxin IVA 250 nM, conotoxin GVIA 5µM and SNX 482 200 nM respectively). The EPSC amplitudes at baseline (before addition of toxins) was normalised to 1 to allow comparison of the effect of the individual toxins between WT and *Cacna1a* knockout experiments.

In wild type neurons it was found that blocking P/Q-type VGCCs with agatoxin IVA reduced the amplitude of the EPSC by 70%. Subsequent application of conotoxin GVIA reduced EPSC amplitude to 9% of baseline and finally application of SNX 482

almost completely obliterated the post synaptic response. In contrast, application of agatoxin IVA to synapses lacking P/Q-type channels did not cause any significant effect to EPSC amplitude. However, blockage of N-type channels with conotoxin GVIA reduced EPSC amplitude by 70% and SNX 482 reduced EPSC amplitude by a further 20%. After application of the three calcium channel toxins, there remained a residual postsynaptic response, at 10% of baseline (Table 5.1).

EPSC amplitude (normalised to EPSC amplitude at baseline)				
	Baseline	Agatoxin IVA	Conotoxin GVIA	SNX 482
Control (n=10) **SD** **SEM**	1	0.3 0.16 0.04	0.09 0.17 0.02	0.02 0.03 0.01
KO (n=6) **SD** **SEM**	1	0.94 0.3 0.18	0.29 0.21 0.03	0.1 0.16 0.02

Table 5.1. EPSC amplitude in WT and KO neuronal cultures at baseline and after sequential treatment with specific VGCC toxins (normalised to EPSC at baseline).

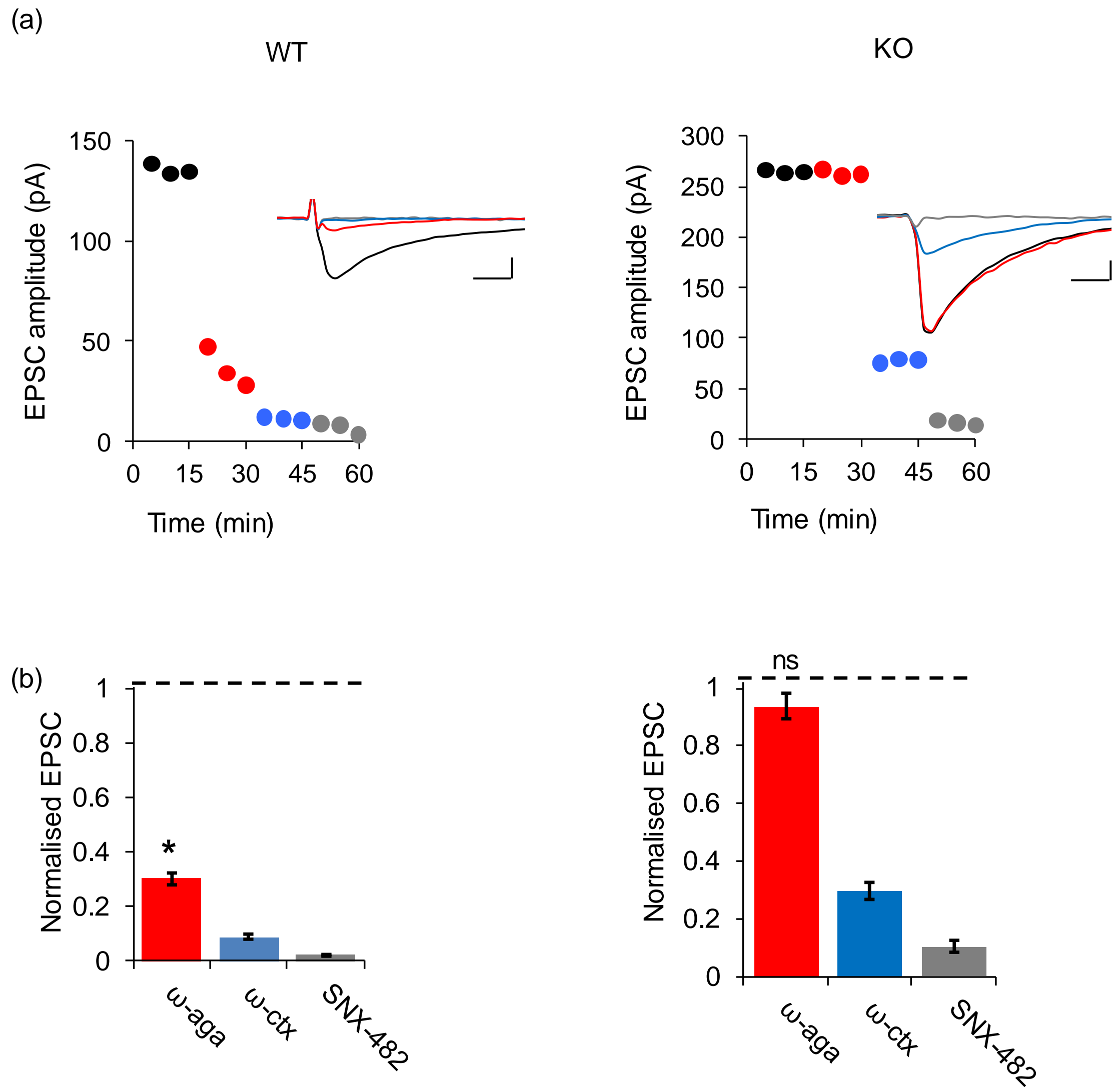


*Figure 5.1 Synaptic transmission in Cacna1a$^{-/-}$ neurons depends on N- and R-type VGCCs. (a) Typical experiments showing evoked excitatory postsynaptic current (EPSC) amplitude plotted against time in WT (left) and Cacna1a knockout (KO, right) neurons before (black) and after sequential application of the P/Q-type blocker ω-Aga IVA (250 nM; red), the N-type blocker ω-Ctx GVIA (5 µM; blue), and the R-type blocker SNX-482 (200 nM; grey). Insets: representative EPSCs before (black) and after addition of each blocker (colours as for scatter plot). Scale bar: 50 pA, 20 ms. (b): normalized EPSC amplitudes in WT (left) and Cacna1a knockout (right) neurons after sequential application of the VGCC blockers (WT, n = 10; KO, n = 6; *, $p < 0.05$).*

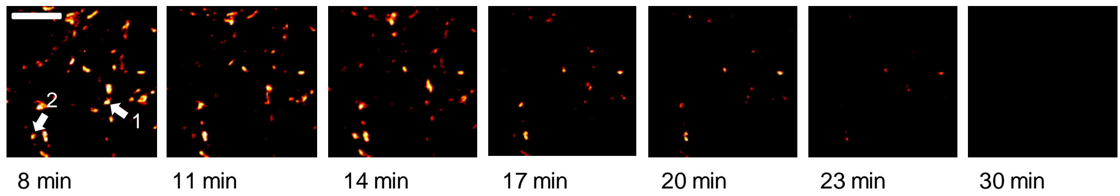
5.3.2 Effect of LEMS IgG in synapses lacking P/Q-type VGCCs

The above experiments demonstrated that neuronal cultures from *Cacna1a* knockout mice lack functional P/Q-type VGCCs and synaptic transmission is largely dependent on current through N-type VGCCs. The next step was to test whether LEMS IgG reduced synaptic vesicle release in these neuronal cultures lacking functional P/Q-type VGCCs.

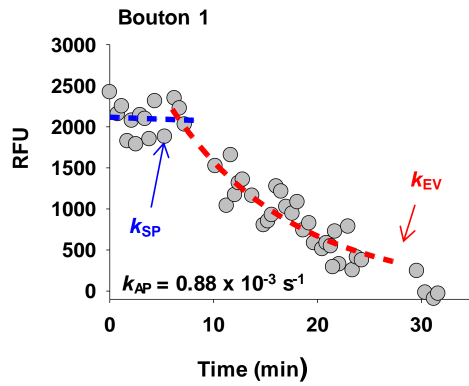
One LEMS IgG sample was selected to use in these experiments. The sample was chosen precipitated a high titre of P/Q-antibodies and also N-type antibodies at a lower titre. This was sample 3 highlighted in Chapter 4. It was hypothesised that if the N-type antibodies in this sample were pathogenic, this would be revealed by difference between LEMS IgG and Control IgG treated Cacna1a KO cultures, which lack P/Q-type channels but retain N-type channels.

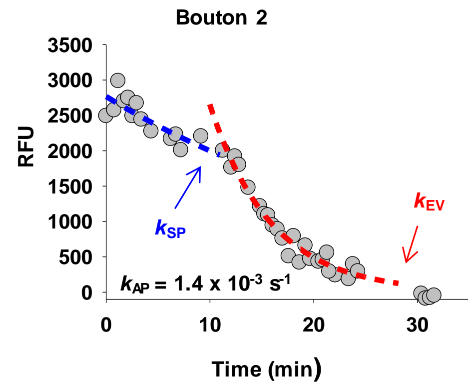
The experiments were conducted in the same way as described in Chapter 4. Representative bouton traces and destaining profiles for WT and KO samples treated with control or LEMS IgG are shown in Figure 5.2.

(a)

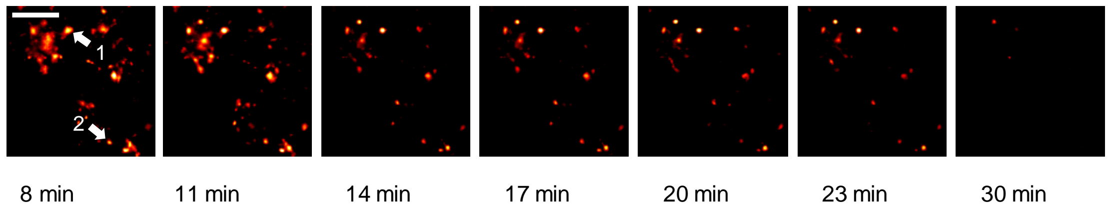


8 min 11 min 14 min 17 min 20 min 23 min 30 min

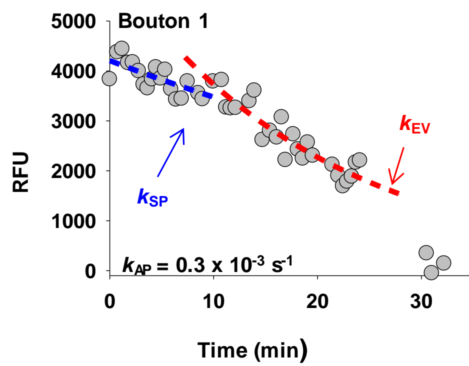


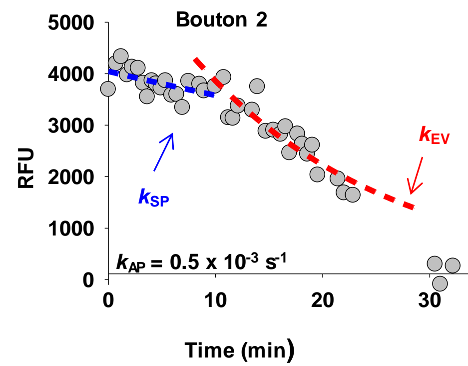


(b)



8 min 11 min 14 min 17 min 20 min 23 min 30 min





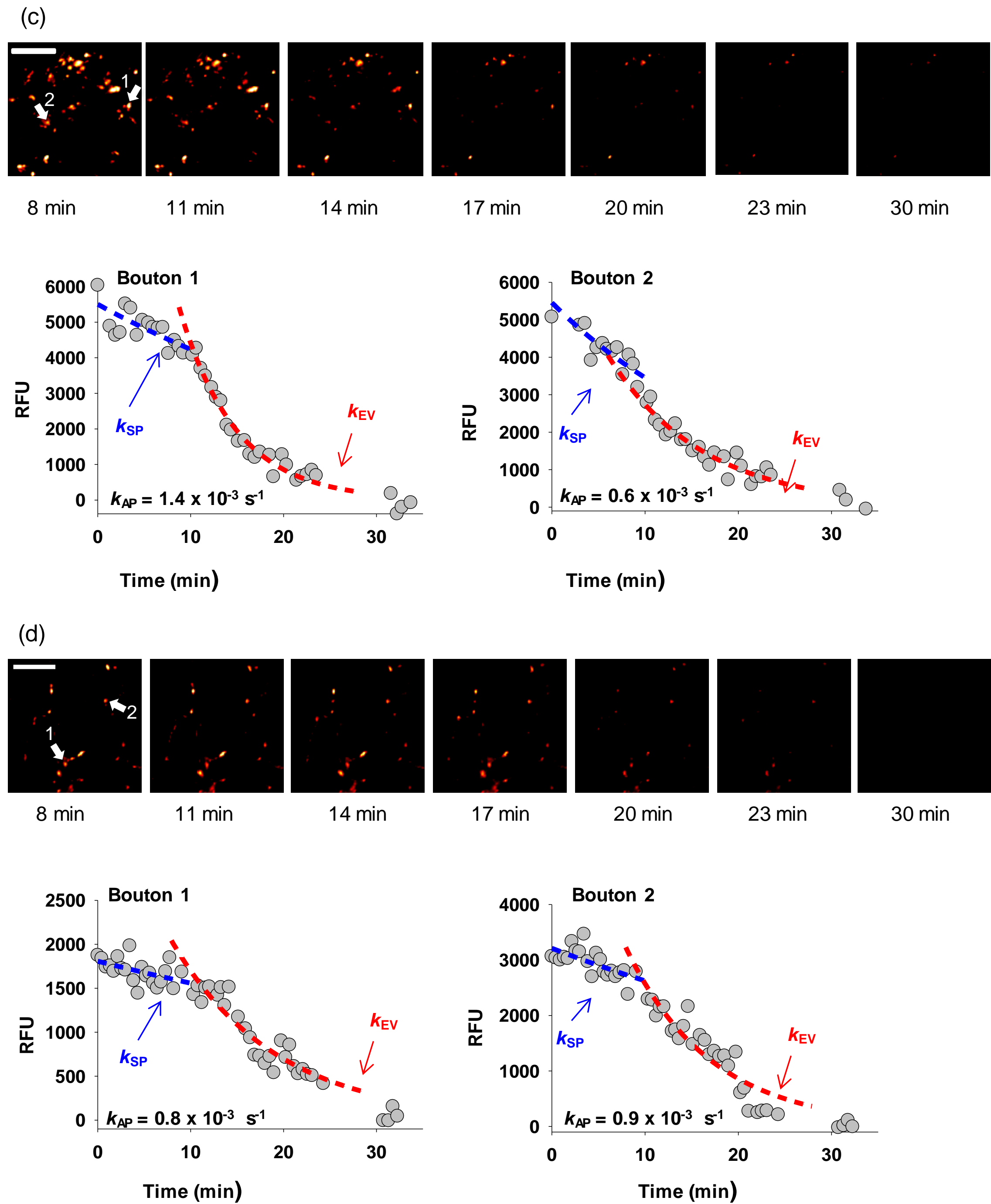


Fig 5.2 (a,b,c,d,) Representative SRC1 imaging experiments in WT cultures treated with control (a) or with LEMS IgG (b) and KO cultures treated with control (c) or with LEMS IgG (d). Fluorescence microscopy images (top) show loss of fluorescence at different time points during the experiment. Fluorescence time-courses in two pairs of

representative boutons (arrows) are shown below. Spontaneous and evoked destaining rates were fitted with monoexponential curves. Scale bars: 10 µm.

