The role of humoral autoimmunity in gastrointestinal neuromuscular diseases

Andrew William Hubball

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The Wingate Institute of Neurogastroenterology
Blizard Institute of Cell and Molecular Science
Barts and The London School of Medicine and Dentistry
Queen Mary, University of London

To Carlton

ABSTRACT

Dysfunction of the gastrointestinal neuromuscular apparatus (including interstitial cells of Cajal) is presumed to underlie a heterogeneous group of disorders collectively termed gastrointestinal neuromuscular diseases (GINMDs). Humoral (antibody)-mediated autoimmunity directed against ligand- or voltage-gated ion channels or associated proteins involved in neuromuscular transmission is associated with several acquired neuromuscular diseases of the periphery and is now implicated in an increasing number of less well-characterised diseases. Anti-channel antibodies have been reported in small numbers of patients with GINMD, particularly in those with GI dysfunction secondary to an underlying disease such as neoplasia or infection. However, little is known of humoral autoimmunity in primary GINMDs.

This thesis investigated the association between anti-channel antibodies and GINMD, and the human GI tract as a potential target of anti-channel antibodies. The presence of anti-voltage- and ligand-gated antibodies was investigated in the serum of patients with primary achalasia, enteric dysmotility and intestinal pseudo-obstruction, and in those with GINMD secondary to Chagas' disease. The functional effect of sera from some of these patients on colonic smooth muscle contractility was also characterised. Finally, the distribution of six voltage-gated potassium channels (VGKCs), which serve essential roles in the peripheral and central nervous systems and are known targets of pathological autoantibodies, were investigated in all layers of the human GI tract.

Our findings suggest that currently recognised anti-channel antibodies are not a common pathogenic factor in primary GINMDs. Anti-channel antibodies, in particular anti-VGKC antibodies, were however found in a significant number of individuals with Chagas' disease. Furthermore, circulating factors in patients with chagasic GI disease altered GI smooth muscle contractility *in vitro* which may have pathological relevance to GI

dysfunction. VGKCs were found throughout the human GI tract, especially in enteric neurons and epithelial cells, which has significance for both normal function and in relation to the GI tract as a target organ for anti-channel autoimmunity.

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ABBREVIATIONS

ACh	acetylcholine
AGID	autoimmune gastrointestinal dysmotility
AINMD	autoimmune neuromuscular disease
ANA	anti-neuronal autoantibody
ANNA-1	anti-neuronal nuclear antibody type-1
ANNA-2	anti-neuronal nuclear antibody type-2
BICMS	Blizzard Institute of Cell and Molecular Science
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
°C	degrees Celsius
cDNA	deoxyribonucleic acid
ChAT	choline acetyl transferase
CHRM2	chromosome 2
CI	confidence interval
CIPO	chronic intestinal pseudo-obstruction
cm ²	centimetres squared
CmTx	conotoxin MVIIC
CNS	central nervous system
COX-2	cyclo-oxygenase-2
CO ₂	carbon dioxide
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
D-IBS	diarrhoea-predominant irritable bowel syndrome
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
DnTx	dendrotoxin

ECG	electrocardiograph
EcoRI	Escherichia coli restriction enzyme one
E.coli	Escherichia coli
ED	enteric dysmotility
EFS	electrical field stimulation
ELISA	enzyme-linked immunoadsorbent assay
ENS	enteric nervous system
Epb	epibatidine
ERG	ether-a-go-go
FCS	fetal calf serum
fM	femtomole
g	gram
GAD	glutamic acid decarboxylase
GFP	green fluorescent protein
GI	gastrointestinal
GINMD	gastrointestinal neuromuscular disease
GORD	gastro-oesophageal reflux disease
h	hours
H ₂ O ₂	hydrogen peroxide
H&E	haematoxylin and eosin
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	human leucocyte antigen
Hz	Hertz
I _A	rapidly inactivating A-type current
IBS	irritable bowel syndrome
ICC	interstitial cell of Cajal
IEC-6	intestinal epithelial cell line 6

IgG	immunoglobulin G
I _K	potassium current
IMS	industrial methylated spirit
iNOS	inducible nitric oxide synthase
K	potassium
K _{Ca}	calcium-activated potassium (channel)
KCl	potassium chloride
KH ₂ PO ₄	monopotassium phosphate
K _V	voltage-gated potassium (channel)
K _V 1	group 1 voltage-gated potassium (channel)
L	litre
LB	lysogeny broth
LEMS	Lambert-Eaton myasthenia syndrome
L-NAME	N-ω-nitro-L-arginine methyl ester
LOS	lower oesophageal sphincter
M _{2,3}	muscarinic receptor 2, 3
mA	milliampere
mAChR	muscarinic acetylcholine receptor
MG	myasthenia gravis
MgSO ₄	magnesium sulphate
min	minute
ml	millilitre
mm	millimetre
mM	millimole
mRNA	messenger ribonucleic acid
ms	millisecond
MuSK	muscle-specific receptor tyrosine kinase
nAChR	nicotinic acetylcholine receptor

NaCl	sodium chloride
NaHCO ₃	sodium bicarbonate
NF	neurofilament
Ng	nanogram
NK	neurokinin
nm	nanometre
NMT	neuromyotonia
NO	nitric oxide
NOS	nitric oxide synthase
O ₂	oxygen
PBS	phosphate buffered saline
PCA	Purkinje cell cytoplasmic antibody
PGE ₂	prostaglandin E2
pM	picomole
PNS	peripheral nervous system
PSA	penicillin, amphotericin and streptomycin
RNA	ribonucleic acid
RT	room temperature
rpm	revolutions per minute
s	second
SCLC	small-cell lung cancer
SID	severe intestinal dysmotility
SSc	systemic sclerosis
STC	slow-transit constipation
TFB	transformation buffer
T	temperature
TTX	tetrodotoxin
μ	micron

µg	microgram
µl	microlitre
UV	ultraviolet
V	volt
VGCC	voltage-gated calcium channel
VGKC	voltage-gated potassium channel
WB	wash buffer
w/v	weight / volume
XhoI	Xanthomonas holcicola I

INTRODUCTION

1

1.1 GASTROINTESTINAL NEUROMUSCULAR DISEASES

Gastrointestinal neuromuscular diseases (GINMDs) are a clinically heterogeneous group of disorders in which symptoms are presumed or proven to arise as a result of neuromuscular (including interstitial cell of Cajal) dysfunction (Knowles *et al.* 2010). This classification requires both symptoms of impaired motor activity and demonstrable abnormalities in motor function, for example manometric abnormalities with or without evidence of transient or persistent radiological visceral dilatation (Wingate *et al.* 2002). These disorders may be due to relatively rare congenital defects, for example Hirschsprung's disease, where the pathophysiologies are to some extent elucidated (Heanue *et al.* 2007), however most GINMDs are acquired in later life and may be primary or secondary to another established disease.

Primary GINMDs include oesophageal achalasia (Hertz 1915), gastroparesis (Shellito *et al.* 1984), enteric dysmotility (ED) (Wingate *et al.* 2002) and chronic idiopathic intestinal pseudo-obstruction (CIPO) (Dudley *et al.* 1958) (for the purposes of this thesis ED and CIPO are referred to collectively as severe intestinal dysmotility: SID), slow-transit constipation (STC) (Preston *et al.* 1986) and idiopathic megabowel (Bodian *et al.* 1949).. Some international consensus exists on the clinico-physiological diagnosis of such disorders (Wingate *et al.* 2002). Histopathological changes in nerves (Schuffler *et al.*

1982), smooth muscle (Smith *et al.* 1997) and interstitial cells of Cajal (ICC) (Huizinga 1998; He *et al.* 2000; Farrugia 2008) have been variably described in most and have received recent attention by an international working group (Knowles *et al.* 2010).

Clinically similar disorders can arise in association with other diseases. Such secondary GINMDs include intestinal pseudo-obstruction occurring as a paraneoplastic syndrome, most commonly in association with small cell lung carcinoma (SCLC) (Schuffler *et al.* 1983; Chinn *et al.* 1988; Sodhi *et al.* 1989; Lucchinetti *et al.* 1998), but also in cases of bronchial carcinoid (Gerl *et al.* 1992), thymoma (Pande *et al.* 1999), ganglioneuroblastoma and neuroblastoma (Schobinger-Clement *et al.* 1999; Martin *et al.* 2008) and ovarian carcinoma (Lee *et al.* 2001). GINMDs may also occur in association with infective systemic diseases such as Chagas' disease (South American trypanosomiasis) (Ferreira-Santos 1961), connective tissue disorders such as scleroderma (Treacy *et al.* 1963; Cohen *et al.* 1972; Battle *et al.* 1981) and some endocrine and metabolic disorders e.g. diabetes mellitus (Werth *et al.* 1992) and amyloidosis (Legge *et al.* 1970).

1.2. THE GI TRACT AS A TARGET FOR AUTOIMMUNITY

A significant proportion of GI diseases have an inflammatory / immune-mediated aetiology e.g. coeliac and inflammatory bowel disease. Although less studied, there is increasing experimental and clinical evidence that some GINMDs could also be immune-mediated. Innate and adaptive immune mechanisms can both modulate neuromuscular function acutely, and lead to changes in sensorimotor function that are sustained after resolution of a discrete inflammatory stimulus including one confined to the mucosa. This evidence, based on extensive study of the *Trichinella*-infected jejunum of small experimental animals (Collins 1996; Barbara *et al.* 1997) is supported by several studies in IBS that demonstrate increased numbers of intra-mucosal inflammatory cells including

mast cells (Barbara *et al.* 2004; Wang *et al.* 2004) and T lymphocytes (Spiller *et al.* 2000; Chadwick *et al.* 2002), as well as increased levels of pro-inflammatory mediators (Gonsalkorale *et al.* 2003; O'Mahony *et al.* 2005). Inflammatory cells have also been shown to infiltrate deeper layers of the muscle wall or the myenteric plexus (hence the terms of *leiomyositis* and *ganglionitis*, respectively) and tend to be associated with more severe motor dysfunction. Similar findings have been made in CIPO (Ruuska *et al.* 2002; Tornblom *et al.* 2002; Knowles *et al.* 2004; Haas *et al.* 2005; Tornblom *et al.* 2007; Knowles *et al.* 2008; Lindberg *et al.* 2009).

Humoral (antibody)-mediated autoimmunity is associated with several well-established acquired neuromuscular diseases (below) and is implicated in an increasing number of less well-characterised disorders (Newsom-Davis *et al.* 1991), particularly of the central nervous system (Lang *et al.* 2003). Although less studied, there is some evolving evidence, especially in secondary GINMDs that the enteric neuromusculature could be a target for autoimmune attack. Here the diversity of ion channels and other receptors, and in this regard, potential epitopes, is only currently being realised (Vianna-Jorge *et al.* 2000; Galligan 2002; Schaufele *et al.* 2005).

1.2.1 NEURONAL ION CHANNELS OF THE GI TRACT

It is likely (based on observations of neurotransmitter heterogeneity and numbers of enteric neurons, akin to the spinal cord (Gershon *et al.* 1981; Furness 2000) that the profile of ion channels in the gut is similar to that of the central and peripheral nervous systems (CNS and PNS respectively), although less well-studied. To date, a diversity of voltage-gated calcium (Starodub *et al.* 1999), potassium (Zholos *et al.* 1999; Hatton *et al.* 2001) and sodium channels (Franklin *et al.* 1993), ligand-gated ion channels (Galligan 2002) and G-protein-coupled (North *et al.* 1985), neurokinin (Portbury *et al.* 1996), adrenergic (Scheibner *et al.* 2002) and serotonergic receptors (Pan *et al.* 1997) have been

identified in the enteric nervous system (ENS) and play important roles in gastrointestinal sensation and motility (Cervero *et al.* 2003) (Table 1.1). Of particular relevance to this thesis are voltage-gated potassium channels (VGKCs) which are described in more detail below.

1.2.1.1 Group 1 voltage-gated potassium channels

Amongst the major classes of K^+ channels, the greatest diversity is to be found amongst VGKCs which activate in response to a change in membrane potential (Hille 2001). A large gene family encodes twelve subfamilies (K_v1-12) of the principle pore-forming α -subunit (Figure 1.1). Structurally four α -subunits, from the same subfamily, assemble into a tetrameric complex together with modifying auxiliary subunits, usually called β -subunits, to form a functional channel (Christie *et al.* 1990; Pongs 1992). Channel diversity is further enhanced by the assembly of α -subunits into heteromultimeric channels with intermediate properties (Christie *et al.* 1990; Sheng *et al.* 1993; Scott *et al.* 1994). A wide range of biophysically distinct VGKCs is thus available to cells, which they may selectively express to meet their functional requirements. VGKCs are involved in numerous processes including setting resting membrane potentials, regulation of cellular excitability and synaptic transmission. The most extensively studied members are the K_v1 or *Shaker*-related subfamily. Numerous studies have investigated the distribution of these channels with a view to establishing their role in normal function and disease states.

Table 1.1: Ion channels identified in the GI tract

Class	Subclass	Ion channel
G-protein coupled	Class A (Rhodopsin – like)	M ₂ / M ₃ mAChR Adrenergic Histamine 1-4 5HT _{1,2,4,6,7} Bradykinin 1-4 NK1-3 Protease-activated (PAR) 1-4 Prostaglandin 1-4 Purinoreceptors 1-11
	Class B (Secretin – like)	Calcitonin CRF 1-2
	Class C (mGluR-like)	Glutamate 1-8 GABA B1-2 Ca-sensing Taste T1-2R
Ligand-gated		nAChR P2X purinergic 5HT ₃ NMDA AMPA Kainate Glycine GABAA
Regulatory and sensory		TRP K _v K _{Ca} KcsA Na _v L, N, P/Q-type VGCC Cl ⁻ ASICs
Growth factor		Trk-A RET

ASIC, acid sensing ion channel; CRF, corticotrophin-releasing factor; K_{Ca}, calcium-activated potassium channel; K_v, voltage-gated potassium channel; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; NK, neurokinin; TRP, transient receptor potential; Trk, tyrosine kinase receptor; VGCC, voltage-gated calcium channel

Figure 1.1: Phylogenetic tree for the voltage-gated potassium channel family. The K_v1 subfamily, studied in this thesis, are outlined. (Gutman *et al.* 2005).

1.2.1.2 K_v1 channels of the *GI* tract

In the CNS and peripheral neuromusculature regional and cellular K_v1 channel distribution is well characterised (Beckh *et al.* 1990; Veh *et al.* 1995; Coleman *et al.* 1999; Chung *et al.* 2001; Glazebrook *et al.* 2002). In comparison, little is known of K_v1 subunit expression in neurons innervating the gut. Existing studies are described below.

Animal studies

VGKC currents contribute to regulating excitability and repolarisation of action potentials in extrinsic sensory neurons innervating the oesophagus, stomach, ileum and colon. As a result, blocking these currents leads to a significant increase in action potential duration. Macroscopically, these currents can be divided into a rapidly inactivating current (I_A) and a delayed rectifier or I_K current. Pharmacological and molecular evidence points to a contribution of K_V1 channels to both of these currents (Yoshimura *et al.* 1996; Glazebrook *et al.* 2002; Dang *et al.* 2004). Functionally within the gut, K_V1 currents are associated with a number of processes including epithelial cell maturation and migration (Rao *et al.* 2002), nutrient absorption (McDaniel *et al.* 2001), modulation of enteric motor nerve excitability (Suarez-Kurtz *et al.* 1999; Vianna-Jorge *et al.* 2000) and generation of smooth muscle ‘slow-wave’ contractions (Thornbury *et al.* 1992; Thornbury *et al.* 1992; Carl 1995). As such, in a similar manner channel characterisation in the CNS and PNS, several studies have aimed to elucidate the specific K_V subunit expression in the gut. In the mouse, Hatton and co-workers reported $K_V1.1$ immunoreactivity expression in murine (as well as guinea pig and canine) myenteric ganglia and ICC (Hatton *et al.* 2001). However, others describe $K_V1.1$ expression limited to enteroglia cells, with no immunoreactivity detected in neurons or ICC (Costagliola *et al.* 2009). Costagliola *et al.* also report $K_V1.2$ expression in enteroglia cells and additionally in enteric neurons. K_V channel currents contribute to the electrical slow wave generation of canine colonic smooth muscle cells (Thornbury *et al.* 1992; Thornbury *et al.* 1992; Carl 1995). Molecular studies of canine GI smooth muscle have subsequently identified $K_V1.2$, $K_V1.4$, $K_V1.5$ and $K_V1.6$ expression (Hart *et al.* 1993; Epperson *et al.* 1999). However, murine colonic smooth muscle cells express transcripts encoding $K_V1.6$, but lack $K_V1.4$ suggesting inter-species variability (Koh *et al.* 1999).

Human studies

To date, a single study has identified K_V1 channel subunits in the human neuromuscular apparatus. Recordings from isolated human oesophageal smooth muscle cells have revealed voltage-gated and Ca²⁺-activated potassium (K_{Ca}) currents. Molecular analysis of the same tissue identified K_V1.2 and 1.5 mRNA expression in both longitudinal and circular muscle layers (the expression of other K_V1 channels was not investigated). Functionally, pharmacological blockade of the muscular voltage-gated potassium current caused an increase in resting tension, suggesting a role in setting resting membrane potentials. However, the agent used in this study (high concentration 4-aminopyridine) non-specifically blocks a number of potassium channel types and so the effect cannot be solely attributed to K_V1 channels (Wade *et al.* 1999).

1.3. ‘CLASSICAL’ ANTIBODY-MEDIATED NEUROLOGICAL DISEASES

A number of peripheral neuromuscular diseases are known to be mediated by specific autoantibodies and are collectively known as autoimmune neuromuscular diseases (AINMDs). Although they do not primarily involve the ENS, they represent models by which the role of autoimmunity in GINMDs can be critically discussed. Best elucidated are diseases in which antibodies target peripheral ligand- or voltage-gated ion channels or associated proteins involved in neuromuscular transmission leading to several diseases e.g. myasthenia gravis, Lambert Eaton myasthenic syndrome and acquired neuromyotonia (Isaac's disease) (Vincent *et al.* 2006). Often termed autoimmune channelopathies, these contrast with genetic channelopathies in which a loss or decrease in function is conferred by gene mutation with similar phenotypic consequences (Terwindt *et al.* 1998).

1.3.1 MYASTHENIA GRAVIS

Myasthenia gravis (MG) is the best understood autoimmune disorder of the nervous system and provides a ‘bench-mark’ against which putative autoimmune disorders may be judged. It is characterised by clinical features of fatigable muscle weakness which may initially affect the ocular muscles and variously spread to facial, bulbar and respiratory muscles and may also involve the limbs (Adams *et al.* 1993). Antibodies targeting post-synaptic nicotinic acetylcholine receptors (nAChRs) present on the surface membrane of muscle are detectable in greater than 85% of MG patients (Almon *et al.* 1974; Lindstrom *et al.* 1976; Vincent *et al.* 1985).

Around 10% of MG patients have a thymoma, and in these cases nAChR antibodies are always present (Vincent *et al.* 2006). Muscle weakness results from the functional loss of post-synaptic nAChRs secondary to receptor cross-linking by divalent antibodies and subsequent receptor internalisation (Drachman *et al.* 1978; Stanley *et al.* 1978) and complement-mediated degradation of the postsynaptic membrane (Engel 1979; Engel *et al.* 1981; Richman *et al.* 1993). Of those in whom anti-nAChR antibodies are not detectable (seronegative MG), around 40% have antibodies to a muscle-specific receptor tyrosine kinase (MuSK) (Wolfe *et al.* 2008) located at the neuromuscular junction (Valenzuela *et al.* 1995). MuSK is known to be important in clustering of AChRs during development, and although its role in adult muscle is less clear it appears to be necessary for maintenance of the post-synaptic membrane (Kong *et al.* 2004).

1.3.2 LAMBERT EATON MYASTHENIC SYNDROME

The Lambert Eaton myasthenic syndrome (LEMS) is a condition of proximal muscle weakness with accompanying autonomic dysfunction including dry mouth, erectile dysfunction, constipation and urinary symptoms. Approximately 50-60% of patients have

an associated small cell lung carcinoma (SCLC). The underlying pathophysiology of LEMS is impaired calcium-dependent release of acetylcholine from motor nerve terminals (Elmqvist *et al.* 1968). Circulating antibodies against voltage-gated calcium channels (VGCCs) are found in around 90% of LEMS patients (Lennon *et al.* 1995; Motomura *et al.* 1997) and result in the down-regulation of functional VGCCs (Pinto *et al.* 1998). VGCCs are composed of a number of pharmacologically and electrophysiologically distinct subtypes. Although antibodies have been described that bind to the L-, N- and P-/Q-VGCC subtypes (Meriney *et al.* 1996), only those directed against the P-/Q-subtype, which is responsible for pre-synaptic neurotransmitter release at the neuromuscular junction, have been shown to be pathogenic (Pinto *et al.* 1998). SCLCs have been shown to express functionally active P/Q-type VGCCs at the surface and it is thought that in these patients the neurological syndrome may result from a cross-reactive immune response generated by tumour antigens (Viglione *et al.* 1995). The aetiology in the remaining 50% of patients without a tumour is unknown.

1.3.3 ACQUIRED NEUROMYOTONIA

Acquired neuromyotonia (NMT) is a disorder of spontaneous and continuous muscle fibre activity characterised by muscle stiffness, fasciculations, weakness and increased sweating (Isaacs 1961). It occurs due to motor nerve hyperexcitability and is associated with autoantibodies against VGKCs (Isaacs 1961; Newsom-Davis *et al.* 1993). Using current assays, antibodies to the K_v1 family of VGKCs are detectable in 40% of patients with NMT (Hart *et al.* 2002).

Acquired NMT is associated with thymoma or SCLC in approximately 20% of cases (Newsom-Davis *et al.* 1993). Anti-VGKCs in NMT do not appear to block channel function directly as short incubation times have no effect. However, prolonged incubation of several hours reduces K⁺ channel current amplitudes without altering gating kinetics

(Sonoda *et al.* 1996; Nagado *et al.* 1999). This effect is also independent of added complement, or accelerated channel turnover, and degradation may occur secondary to cross-linking of channels by divalent antibodies (Tomimitsu *et al.* 2004).