5.3.2.1 TRP

Firstly, the size of the TRP in LEMS treated neurons compared to controls in KO and WT neurons was analysed. There was no significant difference in the size of the TRP in LEMS treated neurons compared to neurons treated with control IgG in both WT neurons (p=0.6) and KO neurons (p =0.9) (Table 5.2, Figure 5.3).

	Control IgG treated WT neurons	**LEMS IgG treated WT neurons**	**Control IgG treated KO neurons**	**LEMS IgG treated KO neurons**
TRP (mean)	2.14	2.5	1.9	1.8
SD	0.97	1.34	1.8	1.1
SEM	0.24	0.61	0.23	0.53
Number of experiments	16	5	12	5
Number of boutons	602	108	300	143
Number of cultures	10	5	9	5

Table 5.2. Mean TRP size in LEMS treated neurons compared to control

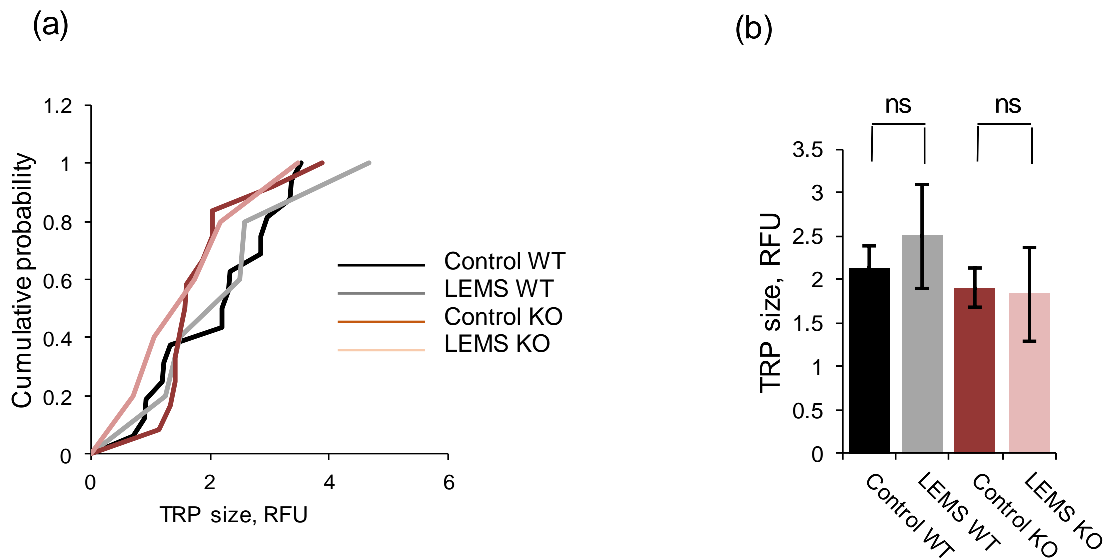


Figure 5.3 Effects of LEMS and Control IgG on relative TRP size in WT and Cacna1a$^{+/-}$ (knockout, KO) neurons. (a) Cumulative distributions of mean TRP size values obtained in individual experiments. Data are from 602 boutons in 15 experiments (WT, Control, black line), 138 boutons in five experiments (WT, LEMS, grey), 303 boutons in 15 experiments (KO, Control, red), and 175 boutons in 6 experiments (KO, LEMS, pink). (b) Mean (± SEM) values for the pooled data. NS, non-significant.

5.3.2.2 k_{SP}

The next step was to analyse the effect of LEMS IgG on spontaneous synaptic vesicle release. LEMS IgG caused a significant increase in the rate of spontaneous synaptic vesicle release in WT neurons ($p<0.01$) but not in KO cultures ($p=0.61$) (Table 5.3, Figure 5.4).

	Control IgG treated WT neurons	LEMS IgG treated WT neurons	Control IgG treated KO neurons	LEMS IgG treated KO neurons
k_{SP} x10^3, s^{-1} (mean)	0.3	0.4	0.3	0.27
SD	0.06	0.04	0.047	0.09
SEM	0.02	0.02	0.02	0.04
Number of experiments	16	5	12	5
Number of boutons	602	108	300	143
Number of cultures	10	5	9	5

Table 5.3. Mean k_{SP} in LEMS treated neurons compared to neurons treated with control IgG at WT and KO synapses

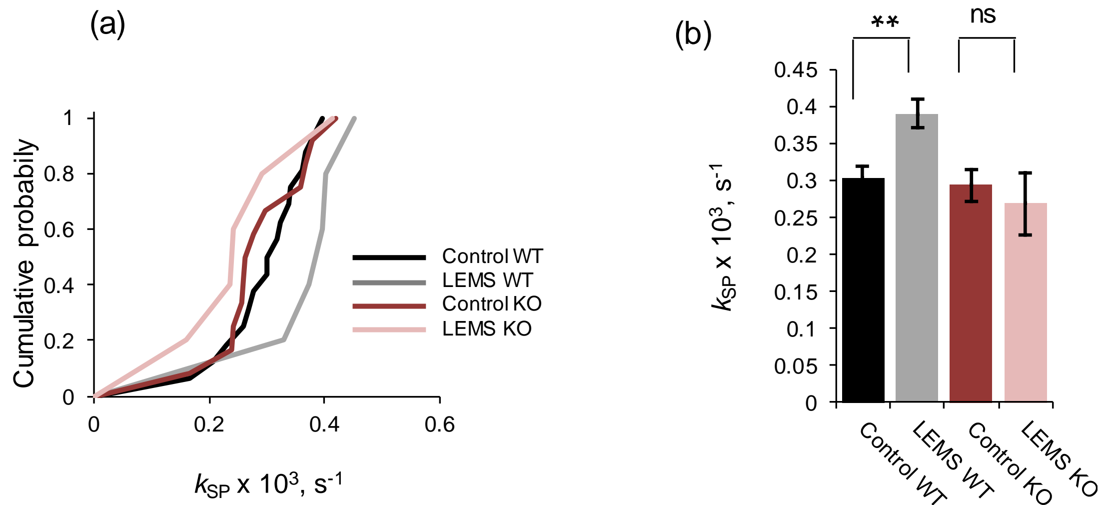


*Figure 5.4 Effects of LEMS and control IgG on k_{SP} in WT and Cacna1a$^{+/-}$ (knockout, KO) neurons. (a) Cumulative distributions of k_{SP} values obtained in individual experiments. Data are from 602 boutons in 15 experiments (WT, Control, black line), 138 boutons in five experiments (WT, LEMS, grey), 303 boutons in 15 experiments (KO, Control, red), and 175 boutons in 6 experiments (KO, LEMS, pink). (b) Mean (± SEM) values for the pooled data. **, $p < 0.01$; ns, non-significant.*

5.3.2.3 Effect of LEMS IgG k_{EV} in neurons lacking P/Q-type VGCCs

Next, the effect of LEMS IgG on the rate of evoked synaptic vesicle release in WT and KO neuronal cultures was analysed. Treatment with LEMS IgG significantly reduced the k_{EV} in WT neurons ($p < 0.01$) but had no significant effect in neurons lacking P/Q type channels ($p = 0.8$) (Table 5.4, Figure 5.5).

	Control IgG treated WT neurons	**LEMS IgG treated WT neurons**	**Control IgG treated KO neurons**	**LEMS IgG treated KO neurons**
k_{EV} x 10^3, s^{-1} (mean)	1.13	0.72	1.17	1.1
SD	0.37	0.19	0.51	0.49
SEM	0.09	0.08	0.15	0.22
Number of experiments	16	5	12	5
Number of boutons	602	108	300	143
Number of cultures	10	5	9	5

Table 5.4 Mean k_{EV} in LEMS treated neurons compared to neurons treated with control IgG at WT and KO synapses

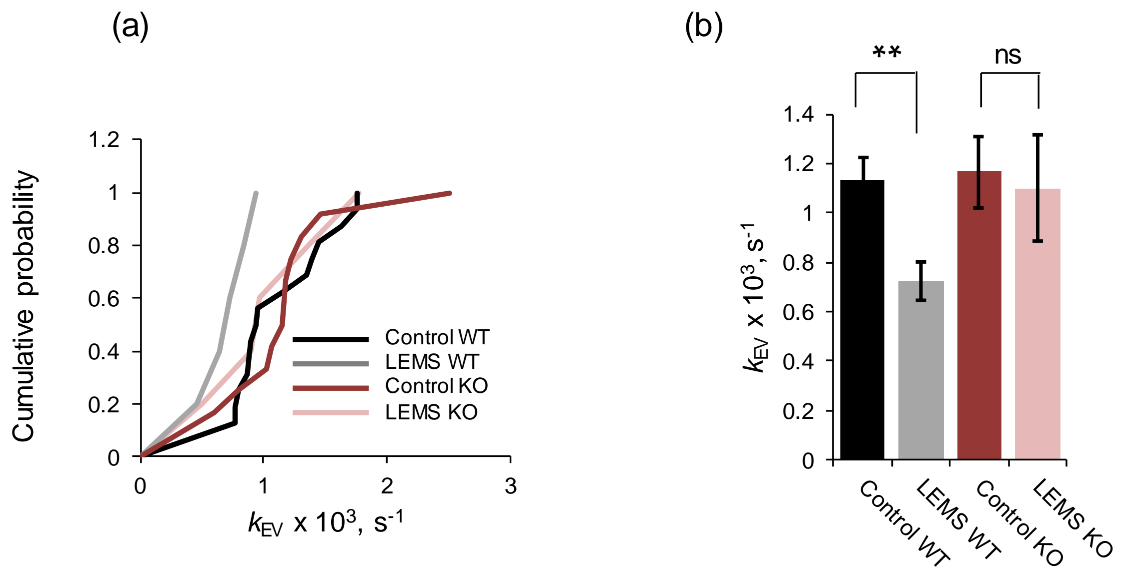


*Figure 5.5 Effects of LEMS and control IgG on k_{EV} in WT and Cacna1a$^{+/-}$ (knockout, KO) neurons. (a) Cumulative distributions of k_{EV} values obtained in individual experiments. Data are from 602 boutons in 15 experiments (WT, Control, black line), 138 boutons in five experiments (WT, LEMS, gray), 303 boutons in 15 experiments (KO, Control, red), and 175 boutons in 6 experiments (KO, LEMS, pink). (b) Mean (± SEM) values for the pooled data. **, $p < 0.01$; ns, non-significant.*

5.3.2.4. k_{AP}

The rate of action potential evoked release in wild type and *Cacna1a* $^{-/-}$ neurons is shown in figure 5.5 and table 5.5. As was shown in the previous chapter, LEMS IgG caused a significant reduction in the rate of action potential evoked release in WT cultures ($p<0.01$).

However, there was no significant effect on the rate of action potential evoked synaptic vesicle release in synapses lacking P/Q type channels ($p = 0.8$) (Table 5.5, Figure 5.6).