1.4 EVIDENCE FOR ANTIBODY MEDIATION OF AUTOIMMUNE DISEASES

A number of clinical and experimental findings may help indicate that a disease has an autoimmune aetiology and these are central to subsequent discussion of the evidence for antibody mediation of GINMDs. Clinical features that are suggestive include onset of disease in adulthood, a history of autoimmunity in the patient or their family, an association with specific alleles of the human leukocyte antigen (HLA) gene complex, association with neoplasia, and clinical improvement following plasma exchange and / or immunosuppressive therapy (Vincent *et al.* 2006) (further discussed below). Such findings are also supported by finding evidence of immune cells in the target tissue although this is not an absolute requirement (Maselli *et al.* 1991; Vincent *et al.* 1999). In experimental terms, proof of disease causation should meet three broad criteria (Table 1.2).

Table 1.2: Proof of disease causation

Detection of relevant autoantibodies	<ul style="list-style-type: none"> • Immunohistochemistry • Western blot • ELISA • Radioimmunoprecipitation assays
Specificity of autoantibodies to the disease	<ul style="list-style-type: none"> • Found in most cases • Rare in controls
Proof of pathogenicity	<ul style="list-style-type: none"> • Response to immunotherapies • Identification of antibody <i>in situ</i> • <i>In vitro</i> effects • Induction of experimental disease by passive transfer and active immunisation

ELISA, enzyme-linked immunoadsorbent assay

1.4.1 DETECTION OF AUTOANTIBODIES

Autoantibodies may be detected by a number of techniques, the choice of which is often limited by the availability of specific antigens. Immunohistochemistry, western blotting, enzyme-linked immunoadsorbent assay (ELISA), radioimmunoprecipitation as well as functional assays are variously employed to measure antibodies in autoimmune neurological disorders (Tozzoli 2007). Each varies in accuracy, sensitivity and specificity and, if possible, a combination of methods should be used to confirm positive results (Vincent 2002). Although not discussed in detail here, it should especially be noted that in comparison with other methods, the sole use of ELISA or immunohistochemistry should be viewed cautiously because of the established risks of non-specific binding (Vincent 2002). Radioimmunoprecipitation assays, although acceptably specific, are

dependent on the availability of suitable radiolabelled antigens of high specificity. The development of human cell lines (e.g. embryonic kidney fibroblasts) transfected to express channels or receptors of interest represents a means by which the binding of antibodies to the external surface of functionally active channels / receptors may be investigated (Blaes *et al.* 2000). This may be done by incubating cells expressing the target of interest with patient serum or by extracting the cells for fluorescent-labelled immunoprecipitation, and is perhaps now the assay of choice in detecting anti-channel antibodies, not only because functionality is strongly implied, but also because such assays may be more sensitive (Leite *et al.* 2008).

1.4.2 SPECIFICITY FOR DISEASE

Pathogenic autoantibodies would be expected to bind to the extracellular domain of a cell-surface antigen relevant to the disease in a significant number of affected individuals, and that such binding is rare in healthy and other disease controls. For instance it has already been noted that specific autoantibodies can now be demonstrated in almost all patients with MG (Chan *et al.* 2007), but are extremely rare in healthy controls and in patients with other autoimmune or neurological diseases.

1.4.3 PROOF OF PATHOGENICITY

The demonstration of circulating autoantibodies, even those specific for relevant cell surface targets does not necessarily prove a direct role in disease causation since these could have arisen secondary to tissue damage with exposure of host epitopes to the immune system (de Scheerder *et al.* 1989). To establish a direct pathogenic role a number of criteria must be fulfilled. These criteria originally established the role of nAChR antibodies in MG and have subsequently been applied to other putative autoimmune

neurological disorders (for comprehensive review see (Vincent 2002). Each is discussed in the following sections:

1.4.3.1 Response to immunotherapies

Clinical improvement to plasmapheresis (plasma separation / exchange), intravenous immunoglobulin treatment or immunosuppressive therapies such as steroids and azathioprine strongly suggests a role for humoral autoimmunity. A further requisite should be that clinical response corresponds with a reduction in the titre of circulating antibodies as is seen in MG (Newsom-Davis *et al.* 1978) LEMS (Newsom-Davis *et al.* 1984) and acquired NMT (Shillito *et al.* 1995).

1.4.3.2 Identification of antibody in situ

Identification of autoantibodies bound at the neuromuscular junction has been demonstrated in MG in association with complement components and the membrane-attack complex (Engel *et al.* 1977). The number of nAChRs was found to be inversely proportional to bound IgG confirming a role in nAChR loss (Engel *et al.* 1987). Following passive transfer to experimental animals, LEMS IgG has been identified at the active zones of the presynaptic motor end-plate membrane (Fukuoka *et al.* 1987).

1.4.3.3 Modulation of antigen expression and / or functional effects in vitro

Incubation with pathogenic IgG leads to altered tissue expression of the antigen and / or function of the antigenic target in cell lines, cell cultures and *ex-vivo* tissue. In LEMS, autoantibodies reduce the number of functional P/Q-type VGCCs on cell lines and cultures (Pinto *et al.* 1998). Similarly, muscle cells grown in the presence of MG IgG have a reduced number of functional nAChRs (Drachman *et al.* 1978). Serum or IgG

from MG patients also inhibits the function of nAChRs on muscle cell lines by preventing the binding of ACh or other agonists (Riemersma *et al.* 1996). Incubation with NMT IgG results in repetitive action potentials in dorsal root ganglion cell cultures and reduced potassium currents in neuronal cell lines (Shillito *et al.* 1995; Nagado *et al.* 1999) mimicking disease electrophysiology.

1.4.3.4 Passive transfer to experimental animals

Injection of sera, plasma or immunoglobulins derived from affected patients into experimental animals results in passive transfer of the disease phenotype. Mice injected with MG IgG show muscle weakness and loss of muscle nAChRs (Toyka *et al.* 1977). The physiological effects on the peripheral and autonomic nervous system of LEMS can also be transferred to mice by injection of LEMS IgG (Prior *et al.* 1985; Lang *et al.* 1987; Waterman *et al.* 1997). Passive transfer of IgG from NMT patients results in increased quantal release of ACh at the neuromuscular junction and prolongation of the action potential (Shillito *et al.* 1995).

1.4.3.5 Induction of experimental disease by immunisation

An animal model of the disease is induced following immunisation with purified antigen. This confirmation of antibody pathogenicity was demonstrated at an early stage of the investigation of MG pathogenesis (Patrick *et al.* 1973). This proof has been difficult to achieve in other AINMDs due to an inability to produce relatively large quantities of pure antigen for immunisation. However, a preliminary animal model of LEMS produced by injecting mice and rats with synaptosomes containing (although not exclusively) L-, N- and P-/Q-VGCCs isolated from the electric organ of the Japanese electric ray, *Narke japonica*, has gone some way to addressing this problem (Kim *et al.* 1998).

These criteria will now be applied to primary and secondary GINMDs. From a perspective of hierarchy of evidence, secondary GINMDs are discussed first.

1.5 EVIDENCE FOR HUMORAL AUTOIMMUNITY IN SECONDARY GINMDS

1.5.1 PARANEOPLASIA

Clinical and histological evidence suggests an autoimmune basis for GINMD-related motor dysfunction associated with neoplasms: (1) histological examination of GI tissue from patients with paraneoplastic motility disorders reveals myenteric ganglionitis / plexitis, suggestive of some form of immune-mediated process. These include lymphocytic and plasmacellular infiltration, enteroglia cell proliferation and neuronal degeneration and loss, progressing to aganglionosis in the most severe cases (Schuffler *et al.* 1983; Chinn *et al.* 1988; Schobinger-Clement *et al.* 1999; Lee *et al.* 2001); (2) patients with SCLC and GI dysmotility often have co-existing neurological disorders including peripheral neuropathies, cerebellar degeneration and limbic encephalitis, which are thought to have an autoimmune origin (Chinn *et al.* 1988; Lucchinetti *et al.* 1998; Vernino *et al.* 1998); (3) several potentially pathogenic ‘paraneoplastic autoantibodies’ reactive against antigens expressed by tumour cells and neurons have been identified, which in combination with neurological disorders, including GI symptoms, strongly suggest the presence of an underlying malignancy (Lucchinetti *et al.* 1998; Pande *et al.* 1999; Schobinger-Clement *et al.* 1999; Lee *et al.* 2001).

1.5.1.1 Detection of autoantibodies

Paraneoplastic GI dysmotility, most commonly secondary to a SCLC, is associated with seropositivity for anti-neuronal autoantibodies. Most common in this context are type 1

anti-neuronal nuclear autoantibodies (ANNA-1, also known as anti-Hu), which recognise neuron-specific RNA binding proteins expressed by central and peripheral neurons, including enteric neurons, and SCLC tumour cells (Bell *et al.* 1976; Lucchinetti *et al.* 1998; Lee *et al.* 2001). Antibodies targeting cell-surface channels have been detected in smaller numbers of cases, including anti-N-type VGCCs with SCLC, retroperitoneal B-cell lymphoma, ovarian and breast carcinoma (Lee *et al.* 2001; Dhamija *et al.* 2008), anti-P/Q-type VGCCs with SCLC (Pardi *et al.* 2002), anti-VGKCs with thymoma (Viallard *et al.* 2005) and anti-ganglionic $\alpha 3$ nAChR antibodies with SCLC, thymoma, bladder and rectal carcinoma (Vernino *et al.* 2000).

1.5.1.2 Specificity for disease

Autoantibodies are commonly found in GI dysfunction secondary to an underlying malignancy. A study of patients at the Mayo clinic with confirmed paraneoplastic GI dysfunction found 10 of 11 patients were seropositive for one or more ‘paraneoplastic autoantibodies, including 8 positive for anti-Hu and one positive for anti-N-type VGCC (Lee *et al.* 2001). In a further study of various types of GI dysmotility, anti-Hu were always indicative of a malignancy (Dhamija *et al.* 2008). It is important to note that GI symptoms frequently precede diagnosis of a tumour, which may remain occult despite intensive evaluation (Lucchinetti *et al.* 1998).

1.5.1.3 Proof of pathogenicity

Response to immunotherapies

Paraneoplastic neurological, including gastrointestinal, symptoms may respond to immunomodulatory therapy. Plasmapheresis leading to a striking improvement in bowel motility and a correlating reduction in circulating antibody levels has been reported in a case of intestinal pseudo-obstruction associated with neuromyotonia and high titre

ANNA-1 and a case of slow transit constipation associated with thymoma and anti-VGKC antibodies (van Vuurden *et al.* 2005; Viallard *et al.* 2005). Intravenous immunoglobulin treatment has also achieved significant improvement in paraneoplastic GI dysmotility associated with anti- $\alpha 3$ nAChR antibodies and ANNA-1 (Dhamija *et al.* 2008).

Modulation of antigen expression and / or functional effects in vitro

On the basis of their intracellular antigenic target (as discussed above), ANNA-1 are unlikely to be directly pathogenic although nuclear epitopes could be exposed by prior inflammation / cell damage. A study based on a small series of patients with paraneoplastic GI dysmotility has suggested that ANNA-1 positive sera are able to trigger neuronal apoptosis *in vitro* suggesting a possible role in enteric neuronal degeneration (De Giorgio *et al.* 2003) (Figure 1.2). Anti-channel antibodies are more likely to exert a pathogenic effect due to the accessibility of the antigenic target. It is known from the study of peripheral neuromuscular disease that autoantibodies against VGKC, P/Q VGCC and $\alpha 3$ nAChRs are pathogenic. However, functional effects of these autoantibodies in the context of paraneoplasia have not been investigated.

Figure 1.2: Pro-apoptotic effects of anti-Hu antibody on cultured enteric neurons.

Representative immunofluorescent, confocal double-labelling photomicrographs of NSE-labelled cultures myenteric neurons (red) and three markers of cellular apoptosis: TUNEL, caspase-3 and APAF-1 (A-F). Exposure of myenteric neurons to commercial anti-Hu antibody (48 hrs) evoked: A: an intense TUNEL-positivity (green) in nuclei of NSE-labelled myenteric neurons (red), B: caspase-3 immunoreactivity (green) and C: APAF-1 (green). In contrast, TUNEL, caspase-3 and APAF-1 were absent in NSE-positive myenteric neurons (red) exposed to fetal calf serum or control (blood donor) serum (D-E, respectively). APAF-1 = apoptotic protease activating factor 1, NSE = neuron-specific enolase, TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labelling. Scale bar = 20 μ m. Courtesy of Prof. R De Giorgio; (De Giorgio *et al.* 2003).

1.5.2 CONNECTIVE TISSUE DISORDERS

Severe bowel dysfunction occurs in a proportion of patients with connective tissue disorders and is most commonly associated with systemic sclerosis (scleroderma, SSc). Any region of the gut may be affected, although oesophageal dysmotility is most commonly recognised (Rose *et al.* 1998). Although in later stages of the disease smooth muscle atrophy and fibrosis are clearly evident, manometric studies suggest that a neuropathic mechanism may be the initial event (Battle *et al.* 1981; Rees *et al.* 1982).

1.5.2.1 Detection of autoantibodies

Scleroderma is associated with anti-nuclear autoantibodies and more recently has been linked with other, non-nuclear antibodies such as anti-endothelial cell, anti-fibrillin-1, anti-metalloproteinases and anti-platelet-derived growth factor receptor (Tan *et al.* 2000; Sato *et al.* 2003; Ahmed *et al.* 2006; Baroni *et al.* 2006). The sera of scleroderma patients has also been found to contain antibodies capable, in immunofluorescent assays, of binding myenteric neurons (Howe *et al.* 1994). Anti-muscarinic AChR (mAChR) autoantibodies have also been identified in patients with SSc and GI dysfunction (see below for further discussion) (Goldblatt *et al.* 2002; Kawaguchi *et al.* 2009).

1.5.2.2 Specificity for disease

Circulating anti-myenteric neuronal autoantibodies demonstrated by Howe *et al.*, using indirect immunofluorescence, were found in 16 of 41 scleroderma patients and 11 of these had clinical or laboratory manifestations of GI dysfunction. However, 19 of 25 patients without anti-myenteric antibodies also had GI dysfunction, casting doubt on a direct causative role (Howe *et al.* 1994). IgG against mAChRs were detected by

radioimmunoprecipitation in 7 of 9 scleroderma patients with gastrointestinal symptoms. The two negative patients had only mild or no GI symptoms (Goldblatt *et al.* 2002).

1.5.2.3 Proof of pathogenicity

Response to immunotherapies

Plasmapheresis combined with immunosuppression resulted in amelioration of severe gastrointestinal symptoms 4 of 15 patients with gastrointestinal symptoms and corresponded with a decline in anti-nuclear antibody titres (Dau *et al.* 1981; Akesson *et al.* 1988).

Modulation of antigen expression and / or functional effects in vitro

IgG purified from scleroderma sera has been shown to inhibit muscarinic cholinergic smooth muscle contraction in isolated colon providing a potential mechanism for GI dysmotility. In this *in vitro* study the putative target was considered to be the M₃ mAChR, as IgG and a specific M₃-receptor antagonist inhibited, in a similar manner, evoked colonic muscle contraction. However, from this observation alone the IgG effect cannot be attributed solely to M₃ mAChR antagonism as the agent used to evoke the contractions, carbachol (a muscarinic receptor agonist) also activates the M₂ receptor subclass. Furthermore, the authors note in their discussion that they have failed to show direct binding to M₃ mAChRs transfected and expressed on the surface of mammalian cells or to virally produced recombinant receptors (Goldblatt *et al.* 2002) (see section 1.5.4 for further description). Nevertheless, the presence of antibodies against the M₃ mAChR was investigated using an ELISA to screen for binding to a synthetic peptide corresponding to the second extraellular loop domain of the human receptor. In a study of 14 scleroderma patients with GI involvement, sera from all were found to bind (Kawaguchi *et al.* 2009). The target autoantigen in scleroderma patients remains to be satisfactorily resolved and this area of study highlights the possible discrepancies

between peptide-based immunoassays and more complex functional or cell-based binding assays.

Passive transfer to experimental animals

Passive transfer of purified IgG from a patient with a specific variant of SSc (CREST syndrome) and anti-neuronal antibodies has successfully produced intestinal myoelectrical disruption in a rat model providing good evidence of pathogenic circulating antibodies. However, the generalisability of this to other variants of SSc is unknown (Eaker *et al.* 1999).

1.5.3 TYPE 1 DIABETES

Measurable disturbances in GI motility, in particular delayed gastric emptying, are a common feature of diabetes mellitus (Keshavarzian *et al.* 1987). Such changes may result from diabetic neuropathy affecting intrinsic (enteric) and / or extrinsic (autonomic) neurons or from changes in intestinal smooth muscle (for review see (Chandrasekharan *et al.* 2007)).

1.5.3.1 Detection of autoantibodies

Autoimmune mechanisms involved in diabetic neuromuscular dysfunction have rarely been investigated. Although autoantibodies to the neuronal and islet cell antigen glutamic acid decarboxylase (GAD) are detectable in around 70% of patients with recent onset type 1 diabetes (Seissler *et al.* 1993), GAD is an intracellular enzyme and would therefore not present a target in intact cells. The presence of functional antibodies in a group of patients with type 1 diabetes and gastrointestinal symptoms has been investigated pharmacologically and identified IgG against the L-type VGCC (Jackson *et al.* 2004).

1.5.3.2 Specificity for disease

Using the techniques described below, 8 of 16 patients had functional anti-VGCC autoantibodies. Of these, 7 had reported symptomatic gastrointestinal dysfunction. Such findings were not found in appropriate positive and negative controls (Jackson *et al.* 2004).

1.5.3.3 Proof of pathogenicity

Modulation of antigen expression and / or functional effects in vitro

Diabetic IgG produced a disruption of migrating motor complexes in intact colonic segments which was similar to the effect produced by an L-type VGCC agonist. Furthermore, the IgG was shown to competitively reduce binding of the specific L-type channel antagonist dihydropyridine (Jackson *et al.* 2004).

Passive transfer to experimental animals

Severe disruption of the migrating motor complex was demonstrated in colons isolated from mice injected with diabetic IgG containing the putative L-type calcium channel agonistic antibodies (Jackson *et al.* 2004).

Thus, experimental evidence for anti-channel autoimmunity in diabetic patients with GI dysfunction exists, albeit from a single study. Whilst the microvascular and neurological consequences of poor glycaemic control are of course significant and account for diabetic complications, the finding of functional anti-channel antibodies in these patients warrants further investigation.

1.5.4 CHAGAS' DISEASE

Infection by the protozoan parasite *Trypanosoma cruzi* causing South American Trypanosomiasis (Chagas' disease) (Chagas 1909) is a significant public health issue in the developing world, affecting 16-18 million of the Latin American population (Kirchhoff 2003). The most damaging and established sequel is that of fatal cardiomyopathy, however the digestive tract is also commonly affected with extensive destruction of ganglion cells (Ribeiro *et al.* 1998; da Silveira *et al.* 2007) leading to altered motility manifest as functional obstruction with eventual progression to hypertrophy of the smooth muscle and marked dilatation (i.e. megaoesophagus and megacolon) (Koberle 1968). Phenotypically, chronic disease manifestations vary geographically, reflecting the heterogeneity of *T.cruzi* strains and their pathogenic sequelae. Megaoesophagus and megacolon are prominent complications in southern countries of South America, whereas these complications are rarer in Central and northern regions (Miles *et al.* 2009).

The role of antibody-mediated autoimmune pathology in Chagas' disease is speculative given that there is more evidence for direct damage caused by the organism itself and by cell-mediated mechanisms (Kierszenbaum 2005). Nevertheless, some autoantibodies to both cell surface and intracellular targets have been detected (Kierszenbaum 1999).

1.5.4.1 Detection of autoantibodies

There is good evidence that circulating autoantibodies present in some patients with Chagas' disease are capable of modifying cellular events downstream of muscarinic cholinergic receptor agonism. Autoantibodies modifying muscarinic cholinergic neuromuscular activity were first identified in Chagas' disease patients with secondary dysautonomic syndrome where patient IgG caused an *in vitro* reduction in cardiac

muscular tone which could be blocked by atropine (Goin *et al.* 1994). Immunoblotting and ELISA subsequently identified the putative antigenic target as the second extracellular loop (the main immunogenic region) of the M₂ mAChR (Goin *et al.* 1997).

1.5.4.2 Specificity for disease

The dysautonomia described in Chagasic cardiomyopathy and GI dysmotility is strongly associated with the presence of autoantibodies as detected by the anti-M₂ peptide ELISA with 90% of patients with a measurable autonomic disturbance testing positive, compared with less than 30% with no disturbance and in no healthy controls (Goin *et al.* 1997; Ganzinelli *et al.* 2009). These autoantibodies have been detected in 84% of patients with chagasic achalasia versus 21% of those with Chagas' disease but no oesophageal sequelae and 28% in idiopathic achalasia (Goin *et al.* 1999). Similarly, in those with chagasic colonic dysfunction, autoantibodies were detected in 87% compared to 24% of those without megacolon (Sterin-Borda *et al.* 2001). However, using the same ELISA, these antibodies have been found in a high proportion of patients with other types of heart disease including 62% of patients with idiopathic cardiomyopathy, 46% with hypertensive heart disease and 48% with rheumatic valvular disease (Fu *et al.* 1993; Zhang *et al.* 2002). The presence of the same autoantibodies in non-parasitic cardiomyopathies increases the likelihood of a secondary phenomenon arising as a result of tissue damage.

1.5.4.3 Proof of pathogenicity

Response to immunotherapies

The chronic phase of the disease may be associated with a low parasitic load suggesting a further immuno-inflammatory response could contribute to disease pathology. In a rat model of chronic disease a short treatment with infliximab (a monoclonal antibody which

neutralises the activity of tumour necrosis factor- α) reduced myocarditis severity, indicating an immuno-pathological role for this cytokine (Perez *et al.* 2009). However, the primary role of parasite induced tissue damage in both the acute and chronic phase of infection, which is limited by host cellular and humoral immune responses, suggests that the use of immunomodulatory therapy would be counterproductive.