	Control IgG treated WT neurons	LEMS IgG treated WT neurons	Control IgG treated KO neurons	LEMS IgG treated KO neurons
k_{AP} x 10^{-3}, s^{-1} (mean)	1.65	0.73	1.86	1.43
SD	0.8	0.34	1.11	1.03
Number of experiments	15	6	15	6
Number of boutons	602	138	303	175
Number of cultures	10	5	9	5

Table 5.5. Mean k_{AP} in LEMS treated neurons compared to neurons treated with control IgG in WT and KO neuronal cultures

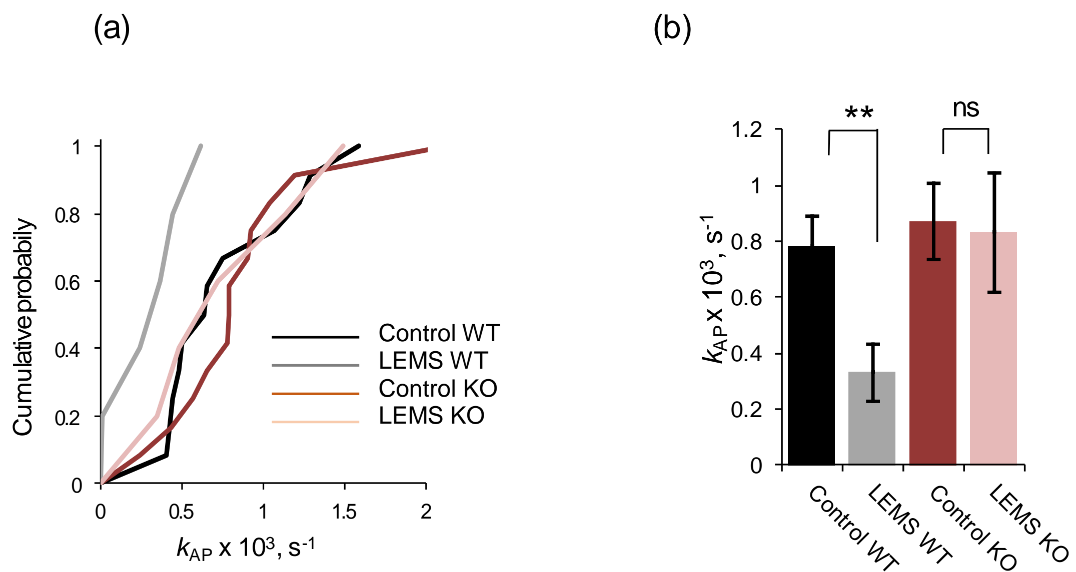


*Fig 5.6 Effects of LEMS and control IgG on k_{AP} in WT and Cacna1a$^{+/-}$ (knockout, KO) neurons. (a) Cumulative distributions of k_{EV} values obtained in individual experiments. Data are from 602 boutons in 15 experiments (WT, Control, black line), 138 boutons in five experiments (WT, LEMS, grey), 303 boutons in 15 experiments (KO, Control, red), and 175 boutons in 6 experiments (KO, LEMS, pink) (b) Mean (± SEM) values for the pooled data. **, $p < 0.01$; ns, non-significant.*

5.4 Discussion

In this chapter, my first task was to characterise the electrophysiology of synaptic transmission in *Cacna1a* $^{-/-}$ neuronal cultures. The calcium channels involved in synaptic transmission were identified by sequential application of toxins to block specific VGCC types while recording EPSC amplitude in whole cell voltage clamp experiments. It was found that ω-Aga IVA had no effect on synaptic transmission in KO cultures but that there was an enhanced dependence on N-type VGCCs. Synaptic transmission in WT cultures was mainly dependent on Ca^{2+} current through P/Q type VGCCs. These data were consistent with similar experiments performed in cultured hippocampal cells by Cao and Tsien (Cao and Tsien, 2010) and with previous work performed in hippocampal brain slices and neuromuscular end plates from knockout mice (Jun et al., 1999; Urbano et al., 2003).

Having confirmed that synaptic transmission in cultures from P/Q- knockout mice was dependent on N- and R-type VGCCs, the effect of LEMS IgG on action potential evoked synaptic vesicle release at these synapses was tested. As was demonstrated for WT rat cultured hippocampal neurons in Chapter 4, LEMS IgG significantly impaired synaptic transmission in WT synapses. However, there was no significant effect of LEMS IgG in synapses lacking P/Q-type channels. This is the first time to our knowledge that the effect of LEMS IgG has been tested in synapses lacking P/Q-type channels.

These findings are consistent with our knowledge regarding which VGGCs are responsible for neurotransmitter release at the NMJ and at central synapses (Protti and Uchitel, 1993; Cao and Tsien, 2010; Luebke et al., 1993; Takahashi and Momiyama,. 1993; Wheeler et al., 1994). The data are also consistent with the work of Pinto et al (Pinto et al. 1998; 2002) in which LEMS IgG decreased current through HEK cells transfected with P/Q-type VGCC subunits but not with N-type subunits. However, transfected VGCCs in HEK cells may not always be an accurate representation of VGCCs in *vivo* due to splice variations or different subunit combinations. Furthermore, the efficiency of transfection may differ for different subunits and thereby alter the size of the currents, raising the possibility of masking a real effect of LEMS IgG on N-type channels if the transfection efficiency is high (Flink and Atchison 2003). The work described here, demonstrating that LEMS IgG has no effect on neuronal synapses that lack P/Q-type channels provides further evidence about the VGCC specificity of LEMS IgG. These data are also consistent with results from passive transfer studies where ω-aga IVA is 30% less effective in reducing the amplitude of Ca^{2+} currents recorded from motor nerve terminals of mice passively injected with LEMS IgG compared to controls (Xu et al., 1998) suggesting a prominent effect on P/Q-type VGCCs.

The conclusions are limited by the fact that only one IgG sample was tested on KO cultures in these experiments. However, as this sample precipitated N-type VGCCs in radioimmunoprecpitation assay, one would expect to see an effect if one existed, as synaptic vesicle release in these synapses is largely dependent on Ca^{2+} entry through N-type channels.

Spontaneous neurotransmitter release was not reduced by LEMS IgG in these experiments. In fact, an increase in the spontaneous vesicle release was seen in WT neurons treated with LEMS IgG. This contrasts with the experiments in WT rat neuronal cultures where LEMS IgG reduced the rate of spontaneous synaptic vesicle release by 24%. The reason for this discrepancy is unclear. The IgG sample that was used in these experiments had not caused a statistically significant reduction in the spontaneous release rate in WT rat experiments and this lack of an effect could be sample specific.

6 Thymectomy in the management of MG

6.1 Background

Advances in the treatments for MG have improved the mortality and morbidity from MG considerably in recent years – the mortality of MG has reduced from over 30% in the 1930s to below 5% now (Christensen et al., 1998). Therapy for MG takes the form of symptomatic treatment with pyridostigmine or immunomodulatory treatment with steroids, steroid sparing agents, plasma exchange and IVIG. Despite these advances in medical therapy thymectomy remains an integral part of the treatment with MG.

Thymectomy has been used in the management of MG since the 1930s when Blalock et al. reported the remission of generalized MG in a 21 year old woman following removal of a thymic tumour (Blalock et al., 1939). Subsequently thymectomy was performed on patients without thymoma (Gronseth and Baron, 2000) and a good outcome was especially described in patients who had a hyperplastic gland.

Thymectomy is always indicated in patients who have a thymoma because of the risk of invasiveness and metastatic spread (Lanska, 1990). However, over 80% of MG cases are non-thymomatous and the evidence for the use of thymectomy in these cases is less certain. There is a clear rationale for the use of thymectomy in MG as the site of anti AChR antibody production is likely located in the activated areas of thymic tissue including B cell follicles and germinal centres (Ambrogi et al., 2011). Although there are no randomised controlled trials to date, one is underway (Newsom-Davis et al., 2008).

There have been many reports of non-randomised studies examining the effect of thymectomy in nonthymomatous MG and in general these studies support its use. In the year 2000, the American Academy of Neurology systematically analysed retrospective non-randomised studies describing the use of thymectomy in MG (Gronseth and Barohn, 2000). In total 28 studies were analysed that described the outcome in thymectomised versus non-thymectomised patients. The studies quoted were inconsistent in terms of definitions of remission, patients included and surgical techniques and no study described formal selection for MG patients undergoing thymectomy. However, in general, positive associations were found in most studies between thymectomy and MG remission/improvement. The relative rate of medication free remission in patients who underwent thymectomy compared to those that did not was 2.1. Persistent positive associations between thymectomy and improved MG outcome were found when single confounding variables such as age, gender and severity of MG were controlled for. However, when multiple confounders were taken into account, the results from different studies were conflicting (Gronseth and Barohn, 2000). The authors could only conclude from this research that the benefit of thymectomy in non-thymomatous MG had not been established conclusively. They recommended thymectomy as an option to increase the probability of remission or improvement in MG – a Class II recommendation.

A previous group in our institution had investigated the use of thymectomy in the management of Myasthenia Gravis (O'Riordan et al., 1998). Fifty-three patients who underwent thymectomy were identified. In general, the outcome was positive with 35% of patients in remission at two years and 46% of patients having mild generalised or ocular symptoms only. The procedure itself was well tolerated, however, 23% of

patients required prolonged intubation and 3.8% of patients required plasma exchange for prolonged myasthenic weakness post operatively. This group found that the beneficial effect of thymectomy was clearest for those patients with thymic follicular hyperplasia. Patients with a thymoma had the poorest outcome. However, thymectomy was felt to be still indicated in these patients because of the risk of tumour invasion, metastases and the poor response to medical therapy (O'Riordan et al., 1998; Lanska et al., 1990).

A longterm follow up study of almost 2000 patients (Grob et al., 2008) found that thymectomy was generally followed by an improvement in myasthenic symptoms. However, it was noted that it may now be more difficult to demonstrate a beneficial effect of thymectomy than before because of concurrent improvement in the nonsurgical management of MG patients (Grob et al., 2008). Thymectomy has also been shown to have a significant impact on quality of life in both physical and psychosocial domains (Ambrogi et al., 2011).

Currently the decision about which myasthenic patients should proceed to thymectomy is often based on the preferences of the treating neurologist. Generally, the indications for thymectomy are early generalised myasthenia and resistant ocular disease. As the natural history of MG is that 20-29% can spontaneously improve, it can be difficult to judge the beneficial effect of thymectomy (Hennessey et al., 2011).

6.2 Specific aims of this chapter

In the absence of randomised data, it is essential to constantly review clinical practice. The National Hospital for Neurology (NHNNN) has a large cohort of patients with MG and performs an average of seven thymectomies a year. The aim of this chapter was to take advantage of this patient cohort and examine the outcome of patients who had undergone thymectomy both in the short and long term.

6.3 Methods

All patients undergoing thymectomy for MG at the NHNN are admitted to the neurological intensive care unit (N-ICU) for perioperative care and thus patients who had undergone thymectomy were identified from the N-ICU log between January 1999 and December 2011. The medical case notes of each patient were reviewed retrospectively and where possible the patients were also reviewed in clinic. A diagnosis of MG was made based on clinical findings and, at least one of the following; EMG findings of increased jitter/block with a decremental response to repetitive nerve stimulation, positive acetylcholine receptor antibodies or a positive erdrophonium (Tensilon) test.

Patient characteristics at baseline were recorded including the severity of MG, immunosuppressive treatment, age at onset of symptoms and duration of MG prior to thymectomy. The duration of admission to ITU, the total hospital stay and any post-

operative complications were recorded for the perioperative period. Post operatively the patients were assessed at six, twelve and twenty four months. When longer follow up was available, the MG status at the latest clinical review was also recorded. MG severity was assessed according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification system. The MGFA post intervention status was used to assess the response to thymectomy at the last clinical review. These measures were specifically chosen as in the past, the lack of universally accepted outcome measures has made it difficult to compare between older studies and a reliance on relatively subjective measures of outcome has hindered the interpretation of previous studies. The Medical Scientific Advisory Board of the MGFA established a task force in 1997 to address these issues. A number of clinical classification systems and outcome measures were defined including the MGFA Clinical Classification and the Post Intervention Status (Table 6.1 and Table 6.2) (Jaretzki et al., 2000).

	MGFA Clinical Classification
Class I	Only ocular muscle weakness
Class II (a*, b)**	Mild weakness affecting other than ocular muscles
Class III (a*, **)	Moderate weakness affecting other than ocular muscles
Class IV (a*, b)**	Severe weakness affecting other than ocular muscles
Class V	Defined by intubation with or without mechanical ventilation
	*a: Predominantly affecting limb, axial muscles or both **b: Predominantly affecting oropharyngeal, respiratory muscles or both

Table 6.1 MGFA Clinical Classification

MGFA Post Intervention Status	
Complete Stable Remission (CSR)	No symptoms or signs of MG for at least 1 year and has received no therapy for MG during that time. No weakness of any muscle on careful examination. Isolated weakness of eyelid closure is accepted.
Pharmacologic Remission (PR)	Same criteria as for CSR except that the patient continues to take some form of therapy for MG. Patients taking cholinesterase are excluded from this category.
Minimal Manifestations (MM)	No symptoms of functional limitation from MG but has some weakness on examination
Improved (I)	A substantial decrease in pre-treatment clinical manifestations or sustained substantial reduction in MG medications.
Unchanged (U)	No substantial change in pre-treatment clinical manifestations or reduction in MG medications
Worse (W)	A substantial increase in pre-treatment clinical manifestations or a substantial increase in MG medications.
Exacerbations (E)	Patients who have fulfilled criteria of CSR, PR or MM but subsequently developed clinical finding greater than permitted by these criteria.
Died of MG	Patients who died of MG, of complications of MG therapy or within 30 days after thymectomy

Table 6.2 MGFA Post-intervention Status

The NHNN is a tertiary referral centre and patients are often referred to a local neurology centre for continued follow up meaning that some patients are lost to follow up. In such cases, the patient's neurologist was contacted where possible.

6.4 Results

6.4.1 Patient characteristics

Eighty-nine patients were admitted to the NHNN for a thymectomy during the study period. Sixty -eight (76%) were female and 21(24%) were male. The average age at surgery was 33 years with a range from 18-74. The average duration of MG prior to surgery was 31.4 months with a range of one month to 120 months. The vast majority (98%) of the patients were positive for anti AChR antibodies. One patient was found to

be positive for MuSK antibodies five years after thymectomy and a further patient was seronegative on routine antibody assay. (Table 6.3)

Prior to surgery, 56% of patients had a history of mild generalised myasthenia gravis (MGFA II), 29% had moderately severe myasthenia (MGFA III) and 3% had severe symptoms (MGVA IV). Ocular symptoms only (MGFA I) were present in 11% of patients. Prior to thymectomy 73% were treated with oral prednisolone at an average dose of 28 mg. Twenty one percent were treated with additional immunosuppressant agents (all of these patients were treated with azathioprine). Twenty four percent were not on any immunosuppressant agent at the time of thymectomy. Tables 6.5 and 6.7 outline clinical severity of MG and treatment prior to thymectomy.

	Total	Thymoma	Hyperplasia	Normal	Atrophic
Number Male *n, (percent)* Female *n, (percent)*	89 21(24%) 68(76%)	21(24%) 6 15	43(48%) 8 35	17(19%) 7 10	8(9%) 0 8
Mean age at surgery *(range)*	32.5 (18-74)	46 (28-74)	29 (19-44)	29.5 (18-56)	27 (22-33)
Mean time from MG diagnosis to thymectomy (months) *(range)*	31.43 (1-120)	10.5 (1-24)	31 (12-120)	29 (2-60)	40 (12-84)
AChR antibody positive (number) (percent)	87 (98%)	21 (100%)	43 (100%)	16 (94%) (1 MuSK)	7 (88%) (1 SNMG)
Mean ICU stay (days) *(SD) (median)*	2.45 (6.4) (1)	2.4 (2.3) (1)	1.4 (0.65) (1)	5.4 (14.6)(1)	1.75 (0.96)(1.5)
Mean hospital stay(days) (SD) (median)	11.2 (17.3)(8)	12.5 (4-27) (2)(8)	8 (5-15) (7.1)(9)	19 (5-150) (39.4)(9)	8.75 (7-12) (3.3)(9)
Thymic enlargement on CT (%)	44(49%)	20(95%)	15(35%)	8/(47%)	1(12%)

Table 6.3 Clinical details of patients undergoing thymectomy

6.4.2 Preoperative imaging

All patients underwent a CT or MRI prior to surgery. Overall half of all patients had evidence of thymic enlargement on CT or MRI scan. All but one of the patients with a thymoma had enlargement of the thymus reported. Seventeen of patients who had thymic hyperplasia had an enlarged gland on radiology but almost half of the patients who were subsequently found to have normal thymic histology also were shown to have an enlarged gland on imaging. One of the eight patients who had an atrophic gland had an enlarged thymus on preoperative imaging.

6.4.3 Hospital stay and complications post thymectomy

All patients underwent extended transsternal thymectomy and all were admitted to ICU post operatively. The mean duration of ICU stay was 2.5 days (SD 6.4). The mean total hospital stay was 11.2 days (SD 17.3). There were no perioperative deaths. Eight patients (9%) had a peri or post-operative complication; three had a pneumothorax, three required a blood transfusion and two patients developed a wound infection. In addition, one patient developed a post thymectomy myasthenic crisis (MC). Seven patients (8%) developed a keloid scar and two patients had persistent sternal pain in the years following thymectomy (Table 6.4).

Complications		Number of patients (%)
Early	Pneumothorax	3 (3%)
	Wound infection	2(2%)
	Blood transfusion	3 (3%)
	Post-operative myasthenic crisis	1 (1%)
Late	Persistent sternal pain	2 (2%)
	Keloid scarring	7(8%)

Table 6.4 Early and late complications after thymectomy

6.4.4. Histological subtype

On analysis of the histological subtype 24% had a thymoma, 48% had a hyperplastic gland and 19% had normal thymic histology. An atrophic gland was seen in 8% of cases. Patients who had a thymoma were older on average (46 years compared to 29, 29.5 and 27 years for hyperplastic, normal and atrophic thymic histology respectively). Patients who had a thymoma detected on imaging, proceeded to thymectomy sooner after a diagnosis of MG compared to non-thymomatus patients (10.5 months compared to 33 months). (Table 6.3).

6.4.5. Clinical outcome after thymectomy

Follow up at 6, 12 and 24 months was available for 86/89 (97%), 73/89 (82%) and 66/89 (74%) patient respectively. Severity of MG as measured by MGFA grade gradually improved throughout the follow up period (Table 6.5.).

	MGFA	Baseline	6 months	12 months	24 months
Thymoma	Asymptomatic	-	8	11	7
	I	2	1	-	2
	II	9	8	6	3
	III	9	2	-	-
	IV/V	1	-	-	-
	Unknown	-	2	4	9
Hyperplasia	Asymptomatic	-	20	23	23
	I	3	2	2	2
	II	26	19	6	6
	III	13	1	2	-
	IV/V	1	-	-	-
	Unknown	-	1	10	12
Normal	Asymptomatic	-	9	9	9
	I	2	2	1	1
	II	11	4	5	5
	III	2	1	-	-
	IV/V	2	1	1	1
	Unknown	-		1	1
Atrophic	Asymptomatic	-	3	3	2
	I	1	1	1	3
	II	6	3	3	2
	III	1	1	-	-
	IV/V	-	-	-	-
	Unknown	-	-	1	1

Table 6.5 MG severity as measured by the Myasthenia Gravis Foundation of America (MGFA) clinical classification at baseline and at 6, 12 and 24 months after thymectomy.

The last clinical review occurred at a mean of 3.8 years post thymectomy (0.5-11 years). Thirty four per cent of patients had achieved complete stable remission at this point, meaning that they were asymptomatic from their MG and did not require ongoing pharmacological treatment. An additional 33% achieved pharmacological remission (PR). Twelve percent of patients had residual myasthenic symptoms/signs that did not

require immunomodulatory therapy – minimal manifestations of disease. An improved status compared to before thymectomy was reported for 13% of patients. One patient died during the follow up period and there was no follow up available for three patients.

Table 6.6 outlines the post intervention status for patients with each histological subtype. When outcomes for different histological subtypes were assessed, a hyperplastic gland was associated with a significantly better outcome compared to other histological diagnoses. Complete stable remission was achieved by 42% of those with a hyperplastic gland compared to 26% for other histological diagnoses ($p < 0.05$).

MGFA post intervention status	All (n=89)	Thymoma (n=21)	Hyperplastic (n=43)	Atrophic (n=8)	Normal (n=17)
CSR	30(34%)	5 (24%)	18(42%)	2(25%)	5(29%)
PR	29(33%)	5(24%)	13(30%)	4(50%)	7(41%)
MM	11(12%)	3(14%)	6(14%)	2(25%)	-
Improved	12(13%)	4(19%)	5(12%)		3(18%)
Unchanged	2 (2%)	1(5%)			1(6%)
Exacerbations	1(1%)				1
Died	1(1%)	1(5%)			
No follow up	3(3%)	2(10%)	1(5%)		

Table 6.6 MGFA post intervention status at last clinical review; CRS: complete stable remission; PR: pharmacological remission; MM: minimal manifestation

There was a trend towards the incidence of CSR increasing with time after thymectomy. Twenty eight percent (12/43) of patients with a follow up duration of two years or less after thymectomy achieved CSR in comparison to 43% of those who had a longer duration of follow up, although this finding did not achieve statistical significance (p=0.06).

The duration of non thymomatous MG prior to thymectomy did not correlate with outcome; 40% of those with a duration of non-thymomatous MG of two years achieved CSR in comparison to 33% (8/24) of those with a disease duration of two years or greater (p=0.19).

6.4.6. Immunosuppressive treatment after thymectomy

Treatment at baseline and during the follow up period is outlined in Table 6.7. Steroid requirements fell continuously during the follow up period but the proportion of patients taking additional steroid sparing immunosuppressant agents increased (Table 6.6.)

	Treatment	Baseline	6 months	12 months	24 months
Thymoma	Prednisolone	12	13	11	8
	No pred	9	6	6	4
	Dose	35mg	20 mg	12 mg	8mg
	Aza	5	8	6	5
	No aza	16	11	11	6
	Other	-	-	-	1
	Unknown	-	2	4	9
Hyperplasia	Prednisolone	30	27	17	13
	No pred	13	15	16	18
	Dose	25mg	20 mg	15 mg	15 mg
	Aza	8	11	8	8
	No aza	35	31	24	21
	Other	-	-	1	2
	Unknown	-	1	10	12
Normal	Prednisolone	15	16	11	8
	No pred	2	1	5	8
	Dose	30 mg	20 mg	8 mg	8 mg
	Aza	4	6	7	5
	No aza	13	11	8	9
	Other	-	-	1	2
	Unknown	-	-	1	1
Atrophic	Prednisolone	8	8	6	4
	No pred	0	0	1	3
	Dose	35 mg	15 mg	10 mg	9mg
	Aza	3	3	3	3
	No aza	5	5	4	4
	Other	-	-	-	0
	Unknown	0	0	1	1

Table 6.7 Treatment at baseline and at 6, 12 and 24 months post thymectomy; Aza: azathioprine; Pred: prednisone

The proportion of patients taking additional oral immunosuppressant agents such as azathioprine or mycophenolate mofetil increased from 21% (19/89) before thymectomy

to 33% (28/86) at last clinical review ($p < 0.05$). However, the proportion of patients not taking any immunosuppressive therapy increased from 27% (24/89 post thymectomy) to 40% (35/86) post thymectomy ($p < 0.05$) (table 6.8).

Treatment at last clinical review	All N= 89	Thymoma N= 21	Hyperplasia N= 43	Atrophy N= 8	Normal N= 17
No treatment	35 (39%)	6 (29%)	21 (49%)	3 (38%)	5 (29%)
Prednisolone alone	23 (26%)	5 (24%)	8 (19%)	4 (50%)	5 (29%)
Oral IS alone	10 (11%)	1 (5%)	5 (12%)	1 (13%)	3 (18%)
Prednisolone and oral IS	18 (20%)	6 (29%)	8 (19%)	-	4(24%)
Unknown	3 (3%)	2 (10%)	1 (2%)		
Dead	1 (1%)	1 (5%)			

Table 6.8 Treatment at last clinical review

6.5 Discussion

Despite a lack of randomised data, thymectomy is frequently used in the treatment of MG. My aim was to perform a retrospective study to assess short and long term prognosis after thymectomy. This study has a number of limitations, not least its retrospective nature and relatively small sample size. However, in general, it was found that thymectomy was associated with a substantial and prolonged improvement in myasthenic symptoms. Severity, as measured by MGFA grade fell progressively

throughout the follow up period. Steroid requirements, both in terms of the proportion of patients requiring steroids and the dose also fell from 6 months post thymectomy. There was a slight increase in the proportion of patients taking additional immunosuppressant agents such as azathioprine and mycophenolate but it was felt that it was unlikely that this would be sufficient to account for the substantial clinical improvement seen.

A significantly greater incidence of complete stable remission (CSR) was seen in patients who had a hyperplastic thymus gland on histology compared to other histological subtypes. Previous studies have also suggested that the presence of thymic hyperplasia correlated with improvement after thymectomy (Jaretzki et al., 1988; Lanska, 1990; Klein et al., 1999, Nicolaou et al., 1996). The benefit of thymectomy in patients with an atrophic thymus is less certain. There were few patients with an atrophic thymus in this study (n=8) but a significant proportion of these achieved either complete stable remission or pharmacological remission. It can be difficult to identify patients with an atrophic gland from radiological features alone; one of the patients with an atrophic gland in this study was thought to have an enlarged thymus on initial imaging. It was therefore interesting to assess the response to thymectomy in the small number of patients in our group who had an atrophic thymus gland. Atrophy of the thymus with an associated reduction in thymic size and activity is generally considered to be an age associated phenomenon (Chen et al., 2011). As the thymus ages, the thymic epithelial space begins to atrophy and the adipose tissue increases with thymic size and activity becoming greatly reduced. However atrophic thymus tissue has been shown to secrete immunoglobulins and AChR antibodies in vitro – particularly with MG duration of two years or less (Katzberg et al., 2001). Although thymic atrophy is

an age associated phenomenon, a significant difference was not detected in the age of the patients with an atrophic gland compared to those with a hyperplastic gland. There have been suggestions in the past that thymectomy of an atrophic gland can be associated with a good prognosis after thymectomy (Shahrizaila et al., 2005; Chen et al 2011). In one study the immune response, as determined by B cell activation, was similar in hyperplastic and atrophic thymuses and that the probability of remission was similar (Chen et al 2011). These data concur with these findings but the number of patients is too small to draw any firm conclusions.

In this cohort, patients with a thymoma did demonstrate improvement after thymectomy, but in general the response was less favourable compared to patients with nonthymomatous MG. This finding is consistent with previous studies (O'Riordan et al., 1998; Budde et al., 2001; Masaoka et al., 1996). Nevertheless thymectomy is still warranted in patients with a thymoma to reduce the risk of metastatic spread or local invasion

Somewhat surprisingly, a difference in outcome was not found in patients with a longer disease duration before thymectomy compared to those with a shorter disease duration. Previously it had been suggested that there was an increased likelihood of benefit from thymectomy when it is performed earlier in the disease course (Monden et al., 1984). However, there is a natural spontaneous remission rate in MG and this is not linear. MG patients in general are more likely to remit earlier in their disease course regardless of therapy. This study is in agreement of that of Budde et al. (Budde et al., 2001) who

found that thymic histology and age at operation were correlated to outcome but disease duration was not.

It is unknown whether MG classification has any bearing on outcome after thymectomy – it is generally thought that patients with mild – moderate generalized MG benefit most from thymectomy. The question of whether ocular MG responds to thymectomy is less certain with some series reporting that patients with purely ocular MG do benefit from thymectomy (Masaoka et al 1996) and other studies showing that ocular MG more resistant to surgery (Budde et al., 2001).

My data did suggest that the incidence of CSR increased with a longer duration of follow up. This did not reach statistical significance but a trend was observed. Twenty eight percent (12/43) of patients with a follow up duration of two years or less from thymectomy achieved CSR in comparison to 43% of patients who had a longer duration of follow up (p=0.06). The continued benefit of thymectomy many years after surgery has been described previously and a peak rate of remission has been seen up to 15 years after thymectomy (Chen et al., 2011; Tomulescu et al., 2011; Masaoka et al., 1996). The slope of the remission curve in thymectomised patients has been shown to increase suddenly between 5 and 6 years (Tomulescu et al 2006, 2011). The average follow up time in this study was comparatively short at 45.6 months. It is possible that, with continued follow up of our cohort, an increased rate of CSR may be demonstrated.

In the past, no significant association between CSR rate at 24 months and sex, age, preoperative use of steroids, histology (in non thymomatous MG) or need of post-

operative intubation has been demonstrated (Ambrogi et al., 2011). However, shorter duration of disease, oropharyngeal involvement, presence of germinal centres in native or ectopic thymus and presence of ectopic thymus were negative predictors (Ambrogi et al., 2011).