Modulation of antigen expression and / or functional effects in vitro

Chagasic IgG has repeatedly been shown to have *in vitro* effects which are mediated via the M₂ mAChR signalling pathway. In oesophageal and colonic muscle preparations, IgG produced concentration-dependent increases in basal tone which were sensitive to M₂ mAChR antagonism (Goin *et al.* 1999; Sterin-Borda *et al.* 2001). It also acted as a partial agonist, mimicking the effect of the muscarinic agonists carbachol and pilocarpine in a non-competitive manner (Goin *et al.* 1994; Goin *et al.* 1997). Radioligand binding assays investigating the interaction of chagasic IgG with the muscarinic receptor indicated chagasic IgG acts as an allosteric antagonist of the muscarinic receptor. It decreased the number of receptors available for binding (B_{max}) without an associated effect on the affinity constant (K_D) of the radioligand (Goin *et al.* 1994; Sterin-Borda *et al.* 2001). Further support of receptor agonism was provided by studies examining downstream effects of muscarinic activation in the presence of IgG. Incubation of cardiac or GI smooth muscle with IgG revealed pertussis toxin-sensitive decreased tissue levels of cyclic adenosine monophosphate (cAMP) and an increase in cyclic guanosine monophosphate (cGMP). This is consistent with muscarinic receptor activation (Goin *et al.* 1997; Goin *et al.* 1999; Sterin-Borda *et al.* 2001).

L-type voltage-gated calcium channel (VGCC) currents in isolated ventricular myocytes are also reduced in the presence of chagasic antibodies, in the absence of other agonists (Hernandez *et al.* 2003). An increase in inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene transcription is observed in response to chagasic IgG application, increasing prostaglandin E₂ (PGE₂) and nitric oxide (NO) production

(Ganzinelli *et al.* 2009). This has pathological relevance to the end-organ inflammation seen in chronic Chagas disease.

Passive transfer to experimental animals

To date, the transfer of disease phenotype to experimental animals by injection of immunoglobulins from affected patients has not been demonstrated.

Induction of experimental disease by immunisation

Active immunisation of mice with M₂ mAChR-derived synthetic peptides led to the production of functional anti-peptide antibodies. In these animals basal heart rate was unaffected, however carbachol induced bradycardia was significantly increased in comparison to controls, and atropine induced tachycardia was blunted suggesting an agonistic function (Peter *et al.* 2005).

1.6 EVIDENCE FOR HUMORAL AUTOIMMUNITY IN PRIMARY GINMDS

1.6.1 IDIOPATHIC ACHALASIA

Idiopathic achalasia of the oesophagus is an idiopathic motility disorder characterised by aperistalsis of the oesophageal body and incomplete lower oesophageal sphincter (LOS) relaxation on swallowing leading to dysphagia, regurgitation, chest pain and weight loss (Willis 1674; Hertz 1915; Castell 1976). Myenteric denervation of the distal oesophagus extending into the stomach is characteristically seen at histological examination (Csendes *et al.* 1992) and is selective for inhibitory nitric oxide (Mearin *et al.* 1993; De Giorgio *et al.* 1999; Zarate *et al.* 2006) and vasoactive inhibitory polypeptide (Aggestrup *et al.* 1983; Sigala *et al.* 1995) releasing motor neurons resulting in unopposed excitatory activity. Familial, infectious, genetic and autoimmune mechanisms have all been postulated to contribute to the development of achalasia (Park *et al.* 2005).

1.6.1.1 Detection of autoantibodies

An autoimmune pathogenesis is suggested by lymphocytic inflammatory infiltrates in the myenteric plexus (Goldblum *et al.* 1994; Clark *et al.* 2000) and disease association with specific HLA alleles (Ruiz-de-Leon *et al.* 2002; Latiano *et al.* 2006). Immunohistochemical techniques have repeatedly demonstrated anti-neuronal antibodies binding both submucosal and myenteric ganglia in the serum of achalasia patients (Storch *et al.* 1995; Verne *et al.* 1997; Moses *et al.* 2003). Autoantibodies against specific neuronal, glial and muscle antigens studied using radioimmunoprecipitation assays have identified nAChR, VGKC and N-type VGCC antibodies in a subset of primary achalasia patients (Kraichely *et al.* 2009).

1.6.1.2 Specificity to disease

Indirect immunofluorescence screening puts the prevalence of anti-neuronal antibodies at 12-64% compared with 0-9% of healthy controls (Storch *et al.* 1995; Verne *et al.* 1997; Ruiz-de-Leon *et al.* 2002; Moses *et al.* 2003; Bruley des Varannes *et al.* 2006). This proportion has been reported to rise significantly in patients with specific HLA genotypes. One hundred percent of female and 67% of male carriers of the DQA1*0103-DQB1*0603 heterodimer with achalasia were found to have antineuronal antibodies (Ruiz-de-Leon *et al.* 2002). Significant associations between DQB1*0602, DRB1*15 (Verne *et al.* 1999), DQB1*0502 and DQB1*0601 alleles (Latiano *et al.* 2006) and achalasia have also been reported, confirming an immunologic predisposition to the disease. However, although anti-neuronal antibodies appear specific to enteric neurons they bind both inhibitory and excitatory myenteric neurons and also submucosal ganglia with equal affinity, thus not accounting for the selective loss of inhibitory motor neurons. Regional specificity, which would also support a pathogenic role is lacking as antibodies label myenteric ganglia from all regions of the GI tract (Storch *et al.* 1995; Verne *et al.*

1997; Moses *et al.* 2003). Whilst reasonably disease-specific i.e. not present in control groups such as megacolon, Hirschsprung's disease, oesophageal cancer and MG (Storch *et al.* 1995), anti-neuronal antibodies have also been found in as many as 50% of patients with gastro-oesophageal reflux disease (GORD) casting doubt on their primary role in disease causation (Moses *et al.* 2003). Furthermore, no correlation has been found between patient age or clinical findings and the presence of anti-neuronal antibodies (Storch *et al.* 1995; Verne *et al.* 1997). A study of the prevalence of specific anti-neuronal / muscle antibodies using radioimmunoprecipitation assays (VGKC, GAD, N-type VGCC and muscle nAChR) found a significant proportion (26%) had one or more. Most strikingly, 21% of primary achalasia patients versus 2.5% of healthy controls had detectable anti-GAD antibodies. Only one of these had documented diabetes. The clinical significance of this finding remains to be ascertained (Kraichely *et al.* 2009).

1.6.1.2 Proof of pathogenicity

Modulation of antigen expression and / or functional effects in vitro

A novel approach using pre-incubation of gastric fundal tissue with sera from patients with achalasia demonstrated a subsequently altered myenteric neurochemical phenotype relevant to current understanding of disease pathogenesis (Bruley des Varannes *et al.* 2006). Specifically, inhibitory nitric oxide synthesising (NOS) neurons were reduced in number, whilst the proportion of excitatory choline acetyltransferase (ChAT) synthesising neurons increased, findings which in keeping with both incomplete LOS relaxation and histological findings. Achalasia sera used for both immunohistochemistry and *in vitro* motility studies revealed a correlation between the decrease in the proportion of NOS immunoreactive neurons and electrical field stimulation induced nitric oxide NO-dependent relaxation in circular muscle strips. The decrease in NOS immunoreactive neurons may be due to an effect of the serum on neuronal NOS (nNOS) expression at mRNA and/or protein level as the changes occurred rapidly (after 16-18 hours of

incubation). Although GORD serum did not alter neuronal chemical phenotype, suggesting a specific effect of achalasia serum, this methodology is not yet well validated and no signs of neuronal degeneration were found as seen histologically in active disease. In addition it is unknown which serum factors are responsible for this effect (Boeckxstaens 2006).

1.6.2 SEVERE INTESTINAL DYSMOTILITY

Severe intestinal dysmotility (SID) describes a group of disorders where the bowel fails to propel its contents in the absence of an obstructed lumen. At the most severe end of this spectrum are patients with episodic or chronic signs mimicking a mechanical obstruction: abdominal pain, distension, nausea/vomiting, and intractable constipation. These are termed chronic idiopathic intestinal pseudo-obstruction (CIPO) (Dudley *et al.* 1958). This diagnosis is based on a combination of the above symptoms with radiological evidence of gut dilatation and air-fluid levels during acute episodes (Gibbons *et al.* 1978; Hirsh *et al.* 1981; Gilchrist *et al.* 1985) and/or abnormal small bowel motility (Stanghellini *et al.* 1987; Di Lorenzo 1999). Enteric dysmotility describes patients with demonstrably abnormal small bowel contractility, but no history of frankly obstructive episodes. These patients may be at an early stage in the progression of CIPO, or perhaps be those in a quiescent period of a relapsing / remitting pseudo-obstruction (Wingate *et al.* 2002; Lindberg *et al.* 2009). Whilst the pathophysiology of these disorders remains poorly understood, CIPO may be classified according to recognised histopathological abnormalities of enteric smooth muscle (myopathies), ENS (neuropathies) and of ICC (mesenchymopathies). Several degenerative and inflammatory myopathic and neuropathic phenotypes have been described in children and adults on the basis of histopathology (Smith *et al.* 1992; Ruuska *et al.* 2002; Knowles *et al.* 2004; Tornblom *et al.* 2005; Knowles *et al.* 2009). Cases of both myopathic and neuropathic CIPO secondary to presumed autoimmune processes have been reported. Histological features

in most cases suggest the disease may be caused by local T-cell-mediated responses directed against enteric neurons or smooth muscle, but there is also evidence for autoantibody-mediated pathogenesis.

1.6.2.1 Detection of autoantibodies

Serologically, CIPO has been associated with anti-nuclear, anti-neutrophil cytoplasmic antibody (ANCA), anti-DNA, anti-smooth muscle and anti-neuronal antibodies (ANAs) (Ruuska *et al.* 2002; Ghirardo *et al.* 2005). Anti-enteric neuronal antibodies have been described in two patients with intestinal obstruction due to acquired aganglionosis. Whilst these antibodies also bound CNS neurons in a pattern characteristic of ANNA-1, and were also indistinguishable on Western blots, immunostaining of enteric neurons displayed a quite different pattern, being mostly cytoplasmic with only a weak nuclear component. Neither patient had CNS involvement and the autoimmune injury appeared limited to the ENS (Smith *et al.* 1997).

1.6.2.2 Specificity for disease

Using indirect immunofluorescence techniques, the largest study to date found 6 out of 25 (24%) patients with CIPO had anti-enteric neuronal antibodies (binding to both neuronal nuclei and cytoplasm) (de Giorgio *et al.* 2008). Other studies have identified specific autoantibodies in small numbers of patients, including one patient with anti-VGKC and one with anti-ganglionic $\alpha 3$ nAChR antibodies. Both of these patients had histologically-proven inflammatory enteric neuropathy, although in these cases diarrhoea was the chief complaint (Tornblom *et al.* 2007). Using immunoprecipitation assays to identify antibodies against $\alpha 3$ nAChRs in patients with various types of dysautonomia, Vernino *et al.* identified antibodies in 3 of 34 patients with a diagnosis of idiopathic GI dysmotility, although at a lower titre than other seropositive patients with more severe,

diffuse dysautonomias, suggesting these patients may have a limited form of autoimmune autonomic neuropathy (AAN) (further discussion below). (Vernino *et al.* 2000). Low, but positive titres of antibodies to P/Q VGCCs were also found in 4 out of 10 patients with CIPO (Knowles *et al.* 2002).

1.6.2.3 Proof pathogenicity

Response to immunotherapies

A number of case reports highlight remission of pseudo-obstructive episodes following immunomodulatory therapy. In these patients abdominal symptoms responded to immunosuppression with steroids and / or cyclosporin, which were started after failure of prokinetic therapy and surgical intervention and often after histological assessment revealed an inflammatory neuropathy or myopathy (Smith *et al.* 1997; De Giorgio *et al.* 2002; Ruuska *et al.* 2002; Dewit *et al.* 2008). Whilst a cell-mediated immune response is clearly present in these cases, the humoral immune system may also be activated, as evidenced by a paediatric case of intestinal pseudo-obstruction associated with anti-smooth muscle and anti-neutrophil cytoplasmic antibodies. In this instance a good clinical response to immunosuppressive therapy was also associated with a reduction in circulating autoantibody levels (Ruuska *et al.* 2002). Whilst not immunomodulatory, a patient with chronic gastroparesis (delayed-gastric emptying) who was seropositive for anti- $\alpha 3$ nAChR antibodies reported significant improvement in GI symptoms after commencing acetylcholinesterase inhibitor therapy, indicating a pathogenic role for these antibodies. (Pasha *et al.* 2006).

Modulation of antigen expression and / or functional effects in vitro

A functional effect of CIPO sera containing ANAs has been suggested by de Giorgio *et al.* (de Giorgio *et al.* 2008). They found evidence that sera containing ANAs stimulates neuronal autophagy (a cellular response to adverse conditions where by injured

organelles such as mitochondria are sequestered in autophagosomes) associated with neuronal stress. This effect was reduced by removal of IgG suggesting a direct role for immunoglobulins. However, this pathway is normally cytoprotective in limiting the production of reactive oxygen species and the pathophysiologic significance in CIPO is unclear.

Passive transfer to experimental animals and induction of experimental disease by immunisation

Using active immunisation and subsequent passive transfer techniques, the pathogenicity of anti- $\alpha 3$ nAChR antibodies has been investigated. Rabbits immunised with a recombinant fusion protein corresponding to the nAChR $\alpha 3$ subunit produced autoantibodies reactive with ganglionic nAChRs with development of a generalised AAN with prominent gastrointestinal dysmotility including gastroparesis and intestinal pseudo-obstruction (Lennon *et al.* 2003; Vernino *et al.* 2003). Severity of findings correlated with antibody levels, and immunohistochemistry revealed reduced numbers of myenteric plexus neurons and reduced immunoreactivity for nAChRs at neuronal surfaces and synapses (Vernino *et al.* 2003). Passive transfer of rabbit IgG containing anti- $\alpha 3$ nAChR antibodies to mice replicated active immunisation findings, confirming the role of antibodies in mediating AAN (Vernino *et al.* 2004). It should be noted, however, that passive transfer of sera of patients with severe autonomic neuropathy and anti- $\alpha 3$ nAChRs, even from those with highest titres of antibodies, failed to replicate these findings. The reason for this discrepancy is unclear (Vernino *et al.* 2004).

1.6.3 SLOW TRANSIT CONSTIPATION

A proportion of patients with severe constipation will have a reduction in the propulsive capacity of all or part of the colon that on the basis of transit studies is defined as slow transit constipation (STC). STC includes a clinically heterogeneous group of patients, the

majority of whom present in childhood and who are nearly always female (Preston *et al.* 1986). Others present in later life with onset of symptoms *de novo* or often attributed to pelvic surgery or childbirth (Knowles *et al.* 1999; Knowles *et al.* 2000). A plethora of aetiologies have been proposed to underlie STC including primary neuromuscular / ICC dysfunction, absorptive, hormonal and psychological abnormalities, with inconclusive results (Knowles *et al.* 1999; Knowles *et al.* 2000). In those presenting with *de novo* adult-onset STC an autoimmune aetiology remains an under-explored possibility.

1.6.3.1 Detection of autoantibodies

Validated immunoprecipitation assays detected high titres of anti-VGKC antibodies in two of 11 sera tested suggesting anti-neuronal antibodies may be important in certain acquired STC patients.

1.6.4 IDIOPATHIC MEGACOLON

Idiopathic megacolon describes a group of patients with intractable constipation associated with rectal or colonic dilatation in the absence of mechanical obstruction or evident cause (Bodian *et al.* 1949). Several pathological abnormalities of nerve, ICC and smooth muscle have been reported including immune-mediated myenteric ganglionitis associated with neuronal degeneration (De Giorgio *et al.* 2002).

1.6.4.1 Detection of autoantibodies

In contrast to acquired megacolon associated with systemic diseases such as Chagas' disease (see above), the possibility of a humoral immune response against enteric neuromuscular elements contributing to the pathogenesis of idiopathic megacolon has not been investigated.

1.7 SUMMARY AND KNOWLEDGE GAPS

The recognition of autoantibody-mediated channelopathies has opened new avenues in the investigation and management of peripheral neuromuscular disorders, whilst in cancer-associated neurological syndromes the detection of antibodies to neoplastic antigens has increased early diagnosis and treatment of the underlying malignancy. It is clear that whilst there is some evidence for autoantibody-mediated GI dysfunction in primary and secondary GINMDs, this is not strong in comparison with AINMDs such as myasthenia gravis, particularly in reference to specificity of antibody detection and proof of pathogenicity. These data are summarised in Table 1.3. In general the evidence is stronger for secondary, in particular paraneoplastic, than primary GINMDs, largely due to a paucity of information regarding the association of anti-channel antibodies with primary GINMDs. In determining a role for humoral autoimmunity in GINMDs the original paradigms generated by the investigation of peripheral neuromuscular disorders such as myasthenia gravis should be applied.

Of direct relevance to anti-channel autoimmunity contributing to GI dysfunction is the lack of data concerning channel expression in the gut. Although investigation of enteric channel expression points towards diversity equal to that of the CNS and PNS, in comparison to these systems knowledge regarding the ENS is in its infancy. This knowledge gap is especially true of the human ENS as initial investigations in small mammals are only beginning to be translated. As well as providing information regarding the functional biology of the gut, these studies will provide an expanding list of candidates for autoimmune interaction.

Table 1.3: Evidence for humoral autoimmunity in AINMD and GINMD

Criteria	Disease			GINMD							
	AINMD			Secondary				Primary			
	MG	LEMS	NMT	PN	CTD	DM	Chagas	IA	CIPO	STC	IM
Clinico-pathological	+	+	+	+	+	+/-	+	+	+	+/-	+/-
Disease specificity *	+	+	+	+	+/-	+/-	+/-	+/-	-	-	-
Pathogenicity criteria †											
Response to immunotherapy	+	+	+	+/-	+	-	-	-	+/-	-	-
Antibody <i>in situ</i>	+	+	-	-	-	-	-	-	-	-	-
<i>In vitro</i> effects	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-	-	-
Passive transfer	+	+	+	-	+/-	+/-	-	-	+/-	-	-
Active immunisation	+	-	-	-	-	-	-	-	+/-	-	-

* cut off of 50% disease cases with antibodies; † defined on the basis of + good quality evidence from more than one study; +/- weaker evidence usually arising from only one study; - no evidence to date. MG, myasthenia gravis; LEMS, Lambert-Eaton myasthenic syndrome; NMT neuromyotonia; PN, paraneoplastic; DM, diabetes mellitus; CTD, connective tissue disorders; IA, idiopathic achalasia; CIPO, chronic idiopathic intestinal pseudo-obstruction; STC, slow transit constipation; IM, idiopathic megabowel.

1.8 AIMS

The aims of this project were:

1. To investigate the presence of autoantibodies directed to enteric ion channels in patients with neuromuscular disorders of the gut leading to severe dysmotility.
2. To investigate whether autoantibodies (if present) are pathogenic.
3. To further elucidate the profile of expressed ion channels in the human enteric neuromusculature.

1.9 HYPOTHESES

1. Autoantibodies to one or more type of ion channel are present in a subset of patients with gastrointestinal neuromuscular disease.
2. As in the CNS and PNS, these autoantibodies are pathogenic and directly contribute to GI neuromuscular dysfunction.
3. Ion channels expressed by the CNS and PNS which are known targets of humoral autoimmune interaction are also present in the ENS.

MATERIALS AND METHODS

2

This chapter covers all specific methods used. Details of routine techniques, such as standard histochemical staining, can be found in the appendices.

2.1 ETHICAL APPROVAL AND COLLABORATIONS

The original application established collaborations with one international and one UK academic institution. These were the *Departamento de Clinica Medica Faculdade de Medicina*, University of Sao Paulo, and the Neurosciences Group, John Radcliffe Hospital, Oxford. The former facilitated collection of sera from Brazilian patients with Chagas' disease and from community controls from several clinics in Fortaleza and surrounding areas for anti-channel antibody analysis. Following the acquisition of Home Office approval, the necessary arrangements were made for the sera to be transported to the UK. The Neurosciences Group provided the facilities and technical support for screening all sera for circulating anti-channel antibodies. The collection and use of sera and tissue studies was approved by the Royal Free Hospital & Medical School Research Ethics Committee in April 2006. Following amendments to the original application, a further site (Homerton University Hospital, London) was added for the collection of human gastrointestinal tissue.

The work contained within this thesis is covered by the following ethics committee references:

06/Q0501/52

06/Q0501/52/AM01

06/Q0501/52/AM02

[See Appendix 1.0 for correspondence and confirmatory documents].

2.2 DETECTION OF AUTOANTIBODIES IN PATIENTS WITH GINMDS

2.2.1 COLLECTION OF PATIENT SERA

Venous whole blood (20 ml) was taken by antecubital fossa venepuncture and centrifuged for 10 min at 3,500 rpm. Serum was removed and stored at -80°C in Eppendorf aliquots (approximately 4 mL / patient) until use.

2.2.1.1 Achalasia

Twenty-one patients with achalasia (14 female, aged 26-72, median 55 years) who had undergone investigation at the Upper GI Unit at the Royal London Hospital were screened for autoantibodies. All patients had undergone manometric investigation of their oesophageal motility and were documented as having ‘established’ achalasia with manometric findings of abnormal peristalsis and incomplete lower oesophageal sphincter relaxation on swallowing (Figures 2.1 and 2.2) (Castell 1976; Pandolfino *et al.* 2005).

2.2.1.2 Severe intestinal dysmotility

Twenty-seven patients with severe intestinal dysmotility (22 female, aged 18-62, median 45 years) who attended the pseudo-obstruction outpatient clinic at the Royal London Hospital were studied. The median number of years from presentation to donation of a blood sample for this study was 10 (range 3-40). Chronic idiopathic intestinal pseudo-obstruction (CIPO) was diagnosed in 7 patients (5 female) on a basis of typical gastrointestinal symptoms (abdominal pain, severe constipation, abdominal distension, nausea and vomiting, weight loss; see Table 2.1), sub-occlusive events with radiological signs mimicking mechanical obstruction (air/fluid levels with or without dilatation on plain abdominal radiograph) and abnormal small bowel manometry (Dudley *et al.* 1958; Wingate *et al.* 2002) (Table 2.2). Twenty patients had abnormal intestinal motility but no history of sub-occlusive episodes and were classified as enteric dysmotility (Wingate *et al.* 2002; Lindberg *et al.* 2009). Abdominal pain and bloating were the most prominent features in both groups. As expected and previously reported (Lindberg *et al.* 2009), nutritional support was required in a greater proportion of patients with CIPO (6/7) than ED (8/20). The predominant manometric abnormality in CIPO patients was a neuropathic motor pattern (as evidenced by abnormal qualitative and quantitative measures of the migrating motor complex (Stanghellini *et al.* 1987). The ED group had an almost equal frequency of neuropathic and abnormal but indeterminate manometric patterns. Histological reports from full-thickness biopsies were available in all CIPO and 9 ED patients who had undergone laparoscopic biopsy (see Table 2.3). A greater number of biopsy samples were reported to be normal in the ED group than the CIPO group. An equal number (3/7) of CIPO patients had a neuropathy as had a myopathy. Alpha-actin deficiency (included by convention as a myopathy (Knowles *et al.* 2010) was the most common finding in the ED group, and two patients had both an alpha-actin deficiency and an inflammatory neuropathy. There was a poor correlation between histological and manometric findings.

Figure 2.1: Oesophageal manometry tracing showing a normal swallow. Trace shows propagation of contraction following a wet swallow (black arrows) and relaxation of lower oesophageal sphincter (red arrow)

Figure 2.2: Oesophageal manometry tracing from a patient with achalasia. Trace shows simultaneous contractions following a wet swallow (black arrows) and failure of relaxation of lower oesophageal sphincter (red arrow)

Table 2.1: Clinical features of severe intestinal dysmotility

Clinical features	N			
	CIPO (n =7)		ED (n=20)	
Symptoms	Presentation	Current	Presentation	Current
Weight loss	6	3	8	3
Dysphagia	0	0	1	2
Nausea	6	3	2	4
Vomiting	2	1	6	9
Reflux	0	0	1	3
Bloating	5	6	15	13
Abdominal pain	6	7	10	17
Constipation	5	2	7	6
Diarrhoea	0	3	0	1
Irregular bowel habit	0	0	0	4
Urgency	0	0	0	1
Incontinence	0	0	0	2
Incomplete evacuation	0	0	0	1
Feeding requirements				
None	1		12	
Enteral	2		2	
Parenteral	4		4	
Both	0		2	

Table 2.2: Investigation findings in patients with SID

Investigation findings	N	
	CIPO (n=7)	ED (n=20)
Radiography (plain radiograph +/- contrast +/- CT)		
Normal	0	6
Dilated small bowel	7	0
Malrotated small bowel	0	2
Dilated large bowel	1*	0
Delayed transit	0	2
Increased transit	0	1
Not reported	0	9
Small bowel manometry		
Neuropathic	5	8
Myopathic	0	1
Abnormal indeterminate	2	10
Not done	0	1†

* One CIPO patient had radiological evidence of small and large bowel dilatation

† Small bowel manometry failed in one patient due to retrograde propulsion

Table 2.3: Histopathological findings in full-thickness jejunal biopsies from patients with SID

Histopathological abnormalities	N	
	CIPO (n=7)	ED (n=20)
Normal	1	4
Visceral neuropathy	3	2
Degenerative neuropathy	1	0
Inflammatory neuropathy	2	2*
Visceral myopathy	3	5
α -actin deficiency	1	4*
Degenerative leiomyopathy	1	0
Inflammatory leiomyositis	0	0
Vascular myopathy	0	0
Hollow visceral myopathy	0	0
Inclusion body myopathy	1	1
Interstitial cell abnormalities	0	0
Total	7	9

* Two patients had both an α -actin deficiency and an inflammatory neuropathy

2.2.1.3 Healthy controls

Ten control samples were obtained from individuals (5 female, age 18 – 70, median 59) attending outpatient clinics at the Royal London Hospital who had no present or past history of GI, connective tissue or autoimmune disorder.

2.2.1.4 Chagas' disease

Sixty-one Brazilian patients with Chagas' disease were tested, forty-five male, aged 28-86, median 50 years. All patients were recruited from clinics in and around the city of Fortaleza, in north-east Brazil (Figure 2.3). Although the strain of *T.cruzi* was not identified in these cases, in this region the sequel of chronic disease is most commonly cardiac damage (see Chapter 1 for discussion of phenotypic variability related to *T.cruzi* strain heterogeneity). However, within this cohort, each chronic manifestation is represented and eight sub-groups were defined using the following criteria: those without organ involvement; those with achalasia, colonic disease or both; those with cardiomyopathy or ECG changes, and those with heart and gut manifestations. These patients were further divided into those that had undergone previous treatment with anti-*T.cruzi* drug therapy with Benzonidazol and those that had not (see Table 2.3). It would have been desirable to obtain more details regarding the past medical history of these patients. However, despite written requests this information was not forthcoming.

Table 2.4: Disease-subgroups of Chagas' disease patients

	Total	Untreated	Treated
Chagas' disease sub-groups			
No sequelae	29	16	13
Achalasia	9	7	2
Colon disease	4	1	3
Achalasia + colon disease	1	0	1
Heart disease	8	7	1
Heart disease + achalasia	5	3	2
Heart disease + colon disease	3	1	2
Heart disease + achalasia + colon disease	2	1	1
	61		

2.2.1.5 Brazilian controls

Twenty control sera samples were obtained from healthy subjects (11 female, aged 22 – 76, median 39 years) from the same communities as those who provided Chagas' disease samples.

Figure 2.3: Map of Brazil. All chagasic blood samples were obtained from clinics serving Fortaleza and surrounding areas in the north-east of the country (circled).

2.2.1.6 Disease controls

Disease control sera were obtained from patients attending the John Radcliffe Hospital, Oxford. These were from patients with: autoimmune autonomic neuropathy ($n = 2$), Lambert-Eaton myasthenic syndrome (LEMS; $n = 2$) and neuromyotonia (NMT; $n = 2$), containing anti-ganglionic $\alpha 3$ nicotinic acetylcholine receptor ($\alpha 3$ nAChR), anti-voltage-gated calcium channel (VGCC) and anti-voltage-gated potassium channel (VGKC) antibodies respectively (see Table 2.4 for details of all subjects).

Table 2.5: Basic clinical data and demographics for patients included in assay studies

Study group	Description	N	Sex F / M	Age median (range)
Achalasia	Incomplete LOS relaxation on swallowing Abnormal peristalsis	21	14 / 7	55 (26 - 72)
Severe intestinal dysmotility	CIPO (n = 7) Enteric dysmotility (n = 20)	27	22 / 5	45 (18 – 62)
Chagas' disease	No sequelae (n = 29) Achalasia (n = 17) Colon disease (n = 9) Heart disease (n = 18)	61	16 / 45	50 (28-86)
Healthy subjects	UK negative controls	10	5/5	59 (18-70)
	Brazilian negative controls	20	11 / 9	39 (22 – 76)
AAN	Positive controls: α 3-nAChR assay	2	1/1	50 (31,68)
LEMS	positive controls: VGCC assay	2	1/1	52 (46, 58)
NMT	positive controls: VGKC assay	2	2/0	42 (34, 50)

AAN = autoimmune autonomic neuropathy, ED = enteric dysmotility, CIPO = chronic idiopathic intestinal pseudo-obstruction, LEMS = Lambert-Eaton myasthenic syndrome, nAChR = nicotinic acetylcholine receptor, NMT = neuromyotonia, VGCC = voltage-gated calcium channel, VGKC = voltage-gated potassium channel

2.2.2 ANTI-NEURONAL AUTOANTIBODY IMMUNOFLUORESCENT ASSAY

2.2.2.1 Background to methodology

Seropositivity for anti-neuronal autoantibodies (ANAs) is a diagnostic marker of occult malignancy in the presentation of paraneoplastic CIPO, which usually precedes the diagnosis of a tumour (Lennon *et al.* 1991; Lucchinetti *et al.* 1998; Lee *et al.* 2001). To confirm the absence of such autoantibodies in our cohort of patients, all those with SID were screened for ANAs. Three types of ANAs associated with paraneoplastic neurological dysfunction are commonly screened for: Anti-Hu, anti-Yo and anti-Ri. Anti-Hu, also known as anti-neuronal nuclear antibody type 1 (ANNA-1), the most important with regards to paraneoplastic GI dysmotility, recognise a family of pan-neuronal RNA binding proteins, thus immunolabelling neuronal nuclei, and are chiefly associated with small-cell lung cancer (SCLC) (Bell *et al.* 1976; Graus *et al.* 1986; Altermatt *et al.* 1991). Anti-Yo, also known as anti-Purkinje cell cytoplasmic antibody (PCA) show cytoplasmic immunoreactivity of cerebellar Purkinje cells and are associated with gynaecological and breast cancer (Greenlee *et al.* 1983; Graus *et al.* 1988). Anti-Ri (ANNA-2), primarily associated with breast cancer, also bind neuron-specific RNA binding proteins but in contrast to anti-Hu, label neuronal nuclei of central neurons only (Budde-Steffen *et al.* 1988; Luque *et al.* 1991). ANAs are detected by standard indirect immunofluorescence where sera are incubated with sections of nervous tissue (typically mammalian cerebellum) and binding detected using an anti-human secondary antibody conjugated to a fluorophore (see section 2.4.1 for general immunohistochemical technical description). The pattern of immunoreactivity (nuclear vs. cytoplasmic) suggests the type of antibody present, which may then be confirmed by western blotting.

2.2.2.2 Indirect immunofluorescence

Sera samples were diluted 1 in 50 in PBS, applied to monkey cerebellar sections (Inova Diagnostics Inc., CA, USA) and incubated for 20 min at room temperature (RT). Anti-Hu and anti-Yo positive control sera (Inova Diagnostics Inc.) were also applied at this time for 20 min, at RT. Sera and control antibodies were rinsed off with PBS and sections soaked in PBS for 20 min. Fluorophore-conjugated anti-human secondary antibody was then applied and incubated for 20 min, at RT, followed by rinsing with PBS and further soaking for 20 min. Sections were mounted in glycerol and stored at 4°C in the dark until reading. Reagents and detailed methodology are found in Appendix 2.0.

Sections were viewed with a Nikon Eclipse 80i fluorescent microscope (Nikon Instruments, Amstelveen, Netherlands) and scored for anti-Hu / Ri or anti-Yo depending on staining pattern. Positive anti-Hu / Ri, weakly positive or equivocal results were sent for western blot confirmation at the Clinical Immunology Service, University of Birmingham, UK.

2.2.3 ANTI-CHANNEL ANTIBODY RADIOIMMUNOPRECIPITATION ASSAY

2.2.3.1 Background to methodology

Over the past 30 years, a number of specific neurotoxins from a variety of vertebrates and invertebrates have been discovered and structurally characterised. In accordance with their amino acid sequence such toxins have differential affinities to channel types and subtypes (Gasparini *et al.* 1998). These toxins have been an invaluable tool in the pharmacological classification of ion channels (Moczydlowski *et al.* 1988; Castle *et al.* 1989; Llinas *et al.* 1989). In autoimmune neurological disease the detection of circulating autoantibodies has largely depended on the availability of such target-specific

neurotoxins which may be radiolabelled for use in radioimmunoprecipitation assays e.g. δ -bungarotoxin for the labelling of nAChRs in the investigation of myasthenia gravis (MG) (Vincent *et al.* 1985), which are used in immunoprecipitation assays. The principle of radioimmunoprecipitation assays is shown in Figure 2.4.

All serum samples were screened for the following autoantibodies: anti- $\alpha 3$ nAChRs, anti-P/Q type VGCCs, anti-VGKC, and anti-glutamic acid decarboxylase (GAD). Assays were performed blind to the clinical diagnosis and at least twice by separate investigators.

2.2.3.2 Preparation of channel extracts

Rabbit brain was used for the preparation of VGKC and VGCC channels. Results have previously been shown to be comparable using human and rabbit cerebellar extracts as an antigenic source for potassium (unpublished data), and calcium channels (Motomura *et al.*, 1997). For use in the assay, the membrane preparation was diluted 1:5 in 2% digitonin solubilisation buffer, incubated for 1 h at 4 °C and centrifuged at 13,000 rpm for 15 min. The supernatants were removed, stored at -70 °C and used as the source of voltage-gated channels in the assay. Cultured neuroblastoma cells IMR32 were used for the preparation of nAChR $\alpha 3$ channel subunits. See appendix 3.1 for detailed methodology.

(a) immunoprecipitation assay **(b) immunoprecipitation assay**

Figure 2.4: Schematic of radioimmunoprecipitation assay. Radiolabelled (^{125}I)toxin bound to extracted channel preparation is precipitated by anti-human IgG in the presence of antibody (b), but remains in solution when autoantibodies are absent (a). IC = ion channel

2.2.3.3 Immunoprecipitation assays

1. Sera were tested for antibodies to $\alpha 3$ nAChRs using immunoprecipitation of neuroblastoma nAChRs labelled with ^{125}I -Epibatidine (^{125}I -Epb) (Perkin Elmer, MA, USA).
2. Sera were tested for antibodies to rabbit brain P/Q-type VGCCs using immunoprecipitation of VGCCs extracted from rabbit cerebellum and labelled with ^{125}I -labelled- ω -conotoxin MVIIC (^{125}I - ω -CmTx) (New England Nuclear Ltd. MA, USA).
3. Sera were tested for antibodies to rabbit brain VGKCs using immunoprecipitation of VGKCs extracted from rabbit cerebellum and labelled with ^{125}I -labelled- α -dendrotoxin (^{125}I - α -DnTx) (Perkin Elmer).
4. Sera were tested for antibodies to glutamic acid decarboxylase (GAD) using a commercially available radioimmunoassay kit (RSR Ltd., Cardiff, UK).

Detailed methodology for channel radiolabelling and immunoprecipitation assays can be found in Appendix 3.2. Briefly, defrosted aliquots of extract were labelled with a saturating (5 fM) concentration of radiolabelled toxin and incubated with diluted sera overnight at 4 °C. To precipitate serum antibodies excess sheep anti-human IgG serum was then added and incubated for 1 hour at RT. The precipitates were pelleted by centrifugation at 13,000 rpm for 5 min, washed in washing buffer (0.1% Triton X100 in 20 mM phosphate buffer, pH 7.4) and counted on an automatic gamma counter (Canberra Packard Cobra II auto-gamma, Meriden ®, CT, USA). Results were expressed as picomoles of labelled toxin binding sites precipitated per litre of serum after subtraction of non-specific binding (calculated from control mean; see Appendix 3.3 for details).

2.2.4 ANTI-M₂ MUSCARINIC CELL-BASED ASSAY

2.2.4.1 Background to methodology

This screening assay utilises cultured mammalian cells that have been transiently transfected to express the target autoantigen of interest (Hart *et al.* 1997). Sera from patients suspected to contain autoantibodies are incubated with transfected cells and any binding revealed using a fluorescently conjugated anti-human secondary antibody. Successful expression of the antigen is verified using a commercial antibody raised against the protein. This assay has a number of advantages over other techniques such as ELISA, immunoprecipitation and other immunohistochemical methods. Firstly, the entire target antigen is translated and expressed in the cell membrane which is closer to *in vivo* conditions. Secondly, this assay can be used to screen for binding to human antigens as opposed to rodent, which is frequently the tissue used in immunofluorescent screening assays. Thirdly, the antigen is specifically expressed at a high concentration, increasing assay sensitivity. Cell binding assays have been employed to detect autoantibodies in a variety of diseases and have been demonstrated to be more sensitive than traditional immunoprecipitation assays in screening for antibodies in well characterised disorders such as neuromyotonia (Kleopa *et al.* 2006; Leite *et al.* 2008).

A number of studies have reported autoantibodies present in Chagas' disease patients with GI disease which in ELISAs bind a synthetic peptide corresponding to a segment of the M₂ muscarinic acetylcholine receptor (M₂ mAChR). We designed and optimised a new cell-based assay to screen for the presence of anti-M₂ mAChR autoantibodies in patients with Chagas' disease using human embryonic kidney cells (HEK 293) transfected to express the M₂ mAChR.

2.2.4.2 Bacterial culture

Escherichia coli (*E. coli*) DH5 α were used for the preparation of plasmid DNA containing the M₂ mAChR. All media components were purchased from Oxoid Ltd., Hampshire, UK and sterilised by autoclaving at 121°C, 15 psi for 20 min. Lysogeny broth (LB; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride) and L agar were used for the routine growth and maintenance of bacteria. L agar was prepared by the addition of agar (1.5% w/v) to LB prior to autoclaving. Sterilised L agar was cooled to 55 °C and heat labile supplements were added (Ampicillin 100 µg/ml), mixed and poured into 9 cm² Sterilin® petri dishes (Sterilin Ltd., Caerphilly, UK). DH5 α was maintained on L agar at 4 °C and subcultured every four to six weeks by overnight growth. Bacterial stocks were stored at –20 °C in 0.4% glucose M9 salts minimal media containing 35 % sterile glycerol (see Appendix 4.1 for details of media compounds and culture methodology).

Growth conditions

E. coli cultures were grown aerobically at 37 °C in 250 ml conical flasks, containing 50 ml of medium with constant shaking at 250 rpm in an orbital shaker. The medium was inoculated with a 1:100 dilution of an overnight (~16 h) culture generated by seeding 2.5 ml of medium with a single colony. Growth was monitored hourly by determining the A₆₅₀ of 100 µl or 1 ml samples respectively, or dilutions thereof, using a Unicam Helios β UV spectrophotometer (Unicam, Cambridge, UK) (see Appendix 4.1 for details).

Preparation of competent cells

Once the culture reached an optical density (at 650 nm) of 0.4–0.5, cells were harvested by centrifugation at 4,000 rpm and 4 °C for 15 min. For volumes of culture less than 1.5 ml, cells were harvested by micro-centrifugation at 13,000 rpm for 5 min at 4 °C. Cells were re-suspended in 4 ml of ice-cold transformation buffer-I (TFB-I). After incubation

on ice for 15 min, the cells were harvested and taken up in 2 ml of ice-cold TBF-II (see Appendix 4.1 for TFB compositions). Competent cells were either used within 3 h or aliquoted (0.2 ml) and stored at -80°C .

2.2.4.3 Bacterial transformation

Plasmid

cDNA for the full length human cholinergic receptor, muscarinic 2 (CHRM2, GenBank NM_001006630) was cloned in-frame with the mammalian cytomegalovirus promoter using the restriction sites EcoRI-XhoI in the polylinker region of pcDNA3 (Invitrogen life technologies, provided by Carl Nelson, University of Leicester). Plasmid DNA was stored at -20°C in ultrapure (Promega UK Ltd., Southampton, UK).

Transformation

Various dilutions of plasmid DNA (1-50 ng in 4 μl) were added to 0.2 ml aliquots of competent cells, gently mixed, and incubated on ice for 40 min, followed by heat-shock at 42°C for 1 min to facilitate uptake of the DNA. After cooling on ice for 5 min, 0.7 ml of pre-warmed LB was added to the mixture and incubated at 37°C for 30–60 min to allow the expression of the antibiotic resistance genes. Finally the cells were harvested by centrifugation at 13,000 rpm for 5 min and 0.7 ml of supernatant removed. The cells were re-suspended in the remaining supernatant and plated out on L agar plates containing ampicillin and incubated at 37°C overnight (see Appendix 4.2 for detailed methodology).

2.2.4.4 Plasmid DNA isolation

Plasmid DNA Miniprep preparations

The QIAprep® Minipreps DNA Purification Kit (Qiagen Ltd., West Sussex, UK) was used according to the centrifugation protocol supplied by the manufacturer for plasmid DNA yields of less than 10 µg. Depending on the copy number of the plasmid, 1-5 ml (high-copy number plasmids) or 10 ml (low-copy number plasmids) overnight cultures were used (see Appendix 4.3 for detailed methodology).

Plasmid DNA Maxiprep preparations

For up to 850 µg plasmid DNA, a 200 ml overnight culture was manipulated using the PureLink™ HiPure Plasmid (Maxi) Filter Purification Kit (Invitrogen Ltd., Paisley, UK) according to the manufacturer's instructions. The DNA pellet was re-suspended in 200-500 µl TE Buffer (in kit) and used in downstream applications or stored at -20 °C (see Appendix 4.3 for detailed methodology).

2.2.4.5 DNA sequencing

DNA digestion with restriction endonucleases

To confirm the presence and check the correct orientation of the CHRM2 insert, the purified plasmid was analysed by DNA digestion. DNA digestion was performed using restriction enzymes (EcoR1 and Xho1) according to the manufacturer's instructions (New England BioLabs UK Ltd., Hertfordshire, UK). Approximately 5 units of enzyme per µg of DNA were incubated at 37 °C for 1–3 h (see Appendix 4.3 for detailed methodology).

Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and visualise DNA fragments, and was performed in a Bio-Rad Mini/Wide-Sub cell (Bio-Rad Labs. Ltd. Hertfordshire, UK).

Agarose gels were 0.7-1.5 %, depending on the size of the DNA fragments, in 0.5x TBE buffer (see Appendix 4.3 for composition), containing 0.5 µg/ml ethidium bromide. Samples were prepared by the addition of 6x loading buffer (Appendix 4.3) and electrophoresed in gels submerged in 0.5x TBE buffer for 45-120 min at 70-100 V (constant). The size marker, Hyperladder I (Bioline, London, UK) was loaded alongside samples for estimating both the size and quantity of DNA following gel electrophoresis. After electrophoresis the DNA bands were visualised using a 2011 Macrovue UV transilluminator (LKB Instruments, Victoria, Australia) (see Appendix 4.3 for solutions and detailed methodology).