In general, thymectomy is a well-tolerated procedure and the 9% perioperative morbidity rate seen in this cohort corresponds well to previous studies (O'Riordan et al., 1998; Takanami et al., 2009). Apart from the immediate risks of any operation including infection, bleeding and risks from general anesthesia, one of the main risks of a thymectomy is damage to the phrenic, recurrent laryngeal and left vagus nerves. Damage to these structures can be devastating, particularly in MG when patients are already prone to bulbar weakness and respiratory failure (Jaretzki et al., 2003). However this was not seen in any of the patients in this study.

One of the patients in the cohort did develop a post-operative myasthenic crisis (MC). However, previous studies have noted a much higher proportion of patients developing MC after thymectomy; up to 19% in some groups (Takanami et al., 2009). The low rate of MC after thymectomy in this cohort was attributed to adequate immunosuppressive treatment before thymectomy and post-operative care in a neurointensive care facility. The main factor that has been found to significantly increase the risk of Myasthenic Crisis after thymectomy is a history of Myasthenic Crisis before thymectomy (Nam et al., 2011). Other factors associated with a post-operative MC include severity of disease, Body Mass Index (BMI) > 28 and duration of symptoms for > 2 years (Leuzzi et al., 2014).

Interestingly, the one patient in our cohort who developed MC was found to have anti-MuSK antibodies four years after thymectomy. This patient had treatment resistant MG, requiring ongoing nocturnal noninvasive ventilation and regular IVIG for many years. She subsequently improved after being commenced on mycophenolate. It was noted previously that patients with MuSK MG tend not to respond to thymectomy (Evoli et al., 2008; Ponseti et al., 2009).

It was found that a significant proportion of patients had an enlarged gland on preoperative mediastinal imaging. As would be expected, all but one of our patients with a thymoma had an enlarged thymus or had an obvious tumour on imaging. Just under half of the patients with a hyperplastic gland had evidence of an enlarged thymus and a significant proportion of those with normal thymic histology also had an enlarged gland. Previously, Pirronti et al. found that CT was 88.5% sensitive for thymoma and 36% sensitive for thymic hyperplasia (Pirronti et al., 2002). Thymic hyperplasia can have various different appearances on CT/MRI. Typically, up to 45% of cases may have normal radiological appearances; 35% are diffusely enlarged and up to 20% may appear as a focal thymic mass (Priola and Priola, 2014). This present study was not intended to review this in detail and the scans were not independently reviewed. However, from my small study it would seem reasonable to conclude that a completely normal CT/MRI of the mediastinum makes a thymoma unlikely.

In recent years, there has been much debate about the surgical technique used in thymectomy. If a thymoma is present, completeness of resection is one of the most

important prognostic factors (Murthy et al., 2009). This is also the case for nonthymomatous MG. In fact, the presence of ectopic thymic tissue has been considered one of the most significant predictors of outcome after thymectomy for MG (Ambrogi et al., 2011; Mori et al., 2007; Roxanis et al., 2002). Removal of as little as 2g of residual thymic tissue at reoperation has been shown to be therapeutic (Masaoka et al., 1982). Hyperplastic areas of thymic tissue are believed to be the site of antibody production and hence removal of as much thymic tissue as possible is considered to be the mainstay of a successful thymectomy (Jaretzki et al., 1998). It is for this reason that transcervical thymectomy is believed to be inferior with a reduced remission rate (Papatestas et al., 1987). The rationale for transsternal thymectomy is based on the thymus arising from several sites thymic tissue can be scattered throughout the anterior mediastinum and even in the retrothyroid space and that the thymus itself is known to consist of multiple lobules that are often separately encapsulated and not necessarily contiguous (Takanami et al., 2009; Jaretzki et al., 2003). In recent years, however, thorascopic thymectomy is increasingly used and has been shown to have a low morbidity and equivalent efficacy to open surgery (Tomulescu et al, 2011). Recovery seems to be faster, pulmonary function is less impaired and the length of post-operative hospitalization is shorter (Palace et al., 2001).

The vast majority of the patients in this study were acetylcholine receptor antibody positive. One patient who was initially thought to be seronegative tested positive to anti MuSK antibodies some years after her thymectomy. Interestingly this is the patient who remained most symptomatic suffering severe respiratory weakness after thymectomy. Conclusions cannot be drawn from a single case but the literature to date does not support the use of thymectomy in anti-MuSK patients (Ponseti, 2009). There

is controversy about whether AChR titres decrease after thymectomy with some reports showing that in the long term titres do not decrease after thymectomy (Tsinzerling et al., 2007) and other reports showing that AChR titres decreased soon after surgery and remained low in patients that showed a good response (Seto et al., 1993)

Apart from a number of cases with recurrent thymoma, none of our patients underwent reoperation for persistent myasthenic symptoms post thymectomy. It is recommended that reoperation should be considered in cases where the patient has severe or worsening myasthenic symptoms 3-5 years following a basic transcervial or standard transsternal thymectomy.

This research shows that transternal thymectomy is generally safe and well tolerated. It was associated with a good outcome in the majority of patients with a reduction in prednisolone dose and an improvement of myasthenic symptoms.

7 Recurrent thymoma – evidence for late recurrence

7.1 Background

Thymomas are the most common tumour of the mediastinum but are still relatively rare with an incidence of 0.15 per 100,000 person years (Engels and Pfeiffer, 2003), representing 0.2%-1.5% of all malignancies (Margaritora et al., 2010) and 20% of all mediastinal tumours in adults (Priola and Priola, 2014). Approximately 10-20% of patients with MG have a thymoma and in turn approximately 20-25% of patients with a thymoma have MG (Lucchi et al., 2009). Histopathologically, thymomas are composed of neoplastic thymic epithelial cells with varying proportions of non neoplastic lymphocytes and are classified and staged according to the WHO and Masaoka systems respectively (Okumura et al., 2002; Masaoka et al., 1981).

As thymomas are rare there is little randomised evidence to guide treatment but a consensus based approach has been used to develop treatment recommendations (Falkson et al., 2009). The choice of treatment depends on the Masaoka stage but surgery forms the cornerstone of treatment regimens. Radiotherapy is considered in the case of Stage III and above tumours and in high risk Stage II tumours (Benveniste et al., 2011). For bulky tumours, chemotherapy as well as radiotherapy may be considered (Falkson et al., 2009). Chemotherapy and radiotherapy are considered in medically and technically inoperable patients (Falkson et al., 2009).

The prognosis of thymoma depends on the tumour stage, WHO histology, completeness of removal and type of treatment (Urgesi et al., 1992; Wilkins et al., 1991, Strobel et al.,

2004). Thymomas are generally characterized by an indolent growth and the overall ten year survival after surgery is estimated at 85.7% (Sakamoto et al., 2011). Despite this good prognosis, recurrence of thymoma following surgical removal is not uncommon; between 7% and 30% of patients develop recurrence of thymoma (Ciccone and Rendina, 2005; Regnard et al., 1997). Recurrence occurs more frequently in stage III or IV thymomas and in epithelial rich histological subtypes (type 3 and 4) (Regnard et al., 1997) but can occur in any disease stage or histological subtype and has been rarely reported after removal of encapsulated thymoma (Regnard et al 1996; Masunaga et al., 1995). The origins of thymoma recurrences are likely to be either tumour remnants due to incomplete surgical resection or pleural seedings that were undetected at the initial operation or occurred due to surgical manipulation (Evoli et al., 1998). Most recurrences occur in the intrathoracic cavity rather than by haematogenous spread (Regnard et al., 1997)

7.2 Specific aims of this chapter

Myasthenic symptoms often remain following removal of a thymoma with over 80% of patients requiring continued immunosuppression during follow up (Evoli et al., 2002). These patients remain under the care of neurologists. In this study, my aim was to highlight the possibility of late thymoma recurrence in patients attending a neurology clinic.

7.3 Methods

A review of the patient records of the dedicated MG clinic in the NHNN was undertaken and cases of late thymoma recurrence were identified and case notes were reviewed. When possible the patients were also interviewed and examined in the clinic. “Late” was defined as recurrence occurring at least six years after the initial tumour resection. This cutoff was chosen based on a study that showed that recurrence was usually observed within a mean of 5 years after resection and our personal experience (Strobel et al., 2008).

7.4 Results

Five patients who attended the specialist MG clinic with a diagnosis of late recurrent thymoma were identified. Four were male. All had a thymoma that was resected by open thoracotomy and all attended the specialist MG clinic for follow up. The average age at onset at initial thymoma resection was 47.6 years (30-60) and all patients were strongly positive for antibodies against the acetylcholine receptor. Four of the patients had myasthenic symptoms at the time of diagnosis of the thymoma whereas the fifth patient developed MG symptoms post operatively.

7.3.1 Clinical Vignettes

Clinical vignettes for each patient are outlined below.

Patient 1: A 26 year old man presented with mild generalised myasthenic symptoms. A thymoma was diagnosed radiologically and he underwent a thymectomy. A WHO grade B1/B2 thymoma completely removed. He was treated with prednisolone and azathioprine and a surveillance CT scan performed five years after the initial thymectomy was normal. Twelve years post initial resection, a CT scan revealed a mediastinal mass. Repeat thoracotomy was undertaken which confirmed the presence of a B3 thymoma with pericardial and pleural metastases. He underwent post-operative radiotherapy. Four years after the repeat thymectomy he developed Morvan's Syndrome – symptoms of which initially responded to plasma exchange. However, he died two years later of metastatic thymoma.

Patient 2: A 46 year old man developed generalized MG and underwent a thymectomy. Histology revealed a stage II, type B2 thymoma, which was completely resected. He remained well for 11 years after surgery when routine surveillance imaging demonstrated thymoma recurrence. He underwent repeat thoracotomy and thymectomy, which once again revealed a B2 thymic tumour. He made a good post-operative recovery but over the following twelve months developed generalised weakness with foot drop and muscle cramps. Electrophysiological tests revealed a pattern consistent with anterior horn cell syndrome and he was diagnosed with amytrophic lateral sclerosis (ALS). He died from ALS related respiratory failure four years later. There was no evidence of thymoma recurrence at this time.

Patient 3: A 51 year old man was incidentally noted to have a large thymic mass on routine chest radiograph. An AB thymoma with clear margins and an intact capsule was excised. At the time of his operation he had no myasthenic symptoms but three years later he developed fatigable weakness. There was no evidence of recurrent thymoma at this time and he was treated with prednisolone. Six years after the initial thymectomy a CT scan demonstrated a diaphragmatic nodule. Histology confirmed an AB thymoma recurrence and he underwent repeat thoracotomy with postoperative radiotherapy. Regular surveillance for the past six years has not demonstrated any further recurrence.

Patient 4: A 40 year old man presented with ocular MG and imaging demonstrated a mass. He underwent thymectomy and histology showed a WHO B1 thymoma with incomplete resection (Masaoka Stage II). He underwent postoperative radiotherapy and remained well for fourteen years at which point he developed chest pain and dyspnoea. Imaging one year previously had been normal but CT at this stage showed a left superior mediastinal mass with pulmonary nodules PET scanning showed appearances consistent with malignancy. Both medastinoscopy and open biopsy were attempted but were unsuccessful. He was deemed not to be a surgical candidate and was treated with cisplatin and ifosfamide chemotherapy. He died eighteen months later.

Patient 5: A 43 year old woman presented with generalised MG and underwent thymectomy. A WHO Type A thymoma was completely excised and she was treated with prednisolone and mycophenolate. Fourteen years after thymectomy a routine CT

scan demonstrated recurrence of the tumour with extensive mediastinal metastases. Pleural biopsy revealed a B1 thymoma. She was not a candidate for surgery and was treated with carboplatin and paclitaxel chemotherapy. Despite this, her disease progressed and she died of malignant meningitis two years later.

The average time from initial resection to diagnosis of recurrence in this cohort was 12.3 years (6-16.25). All patients had ongoing MG symptoms after resection and all required immunosuppression. No patient had a relapse of myasthenic symptoms at the time of recurrence. Outcome was generally poor with three of five patients dying as a direct result of thymoma recurrence (Table 7.1).

Patient	1	2	3	4	5
Age at first presentation	26 (Male)	46 (Male)	51 (Male)	40 (Male)	43 (Female)
Presentation	Generalised MG	Generalised MG	Thymic mass found incidentally on routine CXR	Ocular MG	Generalised MG
Initial histology (WHO type)	B2	B2	AB	B3	A
Masaoka stage	I	II	I	IIB	I
Initial resection complete?	yes	yes	yes	no	yes
Adjuvant therapy	no	no	no	yes radiotherapy	no
Disease-free interval (yrs)	13	12	6	15	14
Repeat surgery	Repeat thoractomy	Repeat thoractomy	Repeat thoractomy, pedunculated nodule removed from diaphragm	no	no
Repeat histology (WHO type and Masaoka stage)	B3 IVA metastases to lung and pericardium	B2 II	AB III	(Mediastinal biopsy attempted but unsuccessful)	B1 IVA metastases to lung and pericardium
Adjuvant therapy	Post-operative radiotherapy	Post-operative radiotherapy	Post-operative radiotherapy	Cisplatin and isfosfamide chemotherapy	Carboplatin and pacitaxel chemotherapy Radiotherapy to spinal mass
AChR titre					
at diagnosis	33	166	-	-	185
At recurrence	Negative	197	221	237	269
Outcome	Died 6 years after diagnosis of recurrence	Developed sporadic ALS and died of ALS-related respiratory failure	No further thymoma recurrence	Died 18 months after diagnosis of recurrence	Died 2 years after diagnosis of recurrence

Table 7.1 Clinical characteristics of patients with late recurrent thymoma

7.5 Discussion

Although thymomas are rare tumours, patients who have had a thymoma resected are commonly seen for many years in neurology clinics. Therefore it is vital that neurologists are alert to the possibility of late thymoma recurrence. There have been some previous reports of late recurrence of thymoma in the literature with recurrences presenting as late as 19 or 20 years post initial thymectomy (Gotti et al., 1995; Lava et al., 1976; Evoli et al., 2002; Naniwa et al., 2002). However, this study is the first to look at a small group of these patients in depth.