DNA sequencing

Final validation was achieved by sequencing the CHRM2 insert from the purified plasmid DNA to confirm that the gene was in frame. DNA sequencing was performed by the in-house service at the Weatherall Institute of Molecular Medicine (WIMM) (John Radcliffe Hospital, Oxford, UK) on a 48 capillary ABI-3730 DNA cycle sequencer (Applied Biosystems, California, USA). Template plasmid DNA (>10 ng depending on the size of the insert being sequenced) was provided in solution to the WIMM sequencing service. Sequencing reactions were performed by using the T7 forward primer: 5'-TAA TAC GAC TCA CTA TAG GG-3' (3.2 pmol/µl) and for the reverse sequence the Sp6 reverse primer: 5'-ATT-TAG-GTG-ACA-CTA-TAG-3' (3.2 pmol/µl).

2.2.4.6 Mammalian cell culture

Human embryonic kidney (HEK293) cells

HEK 293 cells were grown in cell culture flasks containing Dulbecco's modified Eagles Medium (DMEM) (Sigma-Aldrich, Dorset, UK) containing fetal calf serum (FCS) (Sigma-Aldrich) and antibiotics (PSA: penicillin, amphotericin and streptomycin) (Invitrogen, Paisley, UK) (see Appendix 4.4 for compositions) and split at a ratio of 1:8

every three days. On day 3 cells were harvested following removal of DMEM by aspiration, washing in PBS and trypsinising for 1 min at 37 °C. Cells were re-suspended in DMEM containing FCS and PSA, 10 µl extracted, added to an equal amount of Trypan Blue (Sigma-Aldrich) and counted on a haemocytometer. The remaining cell suspension was centrifuged at 1000 rpm for 6 min. After removing the supernatant the remaining cell pellet was re-suspended in DMEM containing FCS and PSA. A volume containing 1.8×10^6 cells was added to DMEM containing FCS and PSA to make a final volume of 12 ml. The remaining suspension was split 1:8 and placed in a new flask for further growth.

Transfection

Cells were seeded onto poly-L-lysine coated cover-slips in 6-well tissue culture plates (3 coverslips / well). 2 ml (3×10^5 cells) of cell suspension were added to each well. Cells were maintained in growth medium overnight at 37 °C. Medium was changed to antibiotic-free DMEM and cells were transfected with the CHRM2-containing plasmid using the transfection agent polyethylenimine (PEI) (Sigma-Aldrich). This was subsequently changed to lipofectamine (Invitrogen) to reduce background staining in the immunofluorescence assay. Cells were incubated for 24 h at 37 °C with antibiotic-free DMEM containing per well: 4 µg DNA and 10 µl lipofectamine. Some cells were transfected with plasmids containing green fluorescent protein (GFP) (AB Vector, San Diego, CA, USA) as a transfection control (see Appendix 4.4 for detailed methodology).

Indirect immunofluorescence

The following day the medium was changed and cells were maintained for a further 24 h at 37 °C. Cover-slips were then transferred to 24-well culture plates washed with PBS and incubated with Chagas' disease or community control serum diluted 1:20 in DMEM containing HEPES and 1% BSA for 1 h at RT. Cells stained with a monoclonal antibody against M₂ mAChR (1:200) (Abcam, Cambridgeshire, UK) served as a positive control. As this antibody is raised against an intracellular epitope, these cells were first fixed with

4% formaldehyde for 10 min and permeabilised with 1% Triton X. Cells were washed three times in DMEM containing HEPES (DMEM wash) and incubated with a fluorescently-conjugated secondary antibody (anti-human for cells stained with serum, anti-mouse for control cells) (Invitrogen Ltd. Paisley, UK) diluted 1:700 for 45 min in the dark at RT. Cells were washed again in several changes of DMEM wash and PBS and mounted on microscope slides using fluorescent mounting medium (Dako, Cambridgeshire, UK) containing the fluorescent DNA stain 4',6-diamidino-2-phenylindole (DAPI; Roche Diagnostics Ltd. Burgess Hill, West Sussex, UK). Slides were refrigerated at 4°C in the dark and allowed to dry over night before reading (see Appendix 4.4 for detailed methodology).

2.3 FUNCTIONAL *IN VITRO* EFFECTS OF CHAGASIC SERA

2.3.1 *IN VITRO CONTRACTILITY EXPERIMENTS*

Background to methodology

Evoking changes in smooth muscle contractility by electrical field stimulation (EFS) is an established tool for the study of contractility (Bulbring *et al.* 1967; Kosterlitz *et al.* 1970). This technique involves maintaining tissue in a physiological solution between platinum ring electrodes and applying pulses of electricity across the muscle to induce a response. Relaxations and contractions are measured by way of an isometric force-transducer to which the muscle is attached and kept under a baseline muscle tension. The muscle is also kept at a constant resting, baseline tension (Figure 2.5). The initial response to EFS may be a contraction or relaxation, depending on whether excitatory or inhibitory neurons form the majority of neurons stimulated. This initial response is usually followed by an after contraction. The pharmacology of the smooth muscle responses may be investigated by adding compounds to the organ bath solution and measuring changes to contractility. The advantages of EFS over simple contractility studies using only a resting

tension is that EFS enables neurogenic responses (which occur during EFS) and myogenic responses to be distinguished.

Chagas' disease and control sera

The effect of sera from three patients with chagasic colonic dysfunction, three with Chagas' disease and no GI sequelae and three healthy community controls on human colonic smooth muscle responses to EFS were investigated as previous studies have shown that chagasic sera may increase the tone of resting smooth muscle.

Human tissue

Resection margin segments of human colon (ascending, transverse or sigmoid) were obtained from patients undergoing surgery for cancer at the Royal London Hospital. The segments were transferred to the research laboratories within 2 h after resection in ice-cold Krebs's solution (containing in mmol L): NaCl 121.5, CaCl₂ 2.5, KH₂PO₄ 1.2, KCl 4.7, MgSO₄ 1.2, NaHCO₃ 25, glucose 5.6) equilibrated with 5 % CO₂ and 95 % O₂. The segments were opened and the mucosa removed by blunt dissection and discarded. Strips (3–5 x 15 mm) from the inter-taenial parts of the segment were cut parallel to the circular muscle fibres. These strips were used immediately or stored overnight at 4 °C in fresh, oxygenated Krebs's solution before use.

Figure 2.5: Organ bath for electrical field stimulation and measurement of smooth muscle contractility. Strips of human colonic smooth muscle maintained in a physiological solution were placed between platinum ring electrodes and electrically stimulated. Smooth muscle contractions were measured by an isometric force transducer (Courtesy of Dr J Broad, Wingate Inst. of Neurogastroenterology, London).

In vitro contractility studies

The strips ($n = 4-16$ from each patient) were mounted in tissue baths containing 5 ml Kreb's solution at 37 °C and gassed with 5 % CO₂ in O₂. The change in the tension was recorded using isometric force transducers (AD Instruments, Chalgrove, UK) on a data acquisition system (Biopac Systems Inc., Goleta, CA, USA). The strips were given 2 g tensions and allowed to equilibrate for 45 min during which time the bath solutions were changed every 15 min. At the end of the equilibration period the strips were stimulated via two parallel platinum ring electrodes connected to a stimulator (STG2008, Scientifica, Uckfield, UK). The stimulation parameters were 50 V (200 mA), 5 Hz, 0.5 ms bipolar pulse duration, for 10 s, every 1 min. These EFS parameters in preliminary experiments were shown to elicit sub-maximal contractions. The effects of sera from patients with Chagas' disease were investigated by adding serum direct to the organ bath, using a single concentration (1%). The effects of control sera from healthy community

controls were also measured in each experiment. The effect of the serum in a single strip was determined from at least three EFS-induced responses. The effect of chagasic sera on cholinergic and nitrenergic pathways were investigated by pre-incubating strips with atropine (a cholinergic antagonist) or N- ω -nitro-L-arginine methyl ester (L-NAME; a nitric oxide synthase inhibitor) respectively. Serum effects were also determined following tissue pre-incubation with the voltage-gated sodium channel blocker tetrodotoxin (TTX). Minimum contact time for pharmacological agent was 30 min.

2.4 EXPRESSION AND LOCALISATION OF ENTERIC K_v1 CHANNELS IN THE HUMAN GI TRACT

2.4.1 INDIRECT IMMUNOHISTOCHEMISTRY

Background to methodology

Immunohistochemistry utilises labelled antibodies as reagents for the optical detection and localisation of antigenic material (usually proteins) in cells and tissues (Coons *et al.* 1950). As such, this method is dependent on the specificity of antibody binding to the chosen epitope. Immunohistochemistry involves a number of steps beginning with the ‘unmasking’ of antigens which may be hidden by the fixation process (e.g. formalin cross-links) with antigen retrieval. A number of techniques such as pressure cooking, protease treatment, and microwaving in an appropriate buffer may be employed, although the molecular mechanisms underlying the process are poorly understood (Shi *et al.* 2001). Following antigen retrieval, the specific primary antibody is applied and incubated with the tissue before washing to remove excess. A species-specific secondary antibody which is conjugated to a tag such as biotin or a fluorophore is applied which binds to the primary antibody. Finally, if required, a detection reagent containing a chromagen is applied that binds to the secondary conjugate and allows visualisation of the antibody complex (e.g. avidin-biotin). Primary antibody specificity should be checked using

control tissue that is known to contain the antigen, and ideally by immunoblot. Negative controls, where the primary antibody is omitted, should also be included in each experiment (Burry 2000).

The composition of all solutions and reagents are detailed in Appendix 5.1.

Clinical material

Sections from formalin-fixed, paraffin wax-embedded tissue samples of disease-free, full thickness human oesophagus, stomach, jejunum, ileum and colon were used in this study. Stomach, small intestinal and colonic tissues were identified and retrieved from the Archive of Pathology at the Royal London Hospital (London, UK). Due to a lack of archived specimens, oesophageal tissue was obtained prospectively (full thickness surplus tissue at oesophageal cancer resections) and processed in the same manner as archived tissue (see Appendix 5.2 for routine tissue processing and section preparation). Prior to immunostaining, a 3 μ m section from each tissue block was cut, collected on coated slides (VWR International, Leicestershire, UK) and stained with haematoxylin and eosin (H&E) (Appendix 5.3). These sections were then inspected using light microscopy to confirm that there were no inflammatory or other histological abnormalities. For each anatomical location studied, anti-channel immunostaining was performed on a minimum of five tissue samples, from different patients.

Control tissue

Formalin-fixed, paraffin wax-embedded murine (strain: C57 black 6) cerebella were obtained from the Blizzard Institute of Cell and Molecular Science (ICMS) Pathology Group, (Queen Mary University, London, UK) control tissue bank. KV1 channel distribution has been previously well-described in this brain region (Veh *et al.* 1995; Chung *et al.* 2001)

Indirect immunohistochemistry

Indirect immunohistochemical staining was performed using a kit-based (Vectastain Universal Elite ABC: Vector Laboratories, Burlingame, CA, USA) avidin-biotin-peroxidase complex method (see Appendix 5.4 for detailed methodology) with commercially available antibodies raised against K_v1 channel α -subunits (Santa Cruz Biotechnology Inc, CA, USA). Tissue sections were de-waxed in two changes of xylene for 2 min and dehydrated in two changes of industrial methylated spirit (IMS) for 2 min. After washing in running tap water for 5 min thermal antigen retrieval was carried out by microwaving sections in a citrate buffer (Vector Laboratories, Burlingame, CA, USA) for 25 min. Sections were allowed to cool for 5 min and washed again in running tap water for 5 min. Endogenous peroxidase activity was blocked by placing sections in methanol containing 3% H₂O₂ for 15 min. After further washing in running tap water for 5 min and soaking in wash buffer (WB; Dako, Cambs, UK) for 2 min, sections were then incubated in normal horse serum (in kit) for 20 min at RT. Excess blocking serum was tipped off and sections were incubated with primary anti-human K_v1 antibodies raised in goat diluted in antibody diluent (Dako, Cambs, UK) for 60 min at RT (see Table 2.5 for dilutions). Sections incubated with antibody diluent only served as negative controls. Primary antibodies were washed off with WB and sections incubated with secondary anti-goat biotinylated antibodies raised in horse (Vector Laboratories, Burlingame, CA, USA) diluted 1:250 in WB, for 30 min at RT. Secondary antibody was washed off with WB and sections incubated in avidin complex solution (in kit) for 30 min at RT, washed again with WB and incubated in diaminobenzidine solution (Bio-Genex-Laboratories, San Ramon, CA, USA) for 5 min. After further washing in running tap water for 5 min, sections were counter-stained in Gill's haematoxylin for 2 min, washed in running tap water for 5 min, dehydrated twice in IMS for 2 min, cleared twice in xylene for 2 min and mounted in Canada balsam (VWR International, Leicestershire, UK).

Light microscopy and qualitative analysis

Sections were analysed at magnifications of x10, x20 and x40 using a light microscope (Leitz Dialux 20, Leica Microsystems UK Ltd., Milton Keynes, UK). The presence and distribution of anti-channel immunostaining was noted and a representative area for each gut region was photographed with a digital camera using the Leica IM50 Image Manager software (Leica Microsystems, UK).

Table 2.6: Anti-K_v1 channel antibody dilutions used for indirect immunohistochemistry

Primary antibody	Dilution
K _v 1.1	1:150
K _v 1.2	1:75
K _v 1.3	1:75 *
K _v 1.4	1:75
K _v 1.5	1:75
K _v 1.6	1:75

All antibodies incubated for one hour at room temperature, except K_v1.3* which was incubated overnight at 4°C

2.4.2 CHARACTERISATION OF ENTERIC NEURONAL K_v1.4 LOCALISATION

The distribution of K_v1.4 immunostaining in enteric ganglia was distinct from other subunits (see Results sections 3.12-3.14). A similar distribution of K_v1.4 containing channels has previously been demonstrated in excitatory neurons of the hippocampal formation in the brain and was localised to axonal segments and pre-synaptic termini (Cooper *et al.* 1998). We therefore investigated whether K_v1.4 is specifically localised to these areas in enteric neurons with indirect immunofluorescent double-staining employing antibodies raised against two corresponding neuronal peptides: neurofilament

(NF) (Diepholder *et al.* 1991; Luider *et al.* 1992) and synaptophysin (Wiedenmann *et al.* 1986; Stefaneanu *et al.* 1988). Neurofilaments belong to the intermediate filament family of proteins and are found specifically in neurons where they form a key component of the axonal and dendritic cytoskeleton (Davison *et al.* 1960; Schlaepfer 1987). Synaptophysin is a glycoprotein present in the membrane of pre-synaptic vesicles (Wiedenmann *et al.* 1985).

Clinical material

Sections from formalin-fixed, paraffin wax-embedded tissue samples of disease-free, full thickness stomach were used in this study. As in the previous immunohistochemical study, tissue was obtained from the Archive of Pathology at the Royal London Hospital (Whitechapel, London, UK). This region was chosen due to tissue availability and high levels of K_v1.4 subunits exhibiting this distribution.

Indirect immunofluorescence

Tissue sections were de-waxed in two changes of xylene for 2 min and dehydrated in two changes of IMS for 2 min. After washing in running tap water for 2 min, thermal antigen retrieval was carried out by microwaving sections in a citrate buffer (Vector Laboratories, Peterborough, UK) for 18 min. Sections were allowed to cool for 5 min, were washed again in running tap water for 2 min and then incubated with a serum-free protein block (Dako, Cambs, UK) for 10 min at RT. Excess protein block was tipped off and sections were incubated with a mixture of anti-K_v1.4 and an anti-neuronal primary antibody diluted in PBS for 24 h at 4 °C (see Table 2.6 for primary antibody details and dilutions). After allowing sections to reach room temperature primary antibodies were washed off by soaking in two changes of PBS for 4 min and incubated with a mixture of appropriate Alexa Fluor® fluorescently-conjugated secondary antibodies (Invitrogen Ltd. Paisley, UK) in the dark for 45 min at RT. Secondary antibodies were washed off in two changes of PBS for 4 min and sections were mounted in fluorescence mounting medium (Dako,

Ely, Cambridgeshire, UK) containing DAPI (1:1000) for nuclear visualisation (see Appendix 5.5 for detailed methodology).

Table 2.7: Antibodies used in indirect immunofluorescence

Primary antibody	Source	Dilution	Secondary antibody
K _v 1.4	Goat polyclonal against internal region of human peptide	1:25	Alexa fluor (568) donkey anti-goat
Synaptophysin	Mouse monoclonal against coated vesicles from bovine brain	1:100	Alexa fluor (488) donkey anti-mouse
NF-2F11	Mouse monoclonal against isolated neurofilament from human brain	1:100	Alexa fluor (488) donkey anti-mouse

Primary antibodies were incubated for 1 h at RT. All secondary antibodies were used at dilution of 1:200 and incubated for 45 min at RT. NF-2F11 = neurofilament, clone 2F11.

Fluorescent microscopy and qualitative analysis

Sections were viewed with a multi-channel fluorescence microscope (Olympus BX61-UCB, Olympus UK Ltd., Essex, UK). Sequential images were captured (Cell* imaging software, Soft Imaging Solutions GmbH, Münster, Germany) and overlaid using Adobe Photoshop (Version 7.0, Adobe Systems Inc., San Jose, CA, USA).

RESULTS

3

3.1 DETECTION OF AUTOANTIBODIES

3.1.1 ANTI-NEURONAL ANTIBODY ASSAY

Twenty seven samples from patients with severe intestinal dysmotility (SID) were screened for the presence of anti-neuronal antibodies, the presence of which may be indicative of a paraneoplastic process. An indirect immunofluorescence assay using monkey cerebellum as substrate was employed to determine the presence of anti-neuronal nuclear (anti-Hu / Ri), or anti-Purkinje cell cytoplasm antibodies (anti-Yo). Commercial anti-Hu and anti-Yo antibodies were used as positive staining controls. Pooled normal serum was used as negative control. Samples which produced positive nuclear staining were sent for western blot confirmation of antigen specificity, as this assay cannot distinguish between anti-Hu and Ri antibodies.

Anti-Hu antibodies produced strong nuclear staining of cells from all three cerebellar layers (granular, purkinje and molecular). Anti-Yo staining was clearly distinguishable from anti-Hu, revealing punctate staining of Purkinje cell cytoplasm only. PNS failed to stain any cerebellar structures. Three samples from patients with SID (1 chronic intestinal pseudo-obstruction and 2 enteric dysmotility) moderately stained cell nuclei of the granular, purkinje and molecular layers and were sent for western blot confirmation. The

remaining 24 samples produced no staining. The three samples sent for confirmation were found to be negative (Figure 3.1). These negative findings minimise the likelihood that these cases of SID had a paraneoplastic basis.

Figure 3.1: Cerebellar anti-neuronal immunostaining. Sections of cerebellum were used as substrate to screen for the presence of anti-neuronal autoantibodies. Anti-Hu positive staining control stained nuclei of granular layer cells, Purkinje cells (arrow) and cells of the molecular layer. Anti-Yo positive staining control specifically stained puncta in Purkinje cell cytoplasm only. Pooled normal serum served as the negative control and failed to stain any cerebellar cells. Sera from two patients with chronic intestinal pseudo-obstruction and 1 with enteric dysmotility moderately stained cells of all three layers and required western blot confirmation. An example from one of these cases is shown in the lower right panel. All three cases were subsequently found to be negative. G = granular layer, P = Purkinje cell, M = molecular layer, CIPO = chronic intestinal pseudo-obstruction. Scale bar = 30 μ m.

3.1.2 ANTI-CHANNEL ANTIBODY RADIOIMMUNOPRECIPITATION ASSAYS

Sera from all patient groups (21 achalasia, 27 SID and 61 Chagas' disease, 20 Brazilian healthy control and 10 UK healthy controls) were screened for the presence of anti-channel antibodies using radioimmunoprecipitation assays. In each assay, with the exception of the glutamic acid decarboxylase assay, where it is considered only necessary to repeat positive results (Powell *et al.* 1996) each sample was tested twice by different investigators blinded to the diagnosis, to confirm the result. Due to limited quantities of chagasic serum samples, it was not possible to screen the full complement of 61 samples in every assay.

3.1.2.1 Ganglionic nicotinic acetylcholine receptor: alpha 3 subunit

Assays performed twice showed good agreement between results: mean difference between assays = 4 pM (95% CI -2 to 9), $p = 0.0007$. All positive and negative control sera were positive and negative in the expected titre ranges. None of the GINMD, Chagas' disease or control sera were found to be positive (Figure 3.2).

3.1.2.2 Glutamic acid decarboxylase

Three sera from Brazil showed precipitation of the complex which was comparable to the 'low positive' calibration control. Two such low positive samples were from patients with Chagas' disease, one with cardiac complications the other with colonic disease, and the third from a healthy community control. A low positive result would suggest that these individuals are predisposed to or have Type I diabetes mellitus. However, these titres are not considered high enough to have a neurological impact (Saiz *et al.* 2008; Liimatainen *et al.* 2010). None of the GINMD or UK healthy control sera were positive (Figure 3.3).

Figure 3.2: Scatter plot of results from anti- $\alpha 3$ nAChR immunoprecipitation assay.

None of the samples from any patient group were positive for anti- $\alpha 3$ antibodies. UK.HC = UK healthy controls, SID = severe intestinal dysmotility, B.HC = Brazilian healthy controls, AAN = autoimmune autonomic neuropathy. Dotted line indicates 100pM, above which samples were regarded as positive.

Figure 3.3: Scatter plot of results from anti-GAD immunoprecipitation assay. Two Chagas' disease samples and one Brazilian healthy control were weakly positive for anti-GAD antibodies. UK.HC = UK healthy controls, SID = severe intestinal dysmotility B.HC = Brazilian healthy controls.

3.1.2.3 Voltage-gated calcium channels (VGCCs)

One of 57 Chagas' disease patients showed strong precipitation of the labelled channel complex in a range comparable to LEMS positive controls (246 pM of MVIIC binding sites per litre of serum). [LEMS median: 194, range 131 to 256 pM]. At the time of venepuncture this patient had not received anti-*T.cruzi* therapy and did not have secondary manifestations of Chagas' disease. No GINMD patient sample or control sample was positive (Figure 3.4).

Figure 3.4: Scatter plot of results from anti-VGCC immunoprecipitation assay. One Chagas' disease serum sample was strongly positive for anti-P/Q type VGCC antibodies. All other samples were negative. LEMS positive controls were positive in the expected titre ranges. UK.HC = UK healthy controls, SID = severe intestinal dysmotility, B.HC = Brazilian healthy controls, LEMS = Lambert-Eaton myasthenic syndrome.

3.1.2.4 Voltage-gated potassium channels (VGKCs)

Assay repeats showed very high agreement: mean difference between assays = 12 pM (95% CI -4 to 28), $p < 0.0001$. Eight of 59 sera from patients with Chagas' disease showed precipitation of the labelled complex that was greater than 100 pM of binding sites per litre of serum. The eight positives did not however define a specific clinical phenotype: 5 were female, 5 had no heart or gut manifestations, one had achalasia and heart disease, one had achalasia only and another had colonic dysfunction only. [Whole Chagas' disease group: median 34, range -16 to 282]. Although consistently positive,

none of the eight were in a range comparable to NMT positive controls [Chagas' disease positive median: NMT median: 1199, range 776 to 1622 pM). All of the samples from patients with primary GINMD were found to be negative as were all UK and Brazilian healthy controls (Figure 3.5).

Figure 3.5: Scatter plot of results from anti-VGKC immunoprecipitation assay. Eight Chagas' disease sera were weak to moderately positive for anti-VGKC antibodies. None, however, were in the same range as the NMT positive controls. UK.HC = UK healthy controls, SID = severe intestinal dysmotility, B.HC = Brazilian healthy controls, NMT = neuromyotonia.

3.1.3 ANTI-M₂ MUSCARINIC ACETYLCHOLINE RECEPTOR CELL-BASED ASSAY

3.1.3.1 Anti-M₂ muscarinic acetylcholine receptor assay optimisation

Cell transfection efficiency was measured by green fluorescent protein (GFP) transfection where protein expression results in cells fluorescing green. This was performed in several assays and was judged as good by the fact the majority of cells in several microscopy views were positive (Figure 3.6). The success of M₂ muscarinic acetylcholine receptor (M₂ mAChR) transfection was confirmed using a commercially available monoclonal antibody raised against the M₂ mAChR in indirect immunofluorescence assays. Only antibodies raised against an internal region of the receptor peptide were available at the time of assay development. It was therefore necessary to first permeabilise transfected cells prior to antibody application in order for the antibodies to gain access to the epitope. Whilst it would have been preferable for cells to have remained intact, cells permeabilised and stained with the monoclonal anti-M₂ mAChR antibody had clearly circumscribed borders suggesting that the cells were not severely disrupted and the receptor was expressed in the cell membrane (Figure 3.6). This procedure was subsequently used as a positive control in every assay. Negative controls consisted of cells incubated with antibody diluent only, and gave only a low background signal.

3.1.3.2 Screening of chagasic sera for anti-M₂ mAChR autoantibodies

None of 57 sera samples from patients with Chagas' disease stained the membranes of M₂ mAChR transfected cells. Similarly, Brazilian healthy community control samples failed to produce positive staining (Figure 3.7).

Figure 3.6: Indirect immunofluorescent staining of transfected HEK293 cells with anti-M₂ mAChR antibody. GFP-transfected cells auto-fluoresced green demonstrating successful transfection. Cells transfected with M₂ mAChR-containing plasmids and incubated with a monoclonal anti-M₂ antibody served as the positive control and revealed membranous immunostaining. Transfected cells incubated with antibody diluent only served as the negative control and produced no staining. Scale bar = 10µm.

Figure 3.7: Indirect immunofluorescent staining of transfected HEK293 cells with chagasic sera. Chagasic serum samples failed to bind M_2 mAChR-transfected cells as demonstrated by indirect immunofluorescence using an anti-human fluorescently-conjugated secondary antibody. Similarly, control serum taken from healthy individuals showed no binding. Scale bar = $10\mu\text{m}$.

3.2 FUNCTIONAL *IN VITRO* EFFECTS OF CHAGASIC SERA

Following the negativity of the M_2 mAChR cell-based assay we investigated the effects of chagasic sera on human colonic smooth muscle contractions. Due to time constraints the work presented here is preliminary. The effects of sera from three patients with chagasic colonic disease on colonic smooth muscle contractility were investigated. These effects were compared with those of three samples from patients with Chagas' disease and no GI disease and from three healthy community controls.

Table 3.1: Subjects investigated for sera effects on colonic smooth muscle contractility

Subject	Age / sex	Details
1	57 F	Chagasic colonic disease
2	53 M	Chagasic colonic disease
3	46 M	Chagasic colonic disease
4	39 M	Chagas' disease, no sequelae
5	37 F	Chagas' disease, no sequelae
6	35 M	Chagas' disease, no sequelae
7	40 M	Healthy community control
8	45 F	Healthy community control
9	33 M	Healthy community control

3.2.1.1 Characterisation of responses of human colonic smooth muscle to electrical field stimulation, atropine and L-NAME

Responses to electrical field stimulation (EFS) in muscle strips from the same patient were heterogeneous and consisted of contractions (55 %; 21 / 38 strips; n = 9 colons), relaxations (32 %) or no response (13 %) and were usually followed by a large after-contraction (Figure 3.8A). Responses could be blocked by the sodium channel blocker tetrodotoxin, 1 μ M (n = 3 colons), indicating that they were neurally mediated (Figure 3.8B). The addition of atropine 1 μ M, a mAChR antagonist, prevented contractions during EFS (n = 3 colons) revealing, or facilitating muscle relaxation, and strongly attenuated after-contractions. L-NAME 0.3 mM, the nitric oxide synthase inhibitor, prevented muscle relaxation during EFS (n = 4 colons) (Figure 3.8B).

Figure 3.8: Human colonic smooth muscle responses to EFS and pharmacological modulation of responses. A: The initial response to EFS was a contraction mediated by acetylcholine, or a relaxation mediated by nitric oxide. Each response was usually followed by a spike representing a large after-contraction. These are mediated by acetylcholine or a neurokinin. (Horizontal bar beneath traces represents the duration of EFS). B: Application of tetrodotoxin prevented EFS responses indicating that they were neurally mediated. Atropine prevented muscle contraction during EFS, revealing a relaxation, and strongly reduced the magnitude of the after-contraction. L-NAME prevented muscle relaxation during EFS and increased basal muscle tension. ACh = acetylcholine, NK = neurokinin.

3.2.1.2 Effect of sera on human colonic smooth muscle responses to EFS

The only consistent, measurable effect caused by addition of serum to colonic muscle stimulated by EFS was a change in baseline muscle tension. Application of 100 μ l (bath concentration 1%) of serum from three patients with chagasic colonic disease caused a consistent reduction in baseline muscle tension ($n = 4$ colons / patient; 12/12 relaxations) without a corresponding effect on the magnitude of responses to EFS (Figure 3.9). The overall reduction in muscle tension (mean $\pm$ SEM) was 36 ± 6 mg ($p < 0.05$, one sample t test; Figure 3.12). Contractions during EFS were 104 ± 13 % of magnitude of contractions before addition of serum; Figure 3.9, which persisted for at least 30 min.

Application of 100 μ l of serum from three Chagas' disease control patients (with no GI sequelae) produced variable responses in baseline muscle tension. The mean effect on baseline muscle tension was not significant (2 ± 15 mg; $n = 4$ colons / patient; 7/12 relaxations; Figures 3.10 and 3.12). The addition of three serum samples from healthy community controls also produced mixed responses, the mean of which was not significant (-7 ± 32 mg; 4 colons / patient; 7/12 relaxations; Figures 3.10 and 3.12).

Figure 3.9: Effects of serum from patients with Chagas' colon disease on human colonic smooth muscle responses to EFS. Following addition of serum from patients with Chagas' colon disease, baseline muscle tension always decreased, although to varying degrees. The magnitude of the response during EFS was unchanged.

Chagas disease control

Healthy community control

Figure 3.10: Effects of serum from Chagas' disease and healthy controls patients on human colonic smooth muscle responses to EFS. Following addition of serum from patients with Chagas' disease and no GI sequelae or from healthy community controls EFS responses were unchanged. Changes in baseline muscle tension varied, but overall were not significant.

3.2.1.3 Effect of chagasic sera on EFS-induced human colonic smooth muscle contractions in the presence of atropine or L-NAME

To investigate whether the effect of serum from Chagas' colon disease involved a mechanism dependent on mAChR activation, as reported in previous studies, the effects of these sera were tested in the presence of the mAChR antagonist atropine. Atropine (1 μ M) was applied to organ baths and allowed 30 min contact time with muscle strips. Serum was then applied as before. In the presence of atropine, serum continued to cause a reduction in baseline muscle tone. Although the relaxation was slightly attenuated, it was consistent, as before (-31 ± 11 mg; $n = 3$ colons / patient; 9/9 relaxations; Figures 3.11A

and 3.12), demonstrating that the observed effect is not dependent on mAChR activation. The response was further examined by application of the nitric oxide synthase inhibitor L-NAME 0.3 mM, to investigate whether the effect was dependent on nitric oxide production. Following 30 min compound contact time, addition of serum reduced muscle tension by 54 ± 11 mg ($n = 3$ colons / patient; 9/9 relaxations) showing that the response is independent of nitric oxide production (Figures 3.11B and 3.12). In further experiments, Tetrodotoxin (TTX), which blocks voltage-gated sodium channels essential for neuronal action potential generation, was applied to muscle to block neuronal activity. Following pre-incubation with $1\mu\text{M}$ TTX, all three Chagas' colon disease serum samples produced a reduction in basal muscle tension (-20 ± 6 mg; $n = 3$ colons / patient, 7/9 relaxations) which although attenuated compared to toxin free preparations, were not significantly different. This demonstrates that although the effect may perhaps in part involve neuronal mechanisms, it is not dependent on neuronal activity.

Figure 3.11: Serum effects on human colonic smooth muscle EFS responses in the presence of atropine and L-NAME. The decrease in baseline tension following the addition of serum from a patient with chagasic colonic dysfunction was maintained in the presence of atropine (A) and L-NAME (B) suggesting that circulating factors were not exerting their effects via muscarinic cholinergic receptor activation or via facilitation of nitric oxide synthesis. Red dashed line represents baseline muscle tension prior to addition of serum.

Figure 3.12: Serum effects on human colonic baseline muscle tension in the presence of atropine and L-NAME. Serum from three patients with Chagas' colon disease produced a significant reduction in colonic smooth muscle tone. Serum from three patients with Chagas' disease but no sequelae variably changed basal muscle tone, however these changes were not significant. Responses to serum from three healthy individuals from the same community were more variable, overall producing a reduction in smooth muscle tone. This effect however was not significant. In the presence of 1 μ M atropine the relaxant effect on baseline muscle tension was preserved, although slightly attenuated, demonstrating the effect is not dependent on muscarinic cholinergic receptor activation. Relaxations continued in the presence of 300 μ M L-NAME, and appeared to be slightly facilitated, revealing that they were not the result of nitric oxide production. Tetrodotoxin (TTX; 1 μ M) slightly attenuated but did not abolish the relaxant effect of serum, demonstrating that although this effect may involve neuronal pathways, it is not dependent on neuronal action potentials.

3.3 K_V1 CHANNEL DISTRIBUTION

The distribution of six members of the K_V1 VGKC subfamily was investigated immunohistochemically in the human GI tract. Antibody specificity and optimal working dilutions were first determined by immunostaining murine cerebellum.

3.3.1 MURINE CEREBELLUM

The distribution of K_V1 channels has been previously established in rodent cerebellum and allowed us to confirm the specificity of our antibodies and determine optimal working dilutions. No staining was seen in the negative control sections where the primary anti-K_V1 antibodies were omitted but the secondary detection system was used as normal, indicating areas of staining seen in test sections were not due to non-specific secondary antibody binding (Figure 3.13). The staining patterns we observed in mouse cerebellum with the anti-K_V1 antibodies (Figure 3.14) were the same as those described in previous reports (Veh *et al.* 1995; Chung *et al.* 2001). K_V1.1 was found in basket cell axon terminals around Purkinje cells and also weakly in the neuropil within the granule cell layer. K_V1.2 immunoreactivity also appeared in basket cell axon terminals, in greater abundance to K_V1.1. Immunoreactivity for K_V1.3 was found in Purkinje cells, Golgi-type II cells of the granular layer and basket cells of the molecular layer. Purkinje cell bodies were stained with anti-K_V1.4, 1.5 and 1.6. K_V1.6 immunoreactivity was also found in basket cells of the molecular layer and Golgi type II cells of the granular layer. No staining was seen in the negative control sections. Human and mouse K_V1 channels are homologous. Therefore, having confirmed that the anti-K_V1 antibodies bound as expected in murine cerebellum, we proceeded to immunohistochemically investigate K_V1 distribution in the human GI tract. For the purposes of this thesis and considering the questionable value of quantitative immunohistochemistry (Polak *et al.* 2003), this is a descriptive study only.

Figure 3.13: Negative immunostaining control of mouse cerebellum. Sections of mouse cerebellum incubated with antibody diluent in the absence of anti-KV1 antibodies showed no staining confirming that the secondary detection system, which was used as normal, was not responsible for any staining subsequently observed. G = granule cell layer, M = molecular layer, P = Purkinje cell. Scale bar = 25 μ m.

Figure 3.14: Anti-K_v1 immunostaining of mouse cerebellum. K_v1.1 and 1.2 localised specifically to basket cell axon terminals around Purkinje cells (arrow and inset, respectively). K_v1.3 was found in Purkinje cell bodies, Golgi type II cells of the granular layer (arrow) and basket cells of the molecular layer (star). K_v1.4 immunoreactivity was also found in Purkinje cell bodies. K_v1.5 strongly stained Purkinje cell bodies. K_v1.6 localised to Purkinje cell bodies, basket cells (star) and Golgi type II cells of the granular layer (arrow). M = molecular layer, P = Purkinje cell layer, G = granular layer; WM, white matter. Scale bar = 25 μ m.

3.3.2 OESOPHAGUS

Oesophageal epithelium

The oesophageal epithelium did not show immunopositivity for any of the six K_V1 channel subunits (Figure 3.15).

Oesophageal neurons

Anti-K_V1.1 strongly immunostained cell bodies of the myenteric plexus. Cell bodies also exhibited moderate cytoplasmic immunoreactivity for K_V1.5. Anti-K_V1.2 and K_V1.4 weakly stained myenteric cell bodies, K_V1.2 exhibited a cytoplasmic distribution and K_V1.4 staining cell nuclei. Anti-K_V1.3 and K_V1.6 immunostaining was not above background levels (Figure 3.16).

Oesophageal smooth muscle

K_V1.5 was found to be localised myocytes of the oesophageal muscularis propria and arterioles. Anti-K_V1.5 immunostaining identified discrete patches of punctate staining, which often appeared to be intracellular, specifically peri-nuclear, in distribution (Figure 3.17). No other channel subunit was identified in smooth muscle cells.

Figure 3.15: Anti-Kv1 immunostaining of oesophageal epithelium. All cells of the squamous epithelium were negative for Kv1.1-1.6 immunoreactivity. E, epithelium; SM, submucosa. Scale bar = 50 μ m.

Figure 3.16: Anti-K_v1 immunostaining of human oesophageal myenteric ganglia.

K_v1.1 was found in neuronal cell bodies in a cytoplasmic distribution. K_v1.2 immunostaining was weak in neuronal cell bodies. K_v1.3 was not detected in oesophageal myenteric ganglia. Neuronal nuclei were weakly immunopositive for K_v1.4 (arrow). K_v1.5 was distributed in the cytoplasm of cell bodies and K_v1.6 was not found to be present. Scale bar = 10 μ m.

Figure 3.17: Anti-K_v1.5 immunostaining of human oesophageal smooth muscle.

K_v1.5 containing puncta were abundant throughout muscularis propria and in arteriolar myocytes. These puncta were often observed to be intracellular, and often peri-nuclear (inset). Scale bar = 10 μ m.

3.3.3 *STOMACH*

Gastric mucosa

K_v1 immunostaining of the gastric epithelium labelled Chief cells (as identified by H&E appearance) where intracellular granular staining was present. Immunostaining for K_v1.1, K_v1.2, K_v1.3 and K_v1.4 was strong compared with K_v1.5 and K_v1.6 which were moderate. Parietal cells and surface epithelial cell staining was not above background levels (Figure 3.18). The muscularis mucosa was also negative (data not shown).

Gastric neurons

K_v1 subunits exhibited a selective distribution in gastric submucosal and myenteric ganglia (Figures 3.19 and 3.20, respectively). Cell bodies and the neuropil of submucosal and myenteric ganglia were weakly immunopositive for K_v1.1. Cell bodies of both plexi intensely stained for K_v1.2. Anti-K_v1.3 immunostaining also localised to cell bodies with the additional observation of nuclear immunostaining. Anti-K_v1.4 failed to stain submucosal cells bodies, however immunostaining of K_v1.4 was scattered around the periphery of myenteric ganglion in a punctate distribution. At light microscopic

magnifications it was not possible to identify these channel-rich structures. Certain myenteric cell bodies were also strongly immunopositive for K_v1.4. K_v1.5 immunostaining was moderate to strong in submucosal and myenteric cell bodies with. K_v1.6 immunostaining was not consistently above background levels. Antibodies to K_v1 subunits did not label circular or longitudinal layers of muscularis externae.

Figure 3.18: Kv1 immunostaining of human gastric epithelium. Cells located towards the base of gastric glands identified as Chief cells were strongly stained with anti-Kv1.1 (inset; dark brown), Kv1.2, Kv1.3 and Kv1.4 antibodies. Chief cells were moderately stained with antibodies against Kv1.5 and Kv1.6. Surface epithelial cells and parietal cells were negative (membranous staining of surface epithelial cells seen in Kv1.6 slide was considered to be artefact). Scale bar = 50 μ m.

Figure 3.19: Kv1 immunostaining of human gastric submucosal neurons. Anti-Kv1.1 weakly stained neuronal cell bodies (arrow) whereas Kv1.2 and 1.3 were strong. Kv1.4 and 1.6 were not found to be expressed by gastric submucosal ganglia (arrow). Anti-Kv1.5 moderately stained cell bodies. Scale bar = 10 μ m.

Figure 3.20: Kv1 immunostaining of human gastric myenteric ganglia. Gastric myenteric neurons were immunopositive for Kv1.1 – 1.5. Anti-Kv1.2, 1.3 and 1.5 antibodies selectively labelled neuronal cell bodies. Kv1.1 was also present in the surrounding neuropil. Kv1.4 exhibited a unique punctuate distribution at the periphery of ganglia and certain neuronal cell bodies (inset). Kv1.6 immunostaining was not above background. Scale bar = 10 μ m.

3.3.4 JEJUNUM

Jejunal mucosa

As in the stomach, K_V1 immunostaining was localised to jejunal epithelial cells (Figure 3.21). Both absorptive enterocytes and goblet cells were immunopositive for both cytoplasm and surface membranes, albeit moderately. Epithelial cells towards the villi tips were more strongly stained than those of the crypts. K_V1.2 was the most abundant subunit in jejunal epithelium. K_V1.1 and K_V1.3 immunostains were moderately positive whereas anti-K_V1.4 and K_V1.5 were weak. K_V1.6 failed to stain above background levels. Anti-K_V1.1, 1.3, 1.4 and 1.6 antibodies specifically immunolabelled a subset of cells, presumed to be enteroendocrine cells (based on their morphology) of jejunal epithelium, particularly in crypts (Figure 3.21, arrows). The muscularis mucosa was negative for all six channel subunits.

Jejunal neurons

K_V1.1 immunostaining was strong in selected ganglion cell bodies of the submucosal plexus and stained the neuropil of myenteric ganglia. K_V1.2 localised specifically to certain cell bodies of both plexuses, with little staining of the neuropil. Submucosal and myenteric cells, including cell nuclei, were strongly positive for K_V1.3. No specific pattern regarding positivity of large vs. small neurons was discernable. As in the stomach, immunoreactivity for K_V1.4 was scattered throughout enteric ganglia in a peripheral punctate distribution with very little staining of cell bodies. K_V1.5 appeared very specific for cell bodies of both ganglia with no staining of the neuropil. Submucosal and myenteric neuronal cell bodies were negative for K_V1.6 (Figure 3.22 and 3.23). Smooth muscle cells of the jejunum appeared negative for all K_V1 immunostaining (not shown).

Figure 3.21: K_v1 immunostaining of human jejunal mucosa. K_v1 subunits were specifically observed in surface epithelial cells, K_v1.2 appearing to be most abundant. Crypt epithelial cells were also stained, although at a lower intensity. K_v1.1 and 1.3 immunostaining were moderate, K_v1.4 and K_v1.5 were weak and K_v1.6 was not above background staining. A subset of cells, presumed by appearance to be enteroendocrine, were strongly stained at their basal aspect by anti-K_v1.1 (inset), 1.3, 1.4 and 1.6 (arrows). Scale bar = 50 μ m.

Figure 3.22: Kv1 immunostaining of human jejunal submucosal ganglia. Jejunal submucosal neurons were moderate-strongly stained with anti-Kv1 antibodies. Kv1.1, 1.2, 1.3 and 1.5 were localised to a subset of neuronal cells bodies. In addition to cytoplasmic staining, Kv1.3 also exhibited nuclear staining. Kv1.4 uniquely immunostained discrete structures around ganglion cells bodies and ganglia periphery, in a punctate distribution (inset). Kv1.6 failed to stain above background. Scale bar = 10 μ m.

Figure 3.23: Kv1 immunostaining of jejunal myenteric ganglia. Anti-Kv1.1 immunostained the neuropil of myenteric ganglia, Kv1.2 and 1.5 localised to neuronal cell bodies and Kv1.3 immunostained both, including neuronal nuclei. Kv1.4 stained discrete fine punctate patches scattered around the periphery of ganglia and certain cell bodies (inset). Kv1.6 failed to stain ganglia. Scale bar = 10 μ m.

3.3.5 ILEUM

Ileal mucosa

The transition between immunopositive and immunonegative cells from villi to crypts was more pronounced in the ileum (Figure 3.24). Ileal enterocytes and goblet cells were strongly immunopositive for K_V1.2 and K_V1.3. K_V1.1 and K_V1.5 also localised to surface epithelial cells although at a lower intensity. As in the jejunum, anti-K_V1.4 and K_V1.6 antibodies only weakly stained epithelial cells. Presumed enteroendocrine cells expressed a high density of K_V1.1, 1.2, 1.3, 1.4 and 1.6 in their basal regions. Cells of the lamina propria and muscularis mucosa were negative.