I have described a series of five patients who developed a recurrent thymoma at a mean of 12.25 years post initial thymectomy. Recurrence of thymoma is a well-reported phenomenon with recurrence rates of 10-30% described in the literature (Regnard et al., 1997, Ruffini et al., 1997). Recurrence rates of 9% have been described when specifically assessing thymoma associated with MG (Evoli et al., 1998; Maggi et al., 2008). Recurrence risk is higher in patients with invasive disease compared to encapsulated thymomas (Ruffini et al., 1997) but this series has demonstrated that recurrence can occur in cases with low grade encapsulated tumours when initial resection of the tumour has been deemed to be complete.

It is known that completeness of resection is one of the main prognostic factors regarding thymoma recurrence. In cases of incomplete resection the use of post-operative radiotherapy in an attempt to reduce the risk of recurrence is well established

but there is no consensus about radiotherapy after a thymoma is completely resected (Ogawa et al., 2002). Currently radiotherapy is not recommended for patients with Stage I disease (Falkson et al., 2009). Recent recommendations suggest that patients with Stage II disease should have radiotherapy if they are considered to have factors indicative of a higher risk of recurrence including invasion through the capsule (Stage IIB), WHO Grade B tumours, if tumour is adherent to the pericardium or if resection is incomplete (Sakamoto et al., 2011). Only one of the patients in our cohort received adjuvant radiotherapy after initial surgery (Patient 3) as he was the only patient with an incomplete resection. A retrospective review has suggested that post-operative radiation can prevent mediastinal recurrence in patients with thymoma that has been completely resected (Ogawa et al., 2002). However, one of our patients had pleural based recurrence and it has been suggested that mediastinal radiation alone will not prevent this (Ogawa et al., 2002). Moreover, the use of adjuvant therapy such as radiotherapy has been shown to have no effect on prolonging the disease free interval in patients with invasive thymomas (Ruffini et al., 1997).

The prognosis of recurrent thymoma can be poor – three of the patients in this study died as a direct consequence of thymoma recurrence. Nevertheless, recurrent thymoma is more likely to be treatable if it is recognized earlier. There are currently no published guidelines regarding the frequency, duration or mode of radiological surveillance for thymoma recurrence (Regnard et al., 1997). CT is said to be equal or superior to MRI in the diagnosis of mediastinal tumours but concerns about the risk of repeated radiation exposure often make the latter the modality of choice, particularly when interval scans are required (Tomiyama et al., 2009). However, there is also a suggestion that PET-CT

is advantageous over CT in follow up after thymoma removal as CT changes may be subtle (Rosado-De-Christenson and Marom, 2008)

In this small case series I tried to ascertain any clinical features that could herald a thymoma recurrence. All the patients continued to have symptoms of generalised MG after thymectomy and required ongoing immunosuppression. This is in keeping with previous studies on thymomatous MG (Margaritora et al., 2010). However, none of the patients had an exacerbation of their myasthenic symptoms when recurrence was diagnosed. This lack of correlation between MG symptoms and tumour recurrence has been described previously (Margaritora et al., 2010). However, paraneoplastic syndromes other than MG have been reported to accompany thymoma and could herald a recurrence. Approximately 10-15% of patients with a thymoma can have a paraneoplastic syndrome other than MG (Evoli et al., 2007). One of the patients in our cohort developed Morvan's Syndrome with associated voltage gated potassium complex antibodies associated with the thymoma recurrence. Recently, acquired neuromyotonia with anti LGI1 and CASPR2 antibodies has been reported as the heralding symptom of recurrent thymoma in a patient with MG (Fleisher et al., 2013). Good's Syndrome (hypogammaglobinemia) has also been reported in association with late recurrence of thymoma (Naniwa et al., 2002).

As thymomas often occur locally, re- operation is advised if the recurrence is deemed to be resectable (Regnard et al., 1997; Maggi et al., 1991). The five and ten year survival rates after re-resection have been found to be between 51% - 71% and 43% respectively (Regnard et al., 1997; Maggi et al., 1991). The prognosis after recurrence of a thymoma

is dependent on total resection of the recurrent tumour and the presence of local recurrence rather than intrathoracic metastases (Ruffini et al., 1997). However optimal surgical treatment of recurrent thymoma may be difficult due to technical difficulties of second operation, possibility of involvement of intrathoracic organs, the possibility of intrathoracic dissemination of the recurrence the possibility of associated myasthenic symptoms (Ruffini et al., 1997). In general, surgery is recommended if possible but there are limited data comparing the results of radiotherapy for treatment of recurrence to surgery (Ruffini et al,. 1997). Total resection seems to offer the best chance of long-term survival but the difference between surgery and radiotherapy in treatment of recurrence has not been found to be significantly different in the past (Maggi et al., 1991; Urgesi et al., 1992). Only two of our patients had resectable disease at recurrence and underwent surgery. A third patient had a repeat thoracotomy but had metastatic disease and so this procedure was largely palliative. The two remaining patients were not candidates for surgery due to widely disseminated disease and were treated with radiotherapy and/or chemotherapy.

This small case series has demonstrated that recurrence of thymoma can occur many years after thymectomy. An important finding was that none of our patients had a flare of their myasthenic symptoms around the time of their tumour recurrence. Prognosis is likely to better if the tumour is diagnosed earlier and so some form of screening is necessary. There are no guidelines regarding this but the practice in our institution is yearly or two yearly imaging with CT or MRI, the latter increasingly becoming the imaging modality of choice due to concerns about radiation. I did not find that AChR antibody levels were appreciably correlated with recurrence although I did not have the full data regarding this for all patients. There has been a search for other serum markers

of thymoma recurrence. Anti titin antibodies are associated with the presence of a thymoma but are also found in non –thymomatous older patients with MG and so are only useful in patients less than 60 (Buckley et al., 2001). In contrast, serum antibodies against cytokines, interleukin alpha and interleukin -12, have been found to be useful in identifying patients with tumour recurrence, especially if imaging is equivocal (Buckely et al., 2001). Anti titin antibodies were found to be elevated in patients with an underlying thymoma even in the context of well controlled MG and immunosuppression. I did not measure these antibodies in our patients but it would be possibly useful in the future.

8 Severe exacerbations of Myasthenia Gravis- management and long-term outcome

8.1 Background

Myasthenic crisis (MC) is one of the most serious complications of MG and occurs on average in 10-15% of myasthenic patients during the course of their disease (Thomas et al., 1997; Berrouschot et al., 1997; Sellmann and Mayer, 1985). A population based study that followed myasthenic patients for an average of 12 years suggested an annual myasthenic crisis incidence of 2.5% (Berrouschot et al., 1997). The incidence does not seem to have changed appreciably over the past four or five decades despite improvements in the immunotherapy of MG (Cohen and Younger, 1981; Ronager et al., 2001). Myasthenic Crisis is traditionally defined as “weakness from acquired myasthenia gravis that is severe enough to necessitate intubation for ventilatory support or airway protection” (Jani-Acsadi et al., 2007; Bedlack and Sanders, 2002). Usually if extubation after a general anaesthetic is delayed for more than 24 hours after surgery, this is also defined as a myasthenic crisis (Bedlack and Sanders, 2002). As the decision regarding whether a patient requires intubation can be quite subjective and individual, this is not a rigid definition and so it can be difficult to compare the treatment and outcome of myasthenic crisis from different studies.

Respiratory insufficiency in MG patients develops as a result of worsening respiratory muscle weakness due to neuromuscular dysfunction-it can be exacerbated or complicated by respiratory tract infection or aspiration pneumonia secondary to associated bulbar dysfunction

Fifty years ago the mortality of MC was typically between 50 and 80% (Rowland et al., 1958) but with improvements in the medical management of these patients, particularly the improvement in respiratory care in the Intensive Care Unit (ICU), mortality is now reported to be less than 5% (Jani-Acsadi et al., 2007; Cohen and Younger, 1981; Alshekhlee et al., 2009). MC typically develops in the first two years after diagnosis of MG (Bedlack and Sanders 2002). On average each episode of MC requires a hospital admission of over a month, more than half of which is spent on the intensive care unit (Thomas et al., 1997).

Patients may also develop acute exacerbations of MG that do not require mechanical intubation and hence do not meet the criteria for a diagnosis of MC but nevertheless require close observation and immediate access to resuscitation facilities, often in an Intensive Care Unit. As MG is increasingly recognised in older patients the proportion of elderly patients with severe exacerbations of MG that are admitted to the ICU has increased.

Little is known about the long-term outcome after an acute severe exacerbation of MG, particularly in the older MG cohort.

8.2 Specific aims of this chapter

The objective was to describe the current management and long term prognosis of severe exacerbations of MG in patients with early and late onset disease. As our institution has a large cohort of myasthenic patients and a dedicated neuro-medical ICU

(N-ICU) it was decided to conduct a retrospective review of all patients admitted with an acute severe exacerbation of MG over an extended period.

I wished to examine the management of acute exacerbations of MG in a N-ICU and to examine both short and long term outcomes in patients with early and late onset MG.

8.3 Methods

All patients admitted to the N-ICU are recorded in the ICU admission log book. All patients admitted to the N-ICU with acute exacerbations of MG between January 1999 and January 2011 were identified from this source and the case notes were retrospectively reviewed.

The severity of MG was classified according to the Myasthenia Gravis Foundation of America (MGFA) clinical grading system (Jaretzki et al., 2000) (see fig 6.1).

Patients were included in the study if they were admitted to the N-ICU because of acutely developing fatigable muscle weakness with severely impaired bulbar or respiratory function (MGFA IVb or V). Patients that were admitted to the N-ICU electively post thymectomy or for routine plasma exchange were excluded.

SPSS (Chicago, IL, USA version 20) was used for statistical analysis. Categorical data were analysed by Fisher's exact test and continuous data were analysed using Student's *t*-test.

8.4 Results

8.4.1 Patient characteristics

Thirty-eight patients (23 female, 15 male) were admitted to the N-ICU with acute exacerbations of MG during the study period. The average age at admission was 57 years (range 19-81 years) and the average age at onset of MG was 53 years (range 17-81 years). Thirty-one patients (82%) were acetylcholine receptor (AChR) antibody positive, four (11%) had antibodies to Muscle Specific Tyrosine Kinase (MuSK) and three patients (8%) were seronegative on routine antibody assay. Twenty-nine (76%) of the admissions were transferred from other hospitals, often from other ITUs.

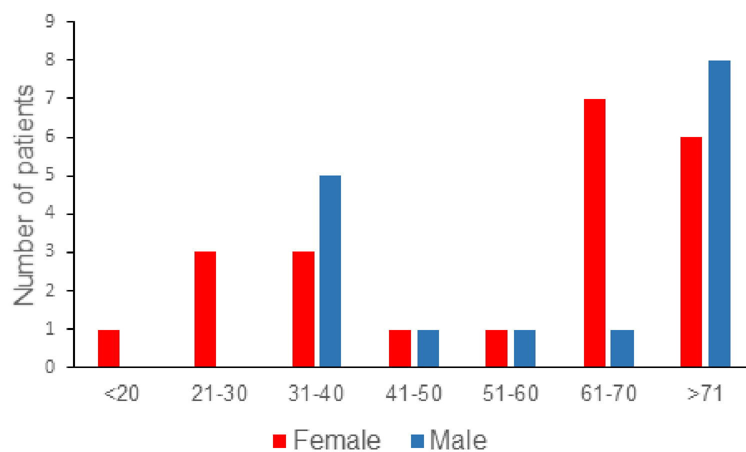


Figure 8.1 Age of patients at time of ITU admission

Twenty four patients (63%) were over the age of 50 years on ICU admission and 11 of these (46%) had no previous diagnosis of MG; the diagnosis was newly made on admission to N-ICU. The average duration of MG in those with an established diagnosis was 4 years (0.5-9 years). In the 14 patients who were under the age of 50 on ICU admission, three (21%) were new presentations of MG. In the 11 patients (29%) with an established diagnosis, the average duration of MG was the same as in the older age group 4 years (0.5-9 years).

8.4.2 MG treatment prior to ICU admission

Sixty three percent (15/24) of those with known MG were treated with prednisolone prior to the acute exacerbation of MG. This figure was similar in the younger and older age groups (7/11 and 8/13 respectively). Younger patients with an established diagnosis of MG were more likely to have been treated with a steroid sparing agent such as azathioprine than the older age group (4/11; 36% compared to 1/13, 8%) although this figure did not reach statistical significance ($p=0.11$). In total, three patients had also received IVIG on at least one occasion prior to ICU admission.

Seven patients had undergone thymectomy at some stage prior to the ICU admission, 4/11 were in the younger age group and 3/13 were in the older age group. All of the older cohort who had undergone thymectomy had a thymoma. One of the younger cohort had a thymoma but the remainder had a hyperplastic gland. The difference between older and younger age groups with regards to thymectomy did not reach statistical significance ($p = 0.28$)

8.4.3 Triggering factors for acute exacerbation

A factor that precipitated the acute exacerbation was identified in 22 patients (58%). One patient deteriorated during high dose steroid treatment. Another patient developed post-operative myasthenic crisis after thymectomy (MCAT). Sepsis was the main precipitating factor identified and was seen in 20 patients (53%). However no factor that precipitated the acute exacerbation of MG was identified in the remaining 16 patients (42%) and the deterioration appeared to be spontaneous.

8.4.4 Treatment in ICU

The mean duration of the ICU stay for the group as a whole was 18.7 days (1-111 SD 21.7) and the mean total hospital stay was 38.9 days (4-157; SD 31.1). Those with a new diagnosis of MG had a trend towards a longer hospital stay than those with an established diagnosis although this did not reach statistical significance (p=0.17). There was no significant difference in ICU stay length in the older group compared to the younger cohort (p=0.42).