Ileal neurons

Immunostaining for K_V1.1 was largely confined to the neuropil of myenteric and submucosal ganglia. K_V1.2 was selectively present in high density in ganglion cell bodies. K_V1.3 exhibited nuclear localisation, appearing highly expressed in submucosal ganglion cell bodies and moderately in myenteric neurons. As in the stomach and jejunum, K_V1.4 immunostaining was scattered throughout ganglia of both plexi in a punctate distribution, which appears unique to this subunit. Certain ileal ganglion cell bodies also appeared weakly K_V1.4 positive. A high density of K_V1.5 subunits was observed in submucosal ganglion cell bodies and a moderate concentration in myenteric ganglion cells. K_V1.6 immunostaining was only weakly positive in submucosal cell bodies and was not present in myenteric cell bodies (Figure 3.25 and 3.26). As in the stomach and jejunum, ileal smooth muscle cells were not immunostained with anti-K_V1 antibodies (not shown).

Figure 3.24: Kv1 immunostaining of human ileal mucosa. Kv1 subunits were localised to ileal enterocytes. Anti-Kv1.2 and 1.3 antibodies strongly stained surface enterocytes and moderately stained crypt cells. Kv1.1 and 1.5 appeared moderately expressed by ileal enterocytes. Kv1.4 and Kv1.6 appeared only weakly expressed in enterocytes. Enteroendocrine cells were intensely stained at their basal aspect by anti-Kv1.1 (inset), 1.2, 1.3, 1.4 and 1.6 (arrows). Lamina propria and muscularis mucosa were negative. Scale bar = 50 μ m.

Figure 3.25: Kv1 immunostaining of ileal submucosal ganglia. Kv1.1 was localised to the myenteric neuropil, whereas Kv1.2 was limited to cell bodies only. Kv1.3 exhibited strong nuclear (arrows) and cell body cytoplasmic staining. Kv1.4 was present in fine puncta adjacent to certain cell bodies (inset) and towards ganglia periphery and weakly in cell bodies. Cell bodies strongly stained for Kv1.5 whereas Kv1.6 was only weakly present. Scale bar = 10 μ m.

Figure 3.26: Kv1 immunostaining of ileal myenteric ganglia. The neuropil was moderately positive for Kv1.1. Kv1.2 appeared highly expressed by some myenteric cells bodies and moderately by others. Kv1.3 exhibited nuclear staining with moderate staining of cell body cytoplasm. Kv1.4 expression appeared restricted to discrete puncta (inset) with occasional weak cell body staining. Kv1.5 moderately stained cell bodies. Ganglia were negative for Kv1.6. Scale bar = 10 μ m.

3.3.6 COLON

Colonic mucosa

The gradient of K_V1 expression in surface compared with deep epithelial cells was most pronounced in the colon (Figure 3.27). Surface enterocytes and goblet cells appeared moderately to strongly positive for all six subunits. Crypt enterocytes appeared largely negative except for enteroendocrine cells which displayed immunoreactivity for K_V1.1, 1.4 and 1.6 (Figure 3.27). Lamina propria and muscularis mucosa were negative.

Colonic neurons

Submucosal ganglion cell bodies and neuropil were moderately immunostained with anti-K_V1.1. In myenteric ganglia this appeared largely restricted to the neuropil where only selected cell bodies appeared positive. K_V1.2 immunostaining was selectively localised to cell bodies which were moderately immunopositive in submucosal ganglia. Selected myenteric ganglion cell bodies were strongly K_V1.2 immunopositive whereas others appeared negative. K_V1.3 localised to neuronal cell bodies of both plexuses, more strongly in the submucosal, with the additional observation of nuclear staining. Submucosal cell bodies were moderately positive for K_V1.4 whereas myenteric cells displayed only weak immunostaining. Punctate staining of K_V1.4 containing structures observed in gastric, jejunal and ileal ganglia were not seen in the colon. In both plexuses K_V1.4 was localised to cell bodies, moderately in submucosal and weakly in myenteric. K_V1.5 immunostaining was strongly present in submucosal and selected myenteric cell bodies. K_V1.6 appeared to be moderately expressed by submucosal cell bodies but only weakly expressed in myenteric cell bodies and neuropil (Figures 3.28.and 3.29, respectively). Colonic smooth muscle appeared negative for K_V1 subunit expression (not shown).

Figure 3.27: Kv1 immunostaining of colonic mucosa. Surface enterocytes appeared positive for Kv1.1-1.6 channel expression, whereas deep epithelial cells appeared negative. Kv1.1, 1.4 and 1.6 were also present in the basolateral membrane of a subset of cells (presumed enteroendocrine, inset and arrows). Muscularis mucosa immunostaining was not above background levels. Scale bar = 50 μ m.

Figure 3.28: Kv1 immunostaining of colonic submucosal ganglia. Kv1.1 immunostaining was present in both neuronal cell bodies and the surrounding neuropil whereas Kv1.2 appeared restricted to cell bodies. Neuronal cell bodies appeared to strongly stain for Kv1.3, both cytoplasmic and nuclear. Immunostaining for Kv1.4 and 1.6 was moderately strong in cell bodies. Kv1.5 immunostaining was strong. Scale bar = 10 μ m.

Figure 3.29: Kv1 immunostaining of colonic myenteric ganglia. Kv1.1 was largely confined to the neuropil. Selected neuronal cell bodies appeared to strongly stain for Kv1.2, 1.3 and 1.5. Kv1.1 was also present in the neuropil. Kv1.3 stained both cytoplasm and cell nuclei. Kv1.4 and Kv1.6 weakly immunostained the neuropil and cell bodies. Scale bar = 10 μ m.

3.3.7 *K_V1.4 LOCALISATION IN HUMAN GASTRIC MYENTERIC PLEXUS*

Following the identification of a unique punctate distribution of K_V1.4 in enteric ganglia, the distribution of this subunit was further investigated using double-labelling immunofluorescent staining in the gastric myenteric plexus.

K_V1.4 immunofluorescent staining

In the same manner as that observed with DAB immunohistochemistry, tissue immunofluorescently stained for K_V1.4 exhibited multiple fine puncta throughout ganglia, often clustered around the ganglion cell bodies and ganglia periphery. Immunofluorescent staining revealed more K_V1.4 containing structures than the DAB method, which, in addition to simple puncta also defined small vesicular structures (Figure 3.30).

Neurofilament and K_V1.4

Neurofilament immunostaining was dense throughout the myenteric plexus, identifying axons and some cell bodies of myenteric ganglia. Where axons were cut transversely, NF staining appeared punctuate, although as larger patches than K_V1.4 puncta. K_V1.4 immunoreactivity was often closely associated with NF and occasionally localised to the periphery of NF-positive structures suggesting this subunit may be localised to a subpopulation of axons (Figure 3.30).

Synaptophysin and K_V1.4

Synaptophysin immunoreactivity was present throughout ganglia, concentrated at ganglia peripheries and surrounding cell bodies. Double-labelling with anti-K_V1.4 revealed frequent but not universal overlap with K_V1.4 puncta, suggesting channels are located at a subpopulation of synaptic terminals (Figure 3.30).

Figure 3.30: Co-localisation of Kv1.4 and neuronal markers in the gastric myenteric plexus. Kv1.4 exhibited punctate distribution throughout myenteric ganglia and also occasional structures which appeared vesicular. Left column: NF immunostaining revealed multiple large puncta within ganglia. Occasional overlap was seen between Kv1.4 and NF puncta (inset), suggesting localisation to a subpopulation of axons and / or dendrites. Right column: Synaptophysin immunoreactivity was also punctate, surrounding cell bodies, and also defined larger, branching areas, presumably representing the divergence of axonal terminals. Kv1.4 and synaptophysin immunostaining frequently overlapped (inset), suggesting localisation to a subpopulation of synaptic terminals. Syp = synaptophysin, NF = neurofilament. Scale bar = 20 μm .

DISCUSSION

4

4.1 ORIGINAL PROJECT AIMS

4. To investigate the presence of autoantibodies directed to enteric ion channels in patients with neuromuscular disorders of the gut leading to severe dysmotility.
5. To investigate whether autoantibodies (if present) are pathogenic.
6. To further elucidate the profile of expressed ion channels in the human enteric neuromusculature.

4.2 SUMMARY OF MAIN RESULTS

The main results of each section of this thesis will be highlighted and discussed in relation to existing studies relevant to this field.

Finding 1: Humoral autoimmunity against currently recognised neuronal antigens is not a common cause of primary GINMD

The presence of circulating autoantibodies against a number of neuronal antigens was determined in 21 patients with primary achalasia and 27 patients with severe intestinal dysmotility (SID) using well validated radioimmunoprecipitation assays. The targets of interest were voltage- and ligand-gated ion channels and a neuronal enzyme which are

known antigens of pathogenic autoantibodies in autoimmune peripheral and central neurological diseases. These were group 1 voltage-gated potassium channels (VGKC / K_v1; acquired neuromyotonia, and limbic encephalitis), voltage-gated calcium channels (VGCC; Lambert-Eaton myasthenic syndrome), ganglionic α 3 nicotinic acetylcholine receptors (α 3 nAChR; autoimmune autonomic neuropathy) and glutamic acid decarboxylase (GAD; epilepsy and cerebellar ataxia). Sera from patients with SID were also screened for the presence of anti-neuronal antibodies (ANAs) which are frequently present in dysmotility secondary to neoplasia. All patients were negative for anti-channel and anti-neuronal antibodies.

Study limitations

It is possible the negativity of our screening could be due to inadequate assay sensitivity or specificity. This is unlikely as the radioimmunoprecipitation assays employed are well-validated (Apiwattanakul *et al.* 2010) and are routinely used in clinical diagnosis. Methodological error is also an unlikely cause of our negative findings as disease control samples included in each assay were consistently positive in the expected titre ranges and each assay was repeated at least twice to confirm results. It is more likely that the converse is true, i.e. the approach taken was too specific and that we might, with hindsight, have used a broader although less specific screening method before embarking on the detailed assays used (see below). Thus, a limitation of our study was the small number of targets screened for. We included antigens which are proven pathogenic targets in peripheral neuromuscular and central nervous system (CNS) diseases, autoantibodies against which have also been previously identified in patients with a secondary GINMD, particularly in association with cancer, and in small numbers of patients with a primary GINMD. Although we included almost all of these antigens, due to time constraints, some autoantibodies were not screened. These included N-type VGCCs, which have been found in primary achalasia, delayed gastric emptying and slow transit constipation (Dhamija *et al.* 2008; Kraichely *et al.* 2009). It is important to point

out that the number of targets for autoantibodies is continually growing, an example being previously 'seronegative' cases of myasthenia gravis now recognised as positive for autoantibodies against a muscle-specific receptor tyrosine kinase (MuSK) (Hoch *et al.* 2001; Wolfe *et al.* 2008). The number of assays available is far behind the number of potential targets, being limited, in the case of radioimmunoprecipitation assays, by the availability of specific toxins to label the channel of interest.

A screening tool which has been used extensively in the investigation of humoral autoimmunity in achalasia is indirect immunofluorescent staining of the target tissue using patient serum (Storch *et al.* 1995; Verne *et al.* 1997; Moses *et al.* 2003). Whilst a positive result does not provide limited information regarding the specific autoantigen, it is a simple means by which evidence for or against 'anti-gut' autoantibodies can be gathered before proceeding to more complex assays to determine the antigen identity. An advantage of this method is that the specificity of the IgG for the gut and gut region may be determined. However, caution should be applied in interpreting results. False-positive results could arise due to non-specific binding, which without a specific antigen in mind is difficult to control for. False-negative results may occur as consequence variable antigen availability following tissue processing and due to temporal or sub-regional variation in antigen expression. There is also a precedent in the study of achalasia for HLA-typing. Certain alleles have been shown to be significantly associated with achalasia and also in this regard with the presence of anti-neuronal antibodies (Ruiz-de-Leon *et al.* 2002), although a correlation with antibodies may not be true for every population (Latiano *et al.* 2006). Nevertheless, HLA-typing of our achalasia patients may have allowed us to better select those in whom the presence of anti-neuronal antibodies is more likely. Identification of a specific HLA allelic association in pseudo-obstruction and enteric dysmotility would lend further support to an autoimmune mechanism, but to date there is no precedent for this.

Significance and interpretations

Primary GINMD are relatively rare disorders and findings of anti-neuronal or anti-channel autoantibodies have previously been restricted to case-reports or small cohorts. The number of cases screened in our study (21 achalasia and 27 SID) represents one of the largest cohorts of primary GINMD studied in this manner to date. A similar study (Dhamija *et al.* 2008) screened 29 patients with GINMD affecting one or more levels of the gut. This study was designed to investigate the profile of autoantibodies useful in the diagnosis of suspected 'autoimmune gastrointestinal dysmotility' (AGID) as all patients had been selected due to the proven existence of one or more autoantibody. AGID represents a group of patients who have a limited form of dysautonomia with prominent GI symptoms of hyper- or hypo-motility and one or more autoantibodies against cell-surface cation channels and includes GI dysmotility secondary to neoplasia, diabetes mellitus and hypothyroidism. It may also occur idiopathically as a manifestation of autoimmune autonomic neuropathies (Lennon *et al.* 1991; Smith *et al.* 1997; Vernino *et al.* 2000). Although the study by Dhamija *et al.* screened 29 cases, only 8 of these could be considered as primary GINMD, the others having neoplasia and / or other disease. Anti-P/Q-type VGCC antibodies were found in a case of primary achalasia, anti-N-type VGCC in a patient with slow small intestinal transit, anti-muscle nAChR in another case of primary achalasia, anti-ganglionic $\alpha 3$ nAChR in two patients with delayed gastric emptying and in a patient with delayed small intestinal and colonic transit, who also had anti-VGKC antibodies, and finally anti-VGKC antibodies in a patient with delayed colonic transit. Whilst useful in defining which autoantibodies are indicative of AGID, the selectivity bias and heterogeneity of findings means that the results of this study cannot be generalised to unexplained GINMD. All of our patients were selected on the basis of a confirmed primary GINMD and had not previously been investigated for anti-channel autoimmunity. All patients with SID were subsequently confirmed to be negative for anti-neuronal antibodies (ANAs) commonly associated with neoplasia (anti-Hu, Yo and Ri). In light of the results of Dhamija *et al.* it is surprising that none of our patients

had detectable autoantibodies. An important reason for this could be the chronicity of the disorders in our patient groups. Dhamija *et al.* do not provide information regarding symptom duration in their patients, however the median duration of follow-up was only 25 months (range 2-127). Two studies have previously screened unselected patients with primary GI dysmotility. The first of these detected high titres of anti-VGKC antibodies in two of eleven patients with colonic slow transit constipation, one gave a 6 year history, the other had had symptoms for only 1 year (Knowles *et al.* 2002). The second study screened 33 patients. The median duration of dysmotility symptoms for this group was 18 years (range 5-60). Two patients with a 9 and 12 year history of diarrhoea-predominant IBS were seropositive for anti-VGKC and anti- $\alpha 3$ nAChR antibodies respectively. In both of these cases a full-thickness jejuna biopsy revealed an inflammatory neuropathic process. Interestingly, none of 4 patients with enteric dysmotility, 8 patients with intestinal pseudo-obstruction and 9 patients with slow transit constipation had detectable autoantibodies (Tornblom *et al.* 2007). The median duration of symptoms of the SID patients of our study, 10 years (range 3-40 years) was similar to that of Tornblom *et al.* It is possible then that autoantibodies in primary GINMD are an early phenomenon which subsequently resolve but lead to chronic disease due to activation of lymphocytes, macrophages/killer cells producing permanent damage. In practise this is difficult to study as the best characterised autoimmune disorders are treated by immunomodulation. However, there is further evidence in support of this. Anti-VGKC antibodies have retrospectively been observed to be monophasic with levels falling over time without immunomodulatory therapy (Buckley *et al.* 2001). Anti-VGKC, -GAD and - N-methyl-D-aspartate receptor (NMDAR) antibodies associated with non-paraneoplastic limbic encephalitis appear to be highest during the early stage of disease and early treatments appear to produce optimal clinical outcomes (Irani *et al.* 2010) Rasmussen's encephalitis which has been associated with anti-glutamate receptor antibodies also responds well to immunotherapy in the early course of the disease, but as the disease progresses this is less effective (Bethan Lang, personal correspondence). Furthermore, in anti-GAD antibody

associated epilepsy, antibody titres are much higher at presentation than seen in chronic disease (Bethan Lang, unpublished observation).

Regarding primary achalasia, a study of a large group (70) of unselected patients revealed a remarkably high prevalence of anti-neuronal antibodies, 25.7% having one or more of anti-skeletal muscle AChR or striational, neuronal voltage-gated cation channel (potassium channel or N-type calcium channel) or GAD antibodies. In addition, 21% had anti-GAD antibodies. The mean number of years from diagnosis to sera collection in this group was only 5 years (Kraichely *et al.* 2009). The mean number of years from diagnosis to sera collection in our achalasia group was 6 years. However, this does not necessarily correlate with disease duration as some of our patients, even at first investigation, were noted to have a manometric pattern characteristic of established achalasia. Data regarding stage of disease at diagnosis in the study of Kraichely *et al.* are not provided. It should be mentioned that none of the positive anti-GAD sera, which were detected using an indirect immunofluorescence assay, were confirmed using the radioimmunoprecipitation assay to confirm specificity (Saiz *et al.* 2008).

Interpreting our results in the light of these previous studies we may tentatively speculate that the failure to detect anti-channel autoantibodies in our GINMD patients may be due to chronicity where antibodies which may present in early disease ‘burn-out’, leaving a more chronic, cell-mediated immune process. To test this hypothesis it would be desirable to study patients in the early stages of disease and follow-up with a longitudinal study. Dhamija *et al.* report a moderate to dramatic improvement in gastrointestinal symptoms in 4 out of 4 seropositive patients treated with immunotherapy.

Summary box: Humoral autoimmunity in primary GINMD

Previous knowledge

- Non-specific anti-neuronal autoantibodies present in significant number
- Anti-channel (VGKC, VGCC, nAChR) antibodies detected in a few cases and may represent a limited form of dysautonomia (AGID)
- Anti-GAD present in significant number of primary achalasia

This study

- Anti-channel antibodies not associated with chronic primary GINMD
- Anti-GAD antibodies not detected in neurologically significant titres in primary achalasia

Finding 2: Autoantibodies which directly bind the M₂ muscarinic acetylcholine receptor were not detected in chagasic patients.

A large body of data from *in vitro* pharmacological experiments supports the existence of circulating autoantibodies in the sera of patients with Chagas' disease and GI involvement, which, in GI smooth muscle, produce effects mediated by activation of M₂ mAChRs. However, the only evidence for direct binding to the receptor previously came from ELISA assays which employ a synthetic peptide representing only a part of the receptor. We designed a cell-based assay to screen for the presence of anti-M₂ mAChR autoantibodies to clarify whether such autoantibodies are able to directly bind to the receptor. We did not detect IgG in the sera of 57 chagasic patients capable of directly binding the M₂ mAChR. Thus anti-M₂ mAChR autoantibodies, if present in this cohort of patients, do not exert their effects by direct receptor binding.

Study limitations

The negativity of our assay could be explained by the fact it was a newly designed assay and was not sufficiently optimised to detect autoantibodies. Control measures were taken however to ensure viability. These controls have previously been employed in the successful optimisation of other cell-based assays used for this purpose. Firstly, transfection efficiency was checked using the well validated method of GFP transfection to produce auto-fluorescent cells (Modarressi *et al.* 2004). This confirmed our method of transfection was robust. Secondly, successful expression of M₂ mAChR was confirmed by immunofluorescently staining transfected cells with a commercial anti-M₂ mAChR monoclonal antibody (Leite *et al.* 2008). It should be noted that only commercial antibodies raised against intracellular epitopes of the receptor were available at this time and as such it was necessary to permeabilise the transfected cells prior to antibody incubation. Whilst not directly comparable to subsequent staining using patient serum on intact cell membranes, this control demonstrated that the receptor protein was successfully expressed by transfected cells and the pattern of staining, despite the permeabilisation process appeared to delineate a circumscribed cell border, suggesting the receptor was reaching the cell membrane. With these conditions satisfied one may feel confident in using the assay to screen sera for antibodies against the target in question. Indeed, this assay is increasingly becoming the assay of choice in detecting autoantibodies as it has been shown to be more sensitive than traditional radioimmunoprecipitation assays and is closer to the *in vivo* conditions where autoantibodies meet membrane bound, fully processed channels and receptors (Leite *et al.* 2008).

Significance and interpretations

The sufficiency of ELISA using a short, linear portion of a receptor to detect autoantibodies has been called into question (Vincent *et al.* 2010). Indeed this issue is highlighted by a similar study of GI dysmotility secondary to scleroderma (Goldblatt *et*

al. 2002). Circulating autoantibodies which modify *in vitro* events associated with muscarinic antagonism have been detected in such patients, and have been postulated, as a result of indirect pharmacological evidence, to exert a functional effect, via the M₃ mAChR. Furthermore, autoantibodies in the sera of patients with scleroderma and GI involvement have been shown to bind a synthetic M₃ mAChR peptide in ELISA. However, the authors of the *in vitro* study note in their discussion that functional these autoantibodies failed to bind mammalian cells transfected with the M₃ mAChR calling into question direct interaction with the receptor itself. Whilst the pharmacological evidence for M₂ mAChR activation in the presence of chagasic autoantibodies is stronger than for M₃ mAChR antagonism in scleroderma, the results of our cell-based assay suggest that anti-muscarinic autoantibodies capable of directly binding the M₂ mAChR are not always present in a significant number of patients with Chagas' disease and GI involvement, as currently claimed.