The FVC was monitored in all patients – typically tracheal intubation was performed in the FVC fell below 15ml/kg, if there was severe bulbar weakness or if there were indications that the patient was tiring. Intubation and mechanical ventilation were required in 24 patients in total (63%) and subsequent tracheostomy was required in 18 cases (47%). The mean duration of ventilation was 28 days (6-102) with a median of 14 days. Seventy one per cent (17/24) of those requiring intubation had a lower respiratory

tract infection compared to 14% (2/14) who did not require intubation ($p < 0.01$). Four patients did not require intubation and mechanical intubation but did receive non-invasive ventilation as bi-level positive airways pressure (BiPAP). There were no significant differences between the two age groups with regard to the need for mechanical ventilation ($p=0.56$).

The vast majority of patients 37/38 (97%) were treated with oral prednisolone with an average dose of 50mg (range 20-80mg) and 87% (33/38) received intravenous immunoglobulin (IVIG). Seven patients underwent plasma exchange as they had ongoing myasthenic weakness. Additional immunosuppression was initiated in the N-ICU in 17 patients (45%). There were no significant differences in the management of the acute exacerbation of MG in the younger age group compared to the older age group. When intubation was required, pyridostigmine was generally discontinued.

Clinical Details	Young onset MG (<50 yrs)	Older onset MG (>50 yrs)
Number Male Female	14 5 9	24 10 14
New Diagnosis	3 (21%)	11(46%)
Established diagnosis Average disease duration Treatment prior to ICU admission Prednisolone Azathioprine IVIG Plasma exchange	11(29%) 3.5 yrs (0.5-14 yrs) 7(64%) 4(36%) 2(18%) 2(18%)	13(54%) 4 yrs (0.5-9 yrs) 8(62%) 1(8%) 1(8%) -
Antibody Status AChR MuSK Seronegative	 11(79%) 3(21%) -	 20(83%) 1(4%) 3(13%)
Mean duration of ICU admission (days)(SD) (median)	22.4 (19.8) (14.5)	16.7 (SD 22.9) (9)
Mean Total Stay (days) (SD) (median)	42.1(28.7) (42.5)	37.1(32.9) (26.5)
Intubation	9(64%)	15 (63%)
Thymectomy Before ICU admission After ICU admission No thymectomy Thymoma	 4(29%) 5(36%) 5(36%) 4(29%)	 3(13%) 1(4%) 20(83%) 4(17%)
Treatment in ICU Intubation Prednisolone IVIG Plasma exchange Other immunosuppressant agent Azathioprine Mycophenolate Rituximab Cyclophosphamide	 9(64%) 114(100%) 13(93%) 3 (4%) 6(43%) 2(14%) - 1	 15(63%) 23(96%) 20(83%) 4(17%) 8(33%) 2(8%) 1 0

Table 8.1 Patient characteristics at time of ICU admission

8.4.4 Mortality in ICU

In total three patients died in ICU. One was a male, aged 74 with multiple comorbidities including a previous stroke, epilepsy and gastric malignancy. He required intubation and ventilation for MC complicated by pneumonia and cardiac failure. He died of sepsis in association with respiratory failure after 30 days in ICU.

The second patient was a 37 year old female with a two year history of seropostitive MG. She had brittle MG requiring azathioprine, prednisolone, plasma exchange and IVIG in the twelve months prior to ICU admission. Despite intubation and mechanical ventilation and treatment with prednisolone, IVIG, plasma exchange and antibiotics for respiratory sepsis, she died 21 days after admission to ITU.

The third patient was a 23 year old female who presented in myasthenic crisis and was found to have a thymoma with positive AChR antibodies. She required intubation for acute respiratory distress and underwent a thymectomy, which revealed a necrotic B2 thymoma. Her illness was complicated by sepsis, gastroparesis, progressive weakness and severe autonomic instability. She was treated with IV methylprednisolone, IVIG, plasma exchange and cyclophosphamide but died 100 days after admission to ICU.

8.4.5 Outcome after discharge from ICU

One patient died after discharge from ICU. He was 40 years old and had a five year history of thymomatous MG for which he had declined a thymectomy. He was intubated and ventilated for four weeks and was treated with prednisolone, IVIG and subsequently plasma exchange. His admission was complicated by Morvan's Syndrome. He was weaned from ventilation successfully and was discharged from ICU. However, he developed respiratory arrest two days after discharge and died due to a pulmonary embolism.

The majority of patients (29/36; 81%) had mild –moderate myasthenic symptoms on discharge from ICU (MGFA Grades I II III). Five patients had severe myasthenic weakness (MGFA Grade 4) and one patient had a tracheostomy in situ for respiratory support (MGFA V).

Seven patients were lost to follow up. One patient died two years after ICU discharge of acute respiratory failure. She had brittle MuSK positive MG and had required frequent IVIG and plasma exchange as well as non-invasive ventilation after ICU discharge. There was follow up data available for the remaining 26 patients for a mean of 4 years (range 6 months -12 years).

Six patients underwent thymectomy after ICU discharge. Five of these were in the younger age group – three had a thymoma and two had a hyperplastic gland. The one patient in the older cohort who underwent thymectomy after ICU discharge was found to have a thymoma. Generally, thymectomy was not performed directly after ICU discharge. Patients were treated with immunosuppressive agents until the MG symptoms were under control and were then subsequently readmitted for thymectomy. An exception was made for a young female patient with severe myasthenic symptoms and a B2 thymoma who presented in myasthenic crisis and had severe autonomic instability.

In general, there was a continued improvement in MGFA status during follow up in both age groups. At the last clinical review which was at a mean of four years after ICU

discharge, 19% (6/31) of patients for whom follow up was available were asymptomatic and 48% (15/31) had either purely ocular symptoms or mild generalised disease.

Clinical Severity (MGFA)	Number of patients ICU discharge		Number of patients Last clinical review (mean time from ICU discharge)	
Age (yrs)	< 50 (n=14)	>50(n=24)	< 50 (n=14) 6.3 yrs	>50 (n=24) 2.7 yrs
Asymptomatic	-	-	2	4
I	1	2	1	1
II	2	14	5	8
III	6	4	1	4
IV	3	2	-	-
V	-	1	-	-
Died	2	1	4	1
Lost to follow up	-	-	1	6

Table 8.2 Clinical outcome after ICU discharge

Sixty five per cent of patients (20/31) remained on oral prednisolone at an average dose of 12.5 mg at last clinical review. Forty eight per cent took additional oral immunosuppression (8/31 on azathioprine, 6/31 on mycophenolate mofetil and 1/31 on methotrexate). Three patients (10%) required IVIG.

Treatment	Number of patients Last clinical review (Mean time from ICU discharge)	
Age (yrs.)	< 50 (n=14) 6.3 yrs.	>50 (n=24) 2.7 yrs.
No immunosuppressive treatment	1	2
Prednisolone Average dose	6 10mg	14 15mg
Azathioprine Mycophenolate Methotrexate Rituximab Regular IVIG	2 4 1 2 2	6 2 1 1
Lost to follow up	1	6
Died	4	1

Table 8.3 Treatment at last clinical review

8.5 Discussion

This study is limited by its small size and retrospective nature. However, it is clear that acute severe exacerbations of MG continue to be associated with significant morbidity and mortality. The mortality rate in ICU in our study was 8% which is higher than that quoted in some previous studies (Thomas et al., 1997; Cohen and Younger, 1981). Our centre is a specialist referral centre and over 70% of the admissions to ICU in our cohort were transfers from different centres. It is possible that our ICU is biased towards admission of more severe cases of MG, which could explain the high mortality rate in our group. However it is clear that with modern intensive care treatment of acute exacerbations of MG the mortality rate has fallen significantly from a rate of approximately 80% in the 1940s (Osserman et al., 1963).

This study has highlighted the need to be vigilant for MG as a differential diagnosis of acute respiratory failure, particularly in older adults. MG is increasingly recognised in the older population- 60% of our cohort were over the age of 50 and MG was newly diagnosed in 40% of these patients. There were no significant differences in short-term outcome between older and younger patients and the long-term prognosis after discharge from ICU was good in both groups.

Predicting the onset of an acute exacerbation of MG can be difficult. There was no obvious precipitant of myasthenic crisis in 42% of our patients. In those patients in whom a precipitant was identified, infection, mainly respiratory sepsis was the most common. Respiratory sepsis was also an overwhelming risk factor for intubation, a factor that has been noted previously (Cohen and Younger, 1981; Ferguson et al., 1982). Infection is a well-known and common cause for acute exacerbations of MG (Kalita et al., 2014; Thomas et al., 1997, Aggarwal et al., 2002). Other precipitating factors noted in the past include inadequate treatment, use of offending drugs and surgery (Bedlack et al., 2002; Kalita et al., 2014) but these factors were not observed in the majority of our patients.

It has been suggested in the past that thymoma may be a risk factor for myasthenic crisis as there is a relatively well-accepted association between thymoma and a more severe myasthenic phenotype (Thomas et al., 1997; Grob et al., 1987). A thymoma was present in 21% of the patients – approximately 10-20% of patients with MG have a thymoma (Lucchi et al., 2009) and so patients with a thymoma were not significantly over represented in our cohort. Previous studies of myasthenic crisis have found higher proportions of patient with thymoma, up to 32% (Thomas et al., 1997).

This study attempted to define some of the factors that are associated with a poorer prognosis in acute exacerbations of MG. As well as being a factor associated with intubation, sepsis, particularly respiratory sepsis was also implicated in the deaths in the three patients who died on ICU. Another comorbidity was pulmonary embolism, which was implicated in the death of the patient who died after discharge from ICU. Similar comorbidities in myasthenic crisis have been reported previously but none were independent predictors of death (Alshekhlee et al. 2009).

In the past intubation but not non-invasive ventilation has been found to be an independent risk factor for mortality (Alshekhlee et al., 2009). Advanced age has been reported to be an independent risk factor for mortality in MG crisis (Alshekhelee et al., 2009). However our data show that older MG patients have a similar prognosis to younger patients in ICU and also in the longer term.

The management of myasthenic crisis has changed considerably in recent years. The mortality of MC is now generally considered to be less than 5% due, in a large part to improvements in the respiratory care of patients. Intubation and mechanical ventilation were required in 63% of our patients. The average duration of intubation was 28 days. An additional four patients were treated with BiPAP. Non-invasive ventilation is increasingly used and has been shown to help reduce the need for intubation in patients with myasthenic crisis (Rabinstein and Wijdicks, 2002). Patients were monitored clinically regarding the need for intubation. Forced vital capacity was routinely measured on the ITU with the aim to perform intubation before significant hypoxia

developed. The average duration of intubation in our patients was longer (on average) than that described in previous studies. It has been described previously that 25% of patients with myasthenic crisis will be extubated after one week, 50% by two weeks and 75% by one month (Thomas et al., 1997). The mean duration of ventilation in our cohort was skewed somewhat by two outliers. Analysing the data in more depth revealed that 30% (7/24) of patients were extubated within one week. As mentioned before, the majority of the patients in our cohort were transferred from other hospitals, suggesting that they may be more severely affected or more treatment resistant than other patients. Intubation for more than three weeks has been associated with a threefold increase in hospital stay and a two-fold increase in functional dependence at discharge (Thomas et al., 1997). Twenty five per cent (6/24) of our patients required mechanical ventilation for longer than three weeks.

IVIG is increasingly used in the management of myasthenic crisis. The vast majority (87%) of our cohort received IVIG. Ten years ago only 11.4% of a similar cohort in our institution received IVIG whereas 60% underwent plasma exchange (O'Riordan et al., 1998a). IVIG and plasma exchange have recently been found to be equally effective for exacerbations of MG but the former is better tolerated and is used increasingly (Barth et al., 2011) and has a lower risk of adverse events such as hypotension, coagulopathy and sepsis (Qureschi et al., 1999).

Little is known about the long-term outcome of patients with acute severe exacerbations of MG following hospital discharge. I have follow-up data for 82% of our patients for a mean of four years and have found that that the vast majority of our patients were

asymptomatic or had mild manifestations of MG at final review. In particular, there were no significant differences in long-term outcome between the older and younger patients. In the past, even if patients survived the initial acute exacerbation, the overall outlook was thought to be poor with up to 30% of patients having repeated myasthenic crisis and almost half of patients continuing to demonstrate loss of muscle strength or becoming functionally dependant (Thomas et al., 1997; Richman and Agius, 2003). Poorer outcomes have been associated with long periods of mechanical ventilation, particularly in older patients (Thomas et al, 1997). The outcomes that are described here are a lot more positive than this and indeed other authors have also described a good long-term outcome in myasthenic patients who survive the initial crisis. Cohen and Younger found that out of 25 patients who survived a myasthenic crisis and were followed up, 32% (8 patients) achieved remission, 32% (8 patients) improved and 24% (6) were "stable"(Cohen and Younger, 1981). Three of our patients had repeated admissions to ITU with severe exacerbations of MG, representing 8% of our total cohort. This is a much smaller proportion than was described in previous studies where 33-43% of patients had recurrent myasthenic crises (Thomas et al., 1997; Berrouschot et al., 1997).

This study suggest that despite the acute risk of mortality associated with severe exacerbations of MG, with intensive treatment, the long- term outcome is favourable. A matched nested case control study from hospital admissions of MG patients has previously shown that patients with MG are less likely to die if they are admitted under the care of a neurologist (Hill and Ben-Schlomo, 2007). Prolonged treatment in a specialised neuro-intensive care environment may be required but even patients who were ill for many weeks on ITU can have a favourable prognosis in the long term.

9 Final Conclusions

In this thesis I set out to tackle a number of unresolved issues regarding the pathomechanisms involved in LEMS and the VGCC specificity of LEMS antibodies. I also decided to take advantage of the large cohort of patients with MG that attend the NHNN to address unanswered questions with regard to clinical aspects of MG including outcome after thymectomy and long term prognosis after a severe exacerbation of MG.

9.1. Immunohistochemistry experiments using LEMS IgG

Initially with regard to LEMS, my first aim was to visualise the binding of antibodies from LEMS antibodies to cell surface antigens on cultured neuronal cells using high-resolution microscopy. Antibodies against LEMS are currently detected by radioimmunopreciptiation assay but the use of cell based assays to detect IgG binding to cell surface antigens in a variety of other autoimmune diseases including Myasthenia Gravis and diseases associated with antibodies to the VGKC complex has enhanced our knowledge about antibody specificity in these conditions.

Hitherto it has been difficult to visualize binding of LEMS IgG to cell surface antigens because of the low density of VGCCs at the cell surface and also because one of the mechanisms of action of LEMS IgG is the cross linking and internalization of these receptors.

In my experiments I mainly used non-permeabilised hippocampal cultures that were incubated with LEMS IgG or control IgG and used anti human IgG Alexa 594 as a secondary antibody. I was able to detect IgG binding to cell surface antigens in all

seropositive LEMS samples tested but non-specific cell surface binding was seen with control IgG. Further experiments attempted to co-localise binding of LEMS IgG to synapses/VGCCs using GFP-tagged VGCC and co-staining with synapsin. Unfortunately I was not able to reliably detect co-localisation of LEMS IgG to synapses in this way. I was also unable to specifically detect binding of one LEMS IgG sample to P/Q-channels using neurons cultured from *Cacna1a* KO mice and their WT littermates. It is possible that the staining we saw was due to the individual patients having additional antibodies in their serum. Patients with SCLC may have antibodies to a variety of antigens as well as VGCCs. Antibodies to GABA(B) receptors have been described in patients with SCLC associated with limbic encephalitis (Boronat et al., 2011). A case of VGCC antibody positive LEMS in association with antibodies against the VGKC complex has also been described (Kalra et al., 2014). It is possible that I was detecting antibodies that are specific for antigens other than VGCCs with this assay.

Further studies using LEMS IgG from a large number of patients are required to determine whether cell surface binding is specific for VGCCs. If these further studies were to be successful it is likely that a cell based assay for LEMS could be developed. This would be extremely beneficial, as not only would it reduce the need for radioactive immunopreciptiation techniques, but it would also allow us to detect the exact antigenic target(s) of LEMS antibodies.

As my efforts to reliably detect specific binding of IgG from LEMS patients to cell surface antigens were unsuccessful, I decided to focus on functional effects of LEMS antibodies.

9.2 Functional effects of LEMS IgG on synaptic vesicle release

9.2. 1 LEMS IgG significantly reduces the rate of action potential evoked synaptic vesicle release

It has been known for some time that LEMS antibodies reduce the amplitude of postsynaptic responses (EPP) at the neuromuscular junction and the current through VGCCs in a wide variety of cell types. It has therefore been postulated that muscle weakness in LEMS results from a decrease in presynaptic neurotransmitter release due to a reduction in Ca^{2+} entry in response to an action potential but the evidence for this to date has been indirect. I tested the effect of LEMS IgG on synaptic vesicle release in hippocampal neuronal cultures using FM dyes.

My fluorescent imaging data show definitively that LEMS IgG significantly decreases AP evoked synaptic vesicle release in rat hippocampal neuronal cultures. A similar effect was seen on spontaneous neurotransmitter release although my experiments were not specifically designed to measure this. All four samples that I tested caused a reduction in spontaneous and evoked transmitter release.

I was not able to correlate the magnitude of the effect in each individual sample to antibody titre. Further experiments with a large number of LEMS samples with different titres would be required to clarify this. I was also unable to demonstrate a

significant effect of “seronegative” LEMS sample on neurotransmitter release. However I only performed a limited number of experiments with this sample and it is difficult to draw firm conclusions regarding the pathomechanism of “seronegative” LEMS.

Nevertheless, my data provide definitive evidence regarding the pathomechanism of antibodies in LEMS. To my knowledge, FM dyes have not been used to investigate the action of LEMS IgG in the past. My successful use of this technique has provided a new tool for studies of the functional effects of LEMS antibodies in the future.

9.2.2 The action of LEMS IgG is specific for P/Q- VGCC

Although it has been known for some time that LEMS antibodies decrease voltage dependent Ca^{2+} entry into cells, it has been less certain which specific Ca^{2+} channel subtypes are affected. There have been conflicting studies in the literature regarding this and in particular it has been uncertain whether LEMS IgG affects N-type VGCCs. This is particularly relevant as up to 33% of patients with LEMS have antibodies against N-type channels. The available literature also does not rule out the hypothesis that different antibodies affect P/Q-type VGCCs and neurotransmitter release.

I studied the effect of a LEMS sample that had antibodies against both P/Q-and N-type VGCCs on synaptic vesicle release in neuronal cultures from a *Cacna1a* knockout mouse that lacks P/Q-type Ca^{2+} channels. LEMS IgG significantly reduced action potential evoked neurotransmitter release in WT synapses but had no significant effect in KO samples, implying that the effect of LEMS IgG is specific for P/Q type channels. To my knowledge, this was the first time that LEMS IgG was tested in synapses lacking P/Q-type channels.

The LEMS sample I used had antibodies detected against both P/Q- and N- type VGCCs on radioimmunoprecipitation assay so if there were an effect on N-channels I would expect to see it in these experiments. However, further experiments with LEMS samples from different patients are required to corroborate our findings.

9.2.3 Limitations of our experiments

There are a number of limitations to my experiments that must be considered when interpreting the data. I used a model system of central synapses derived from hippocampal neuronal cultures. Ideally, I would have chosen to test the effect of LEMS IgG on synaptic transmission at the neuromuscular junction. There were a number of practical reasons why this was not feasible. There are conflicting data as to whether LEMS IgG can exert its effect acutely when applied acutely to neuromuscular junction preparations. In general, when NMJ preparations are used, IgG is passively transferred rather than being acutely applied. This option was not available to me as I used *Cacna1a* KO mice. These mice become progressively ataxic from postnatal day 10 and thus would not survive long enough to allow us to passively transfer the IgG. However, hippocampal neuronal cultures have been validated previously in experiments using FM dye (Ermolyuk et al., 2013) and furthermore, the degree of contribution of hippocampal P/Q-type VGCCs to synaptic vesicle release is similar to that at the neuromuscular junction (Cao and Tsien, 2010; Protti et al., 1996; Uchitel et al., 1992).

Another potential weakness of my study was the limited number of IgG samples used. Ideally a large number of LEMS samples would be used to corroborate our findings.

However LEMS is a rare disease and thus the sera available was limited. However, I saw consistent results with all four LEMS samples we tested. Nevertheless, repeating the above experiments with a larger number of samples is a next step for this project.

9.3 Long-term prognosis in MG

9.3.1 Trans-sternal thymectomy is associated with a good prognosis in the majority of patients with MG

Thymectomy has been used since the 1930s for the treatment of MG. It is always indicated when a patient has a thymoma because of the risk of local invasion and metastatic spread but its use in non-thymomatous MG, although widespread, remains controversial. Persistent positive associations have been found in retrospective non-controlled studies between thymectomy and improvement of myasthenic symptoms. A randomised controlled trial comparing thymectomy combined with prednisolone therapy to treatment with prednisolone alone is currently underway and was designed to answer three questions (Newsom –Davis et al. 2008):

(1) Whether thymectomy results in a greater improvement in myasthenic weakness;

(2) Whether thymectomy allows a lower dose of prednisolone and

(3) Whether thymectomy enhances quality of life by reducing adverse events associated with therapies.

This trial is currently closed to recruitment and results are expected in the next few years. However, as the NHNN has a large cohort of patients with MG, I decided to perform a follow up study on all patients who had undergone thymectomy over the

previous twelve years. Eighty-nine patients were identified, of which 24% had a thymoma, 48% had a hyperplastic gland and 19% and 8% had normal and atrophic thymic histology respectively. I was interested firstly, whether trans-steranl thymectomy was well tolerated and I found that it in general it was a safe and well tolerated procedure. Early complications were rare and were not associated with long-term morbidity with the exception of one patient who developed a post-operative myasthenic crisis. My main focus of interest, however, was whether thymectomy was associated with an improvement in myasthenic symptoms and a decreased steroid requirement in the long term. I found that 34% and 33% of patients achieved CSR and PR respectively at last clinical review, which was a mean of 3.8 years post thymectomy. Patients with a hyperplastic gland had a significantly higher chance of achieving CSR compared to other histological subtypes. There was a trend towards the incidence of CSR increasing with time after thymectomy. These data are in line with previous studies (Jaretzki et al., 1988; Klein et al., 1999, Chen et al., 2011; Tomulescu et al., 2011; Masaoka et al., 1996). Prednisolone doses progressively decreased during follow up and although the use of immunosuppressants such as azathioprine and mycophenolate mofetil increased, I felt that this was unlikely to account for the clinical improvement seen.

My study is limited by its retrospective nature and the relatively small number of patients. However, whilst the results of the randomised controlled trial are awaited, my data add to the previous studies regarding the effectiveness of thymectomy in MG and suggest that thymectomy is a safe well tolerated procedure that can result in a good long –term outcome in MG – particularly in those patient who have a hyperplastic gland detected on imaging.

9.3.2 Recurrence of thymoma can occur many years after initial surgery

Thymomas are rare tumours but approximately 15% of patients with MG have a thymoma and conversely 20-25% of patients with a thymoma have MG (Lucchi et al., 2009). Myasthenic symptoms often remain after thymectomy and patients remain under the care of neurologists. My aim in this chapter was to highlight the risk of late recurrent thymoma as it is a rarely reported but important phenomenon. I described six patients who had recurrence of a previously resected thymoma between six and fifteen years after their original surgery. Initial resection was complete in four of these patients and the remaining patient had received post-operative radiotherapy. I demonstrated that prognosis was poor after thymoma recurrence, three of our patients died as a direct recurrence of recurrence of their tumour. There are currently no published guidelines regarding the frequency, duration or mode of radiological surveillance for thymoma recurrence (Regnard et al., 1997). I did not demonstrate any reliable clinical clues that could alert the clinician to the possibility of recurrence; none of our patients had an exacerbation of myasthenic symptoms at the time of their recurrence and I did not find that AChR antibody levels were appreciably correlated with recurrence although we did not have the full data regarding this for all patients. There are data suggesting various other antibodies including antibodies against cytokines, interleukin alpha, interleukin - 12 and anti titin antibodies that may be useful in identifying patients with tumour recurrence, especially if imaging is equivocal (Buckely et al., 2001). I could not measure these antibodies in the patients in my study as it was retrospective but it would be useful in the future to validate the use of these antibodies in identifying patients at risk of thymoma recurrence. In the meantime I have suggested that post-operative

radiological surveillance after thymectomy is necessary and should continue indefinitely.

9.3.3 Severe exacerbations of MG are associated with a good prognosis in the majority of patients

The most serious complication of MG is MC- defined as respiratory failure due to myasthenic weakness – approximately 10-15% of patients with MG will develop this complication during the course of their disease (Thomas et al., 1997; Berrouschot et al., 1997; Sellmann and Mayer, 1985). Fifty years ago the mortality of MC was between 50 and 80% (Rowland et al., 1956) with advances in immunomodulatory treatments and in particular, improvements in ventilatory care, mortality has now improved to approximately 5% (Jani-Acsadi et al, 2007; Cohen and Younger 1981; Alshekhlee et al 2009). MG is increasingly recognised in older adults and hence, the proportion of older patients admitted to ICU with severe exacerbations of MG is set to rise. Little is known about the long- term prognosis of MG after discharge from ITU, particularly in older patients.

My aim was to examine the current management of severe exacerbations of MG in the ICU and, in particular to study the long-term prognosis in older and younger patients.

I identified 38 patients, 63% of whom were over the age of 50 at admission to ICU – 46% of this group were newly diagnosed with MG during the admission to ICU. Thus, my study highlighted the need to be aware of MG in the differential diagnosis of respiratory failure especially in older patients. The mortality rate in my study was relatively high compared to other studies at 8%- this is likely due to the fact that our

centre is biased in favour of seeing patients with more severe exacerbations of MG as over 70% of patients were transferred from other hospitals, often from other ICUs. In 42% of our patients there was no obvious identifiable reason for the acute deterioration in symptoms of MG- however sepsis, particularly respiratory sepsis was seen in 53% of patients and was a significant risk factor for intubation. I highlighted differences compared to previous studies in our institution regarding the acute treatment of exacerbations of MG- over 85% of our patients received IVIG compared to only 11.4% of a similar cohort ten years ago (O'Riordan et al., 1998a). Although 8% of our patients required repeated admissions to ITU for recurrent exacerbations of MG, I found that overall the long-term outcome was good. I had follow up data for over 80% of our patients for a mean of four years and found that the majority were asymptomatic or had only mild symptoms of MG at final review. In particular there were no significant differences in outcome between older and younger patients in our cohort. Hence, severe exacerbations of MG can be associated with a good prognosis in older patients even if a prolonged ITU admission is required. Further prospective studies with larger numbers of patients are required to identify factors that are associated with a good prognosis.

9.4. Final summary

This thesis has built on previous knowledge regarding the benefit of thymectomy in MG and provides new insight into the long term prognosis after a severe exacerbation of the disease requiring ICU admission. Further prospective studies are needed to clarify our knowledge in this area.

This work also sheds new light on the pathomechanisms of LEMS IgG, showing that LEMS IgG reduces the rate of synaptic vesicle release and that this effect is specific for P/Q- VGCCs. Further experiments using samples from a greater number of patients are required to fully examine the mechanism of action of LEMS IgG which should help identify new targets for treatment of this autoimmune disease.

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