An important consideration in interpreting our results is the origin of our cohort of serum samples which came from individuals living in the north-east of Brazil. All previous studies relevant to this subject have examined the effects of sera collected from patients residing in Argentina (specifically in the Beunos Aires area). This is significant as clinical manifestations of chronic Chagas' disease are distinct in different geographical regions, megaviscera being more prominent in the southern countries of South America. It is now known that *Trypanosoma cruzi* (*T.cruzi*) is a heterogeneous organism, consisting of several strains (Miles *et al.* 2009), and whilst host factors must also play a role, differences in infecting genotypes appear to govern chronic disease sequelae (Figure 4.1). It is then likely that the patients in the present cohort were infected by a genotypically different organism to those studied previously, and although patients studied here and previously had GI involvement, the failure to detect anti-M₂ mAChR autoantibodies in our cohort may be due to a different immune response which is consequent of a dissimilar infective event. To help answer the question of whether the

present cohort have autoantibodies which activate muscarinic pathways without binding receptors directly or have another mechanism of action, we proceeded to investigate the functional effects of the sera from our cohort on GI smooth muscle.

Figure 4.1: Geographical distributions of *T. cruzi* lineages (Tc I – VI). Sera analysed in this thesis were taken from patients residing in and around the city of Fortaleza, in the north-east of Brazil (indicated in the upper left panel). It is possible that individuals in this cohort were infected with any of four strains of *T. cruzi* (Tc I – IV), which may be transmitted via domestic or sylvatic triatomines (haematophagous insect vectors, a subfamily of the genus *Reduvius*). Adapted by permission from Macmillan Publishers Ltd: Nature (465, S8-9), copyright (2010).

Summary box: Anti-muscarinic humoral autoimmunity in Chagas' disease

Previous knowledge

- Autoantibodies present in high proportion of individuals with Chagas' disease are capable of modulating muscarinic GI neuromuscular activity
- Autoantibodies bind synthetic M₂ peptide in ELISA suggesting direct receptor modulation

This study

- Autoantibodies capable of directly binding M₂ mAChR expressed by mammalian cells were not detected in patients with Chagas' disease suggesting an alternative mechanism of receptor activation or absence of anti-M₂ mAChR antibodies

Finding 3: Circulating factors present in patients with Chagas' disease modulate colonic smooth muscle contractility through non-cholinergic / non-nitroergic pathways

A number of studies have implicated chagasic IgG in activation of the M₂ muscarinic cholinergic signalling pathway with subsequent end organ effects (Goin *et al.* 1994; Goin *et al.* 1994; Goin *et al.* 1997; Sterin-Borda *et al.* 1997; Hernandez *et al.* 2008; Ganzinelli *et al.* 2009) including an increase in GI smooth muscle tone (Goin *et al.* 1999; Sterin-Borda *et al.* 2001). It should be noted that the majority of these studies originate from a single research group, and all studies have used the same ELISA to detect anti-M₂ mAChR autoantibodies. Regarding the investigation of serum effects on colonic smooth muscle tone, *in vitro* experiments have used muscle taken from either normal rat colon or human chagasic megacolon samples, reporting very similar responses to incubation with chagasic serum in both tissues. This result is curious as inter-species variability in colonic muscarinic receptor profile appears to exist (Gomez *et al.* 1992) and chagasic megacolon is characterised by extensive damage to the neuromuscular apparatus including

significant inflammatory enteric neuronal degeneration, muscular inflammatory cell infiltration and muscular fibrosis (Corbett *et al.* 2001; da Silveira *et al.* 2007). Our cell-based assay designed to detect anti-M₂ mAChR IgG failed to detect these autoantibodies in a cohort of Chagas' disease patients. We therefore undertook the investigation of the effect of chagasic serum on colonic smooth muscle taken from patients without Chagas' disease.

The overall observed effect was a decrease in baseline smooth muscle tension. This effect was maintained in the presence of the muscarinic antagonist atropine and the nitric oxide synthase inhibitor L-NAME, demonstrating that it was not dependent on muscarinic cholinergic receptor activation or nitric oxide production. This finding represents a novel chagasic serum effect on colonic smooth muscle contractility.

Study limitations

The most important limitation of our findings is the fact that we have investigated the effect of serum from only three patients with chagasic GI disease. For this reason we cannot generalise this finding. Work is on-going to characterise more samples. We did however investigate the effects of disease control serum (from patients with Chagas' disease but no GI involvement) and community healthy control serum (from other individuals living in north-eastern Brazil without Chagas' disease), neither of which showed a comparable effect on smooth muscle tone. It would have also been desirable to purify IgG from the sera to determine if this effect was antibody mediated or due to other circulating factors. However, previous work has shown almost identical effects of whole serum and purified IgG on cholinergic muscle responses (Goin *et al.* 1994). As this was only a preliminary study, we used a single concentration of sera. Work is also on-going to determine a concentration-dependent response which would add weight to the evidence for a direct pathogenic role.

Significance and interpretations

Previous studies reported that the addition of IgG autoantibodies from patients with Chagas' disease to *in vitro* preparations of GI smooth muscle resulted in M₂ mAChR agonism. Initially the IgG were found to non-competitively replace the binding of the cholinergic agonist carbachol, producing partial receptor agonism in an allosteric manner (resulting from binding at a site other than the primary, orthosteric binding site). This effect was also blocked by the muscarinic antagonist atropine. More recent studies report that the same autoantibodies may mediate their effect by positive modulation of acetylcholine activity at the M₂ mAChR (Hernandez *et al.* 2008). Whatever the proposed mechanism of action, with the exception of the dominant excitatory activity in the lower oesophageal sphincter in achalasia, the pathologic relevance of this effect in chagasic GI disease, which is characterised by inflammatory neuro-degeneration and pathological dilatation, remains unclear.

Our findings are novel in three respects. Firstly, we have shown an effect of chagasic sera on non-infected human colon. Secondly, we have identified a relaxant effect of chagasic sera on colonic smooth muscle which may represent early stages in the development of a megacolon. Thirdly, we have shown that this effect is not dependent on muscarinic cholinergic receptor activation.

Previous studies only looked at smooth muscle under a resting tension which does not provide information regarding whether activity is mediated by an alteration in neuronal or muscular activity. We examined the effect of chagasic sera on GI smooth muscle under electrical field stimulation (EFS) which allows neuronally mediated changes in muscle tension to be observed whilst also monitoring the changes in baseline muscle tone. We found that muscle responses during EFS were unchanged following addition of chagasic sera suggesting that the reduction in tone was not secondary to an alteration in neuronal activity. This evidence was strengthened by the observation that the effect was not

prevented by pre-incubation of muscle preparations with the voltage-gated sodium channel blocker tetrodotoxin, which prevents action potential generation (Narahashi *et al.* 1960; Kao 1964). Furthermore, we found that the effect was not dependent on production of nitric oxide, the principle neurotransmitter of inhibitory motor neurons, or mAChR activation. The decrease in baseline muscle tension is therefore likely to be the result of a direct effect on the muscle. A candidate target for mediation of such an effect is the L-type voltage-gated calcium channel (VGCC), which is the predominant channel responsible for calcium entry into intestinal smooth muscle cells necessary for contraction (Farrugia *et al.* 1995; Liu *et al.* 2001). The L-type VGCC antagonist, nifedipine, has been shown to reduce the basal tone of human colonic circular muscle *in vitro* (Ghia *et al.* 2004). Furthermore, in isolated cardiac muscle cells, L-type Ca^{2+} current inhibition was observed in the presence of chagasic antibodies (Hernandez *et al.* 2003). Smooth muscle relaxation in response to serum factors may represent an early stage in the development of dysmotility which may progress to megacolon.

Finding 4: Humoral anti-neuronal autoimmunity is present in some individuals with Chagas' disease

Anti-channel autoantibodies were identified in patients with Chagas' disease. Anti-VGCC antibodies were identified in one of 57 patients, although this patient had no secondary disease manifestations, antibody titre was comparable to LEMS disease control. Eight chagasic patients were found to have moderate titres of circulating anti-VGKC antibodies. Of these, five had no chronic disease sequelae, one had achalasia and heart disease, one had achalasia only and one had colonic dysfunction. Low titre anti-GAD antibodies were found in one patient with cardiac complications, although as previously stated, these are unlikely to have neurological significance

Significance and interpretations

The finding of anti-VGCC and VGKC antibodies in Chagas' disease was unexpected and is the first time this has been described. Whilst the presence of these autoantibodies may occasionally be found in individuals with no overt neurological disease, the presence of anti-VGKC antibodies in 8 (13.6%) of chagasic individuals is notable. The majority of these cases (5/8) at the time of venepuncture had no sequelae of their infection. Of interest to this thesis the remaining three patients had GI disease of oesophagus and / or colon. In the classical anti-VGKC-mediated diseases, such as neuromyotonia, GI features have not been especially reported (Isaacs 1961; Benatar 2000). However, anti-VGKC disorders are being increasingly recognised and three previous studies have described VGKC antibodies in relation to gut dysfunction. The first associated anti-VGKC autoantibodies with slow transit constipation arising *de novo* in adults (an uncommon presentation of a disorder which usually presents in childhood) (Knowles *et al.* 2002). Another describes VGKC antibodies occurring in a case of paraneoplastic (thymoma) associated intestinal pseudo-obstruction. Importantly, improvement in gastrointestinal symptoms following plasma exchange correlated with a decrease in VGKC antibody levels, suggesting for the first time a real pathological significance (Viallard *et al.* 2005). A study of 80 patients who were VGKC autoantibody positive found gastrointestinal symptoms in at least 12 (17%) (the true figure may have been higher because some medical records studied retrospectively were incomplete) (Rueff *et al.* 2008). All patients included were identified on the basis of previous investigation for a suspected autoimmune neurological disorder, possibly paraneoplastic. It is not known whether any of these patients were initially referred because of isolated GI symptoms and what proportion were subsequently found to have an underlying neoplasm. However, this study further establishes an association between VGKC antibodies and GI symptoms.

Damage to the enteric nervous system is an established feature of chagasic GI disorders, and is characterised by neuronal degeneration and loss, connective tissue fibrosis,

inflammatory cell infiltration, decreased numbers of interstitial cells of Cajal and decreased enteric glia (Koberle 1968; Hagger *et al.* 2000; Iantorno *et al.* 2007). The production of autoantibodies in Chagas' disease has previously been attributed to molecular mimicry, where a cross-reactive immune response to a parasitic epitope and native antigen occurs, or due to a secondary immune response to tissue damage where 'cryptic' antigens which are normally hidden from the immune system are revealed (Kierszenbaum 2005). Our findings of anti-VGKC autoantibodies in patients with Chagas' disease, which is known to specifically lead to enteric neuronal pathology, together with our findings of K_V1 channel expression in the gut would fit the hypothesis that these autoantibodies may occur as a result of tissue damage exposing antigens that are normally hidden to the immune system. In support of this was the identification of anti-VGKC antibodies in a patient with diarrhoea-predominant irritable bowel syndrome (D-IBS) which had begun after infectious enteritis. This patient was also found to have inflammatory cell infiltration (lymphocytic ganglionitis) and signs of enteric neuropathy (Torblom *et al.* 2007). Against this is the fact that the majority of the patients in which we detected anti-K_V1 channel antibodies had no disease sequelae, and others had only heart disease. However, even those without overt secondary disease would almost certainly have some parasite induced tissue damage that was perhaps at that time sub clinical (see Finding 5 for further discussion).

Summary box: Anti-channel humoral autoimmunity in Chagas' disease

Previous knowledge

- No previous description of anti-channel autoantibodies in Chagas' disease

This study

- 8/59 (13.6%) patients positive for anti-VGKC autoantibodies but variable clinical phenotype

Finding 5: *Shaker*-related voltage-gated potassium channels are expressed by the human gastrointestinal neuromuscular apparatus.

Due to the increasing evidence of anti-VGKC autoimmunity in certain types of GI dysfunction we set out to investigate the presence of these channels in the GI neuromuscular apparatus. The confirmation of their expression in the human GI tract has potential physiological significance and in the context of this and other studies, pathogenic implications.

Due to the specificity of α -dendrotoxin, anti-VGKC radioimmunoprecipitation assays screen for autoantibodies against K_V1.1, 1.2 and 1.6 channel subunits (Stuhmer *et al.* 1989; Grissmer *et al.* 1994). However, due to the assembly of heteromultimeric channels (MacKinnon 1991; Parcej *et al.* 1992) and the heterogeneity of diseases associated with anti-VGKC autoimmunity (Rueff *et al.* 2008), it is likely that other members of this subfamily also have pathogenic importance. Therefore, we investigated the expression of six members of the K_V1 subfamily (K_V1.1-1.6) in all layers of the human oesophagus and gastrointestinal tract immunohistochemically. The results showed that K_V1 channels are abundant throughout the human gastrointestinal tract and are expressed by epithelial cells and enteric neurons of the myenteric and submucosal plexi in a regionally specific pattern.

Epithelial K_V1 channel subunit expression varied according to the region studied. Oesophageal squamous epithelial cells were not immunopositive for any of the six K_V1 channel subunit. In gastric epithelium K_V1 expression was restricted to chief cells which are responsible for production and secretion of pepsinogen. Jejunal, ileal and colonic enterocytes expressed K_V1 subunits to varying degrees, the concentration of channels appearing to be greatest in mature enterocytes towards villi tips and colonic surface epithelium with relatively little expression in the crypts. In addition, cells identified by

H&E appearance as enteroendocrine expressed a high abundance of channels in their basal aspect.

Within the enteric nervous system each K_v1 channel subunit exhibited a stereotypical pattern of distribution, localising specifically to neuronal cell bodies and / or ganglia neuropil, and these patterns were largely consistent throughout the gut. Of the six subunits studied, K_v1.4 was especially notable for its distinctive punctate distribution within enteric ganglia and this was further investigated using double-labelled immunofluorescent staining (see Finding 6).

Study limitations

This study was dependent on antibody specificity and, as with any immunohistochemical investigation, results could be due to non-specific tissue binding. This possibility is unlikely as we first confirmed that our antibodies stained cerebellar tissue in previously described patterns (Veh *et al.* 1995; Chung *et al.* 2001). These measures confirmed that the antibodies used were specific for these channels. The expression of cell surface proteins may be temporal, altering according to internal or external circumstances, and may also differ between individuals. It is therefore necessary to confirm findings in a number of different cases in order to describe ‘normality’. For each gut region we studied K_v1 channel distribution in at least five different individuals and found our results to be consistent. A limitation of this however was the source of tissue samples. The availability of disease-free tissue is, for obvious reasons, limited. The majority of tissue was obtained from cancer resection margins. Whilst all sections were histologically normal, with no evidence of cancer or inflammation, the possibility that channel expression is altered secondary to such disease processes cannot be ruled out. However, the difficulties inherent in studying normal human microanatomy mean that these results represent a significant step forward in mapping ion channel distribution in the human gut.

Significance and interpretations

VGKC channels are involved in numerous processes in both excitable and non-excitable cells, but are best known in relation to setting resting membrane potentials, regulating cellular excitability and synaptic transmission. The K_V1 or *Shaker*-related subfamily is the most extensively studied and the distribution of these channels in the CNS and periphery in relation to their role in normal function and disease states is fairly well elucidated (Beckh *et al.* 1990; Veh *et al.* 1995; Coleman *et al.* 1999; Chung *et al.* 2001; Glazebrook *et al.* 2002). In comparison, knowledge regarding K_V1 channel expression in the GI tract was limited. Studies of small mammals had begun to identify K_V (including K_V1) currents associated with epithelial cell maturation, migration and nutrient absorption (McDaniel *et al.* 2001; Rao *et al.* 2002). Cells from the IEC-6 cell line derived from rat intestinal enterocytes express K_V1.1, 1.2, 1.4, 1.5 and 1.6 mRNA (McDaniel *et al.* 2001). Furthermore, as these cells migrate and differentiate towards a mature phenotype the expression of K_V1.1 and 1.5 increases (Rao *et al.* 2002). Our finding of K_V1 channel expression in mature, differentiated enterocytes of the human GI tract is in agreement with these findings and further suggests that increased K_V1 channel expression occurs with human enterocyte maturation.

Modulation of enteric motor nerve excitability (Suarez-Kurtz *et al.* 1999; Vianna-Jorge *et al.* 2000) and generation of smooth muscle ‘slow-wave’ contractions (Thornbury *et al.* 1992; Thornbury *et al.* 1992; Carl 1995) have been suggested to involve K_V1 channel activity. Previous work has also elucidated K_V current contributions in extrinsic sensory neurons innervating the oesophagus, stomach, ileum and colon (Yoshimura *et al.* 1996; Glazebrook *et al.* 2002; Dang *et al.* 2004). Examining the intrinsic innervation of the gut, Hatton and co-workers reported K_V1.1 expression in murine, guinea pig and canine myenteric ganglia and ICC (Hatton *et al.* 2001). However, others describe murine K_V1.1 expression limited to enteroglial cells, with no expression detected in neurons or ICC (Costagliola *et al.* 2009). Enteroglial cells and enteric neurons may also express K_V1.2

channel subunits (Costagliola *et al.* 2009). These points have yet to be resolved. In the human gut we did not find evidence of K_V1 channel protein expression in enteroglia cells or cells resembling ICC. Other currents have been shown to contribute to the activity of these cells, for example ether-a-go-go-related (ERG) K channel and VGCCs in ICC (Huizinga *et al.* 2004). Our study suggests that K_V1 channels are widely expressed by enteric neurons of both the myenteric and submucosal plexi and that they have an overlapping yet differential subcellular distribution. This would be expected from heteromultimeric assembly and has been previously shown in the CNS, where K_V1 channel subunits have overlapping but also unique patterns of subcellular distribution, depending on the region and also neuronal subtype studied. This produces a highly complex arrangement, presumably mirroring functional diversity (Wang *et al.* 1994; Veh *et al.* 1995). In contrast to the CNS, K_V1 subunits in the human ENS appear to be stereotypically directed to subcellular locations and this was consistent between submucosal and myenteric neurons and throughout the gut (with the exception of some subunits in the oesophagus and colon). As the specific expression of K_V1 subunits in subcellular compartments would help determine the unique firing pattern and thus function of a neuron it is perhaps not surprising that CNS neurons, which are highly diverse, do not exhibit stereotypical patterns of channel expression. Whilst the gut has a large population of neurons (comparable to that of the spinal cord) (Furness 2000), the number of functions and thus complexity is considerably less than that of the CNS. Whilst further studies of K_V1 expression by specific neuronal subtypes are required, the stereotypical distribution of K_V1 channel subunits in the human ENS is at least suggestive of specific functionality.

In canine colonic smooth muscle molecular studies have identified K_V1.2, K_V1.4, K_V1.5 and K_V1.6 mRNA (Hart *et al.* 1993; Epperson *et al.* 1999). Murine colonic smooth muscle cells express transcripts encoding K_V1.6, but lack K_V1.4 suggesting there may be inter-species variability (Koh *et al.* 1999). A single study has previously identified K_V1

channel subunits in the human neuromuscular apparatus. Recordings from isolated human oesophageal smooth muscle cells have revealed a voltage-dependent potassium current and K_V1.2 and 1.5 mRNA expression in both longitudinal and circular muscle layers were detected (the expression of other K_V channels was not investigated). Functionally, pharmacological blockade of the muscular voltage-gated potassium current caused an increase in resting tension, suggesting a role in regulation of muscular contraction. However, the agent used in this study (high concentration 4-aminopyridine) non-specifically blocks a number of potassium channel types and so the effect cannot be attributed to K_V1 channels alone (Wade *et al.* 1999). Our results demonstrate human GI smooth muscle to be largely devoid of K_V1 channel expression further suggesting inter-species variability in GI K_V1 channel expression. The exception to this and in agreement with the findings of Wade *et al.* was the finding of K_V1.5 expression in oesophageal myocytes. The expression of K_V1.5 in GI smooth muscle was peculiar to the oesophagus. Here, channel expression was mainly, although not exclusively, perinuclear in distribution. In some myocytes, K_V1.5 was also present in the cell cytoplasm.

The observation that K_V1.5 is expressed by oesophageal smooth muscle is interesting in relation to our finding of anti-VGKC autoantibodies in patients with Chagas' disease (see Finding 4). Chronic Chagas' disease primarily affects the heart and the gut, specifically regarding the latter, the oesophagus and colon. Why these organs are specifically the focus of disease pathology remains unknown. It is known however, that K_V1.5 is also expressed by human cardiac muscle (Snyders *et al.* 1993; Wang *et al.* 1993), where it is responsible for the ultra-rapid delayed-rectifier current essential for atrial repolarisation (Feng *et al.* 1997). K_V1.5 is therefore a channel subunit common to both the heart and the oesophagus. One may tentatively speculate that circulating anti-VGKC autoantibodies, which were present in 14% of our cohort and are known to impair K_V1 channel function, may contribute to chagasic oesophageal and heart disease by impairing K_V1.5 channel function. In support of this hypothesis is the fact that down-regulation of expression of

this channel has been found to be associated with arrhythmia (Van Wagoner *et al.* 1997; Lai *et al.* 1999; Brundel *et al.* 2001) which is a common feature of chagasic heart disease (Elizari 2002). Due to their function of regulating cellular excitability, impairment of K_v1.5 oesophageal smooth muscle would be expected to interfere with oesophageal contractility, and this may be relevant to the loss of normal peristalsis and failure of smooth muscle relaxation characteristic of oesophageal achalasia. We did not observe K_v1.5 immunostaining in colonic smooth muscle. We did however observe K_v1.5 in colonic submucosal and myenteric neurons. Whilst these findings are interesting and deserve further investigation, the inflammatory fibrotic reaction which damages the cardiac muscle and conduction network and the enteric nervous system remains the most striking feature of this disease. Not all infected individuals, progress to the chronic, symptomatic stage of the disease. About two-thirds of patients remain in an asymptomatic ‘indeterminate’ phase and no determinants of progression to the symptomatic phase have been identified (Lescure *et al.* 2010) As discussed in Finding 4, five of the eight patients in which we detected anti-VGKC autoantibodies were asymptomatic. It would be of interest to follow these patients to see if they go on to develop symptomatic disease, as perhaps in some individuals anti-VGKC autoantibodies represent a marker of disease progression, either as a pathological factor, or as a marker of more severe tissue damage which reveals these antigens to the immune system. In either case, the finding would be of significance in guiding a treatment regime which currently lacks definition (Lescure *et al.* 2010).

Having now demonstrated the presence of K_v1 channels throughout the human enteric nervous system, the finding of anti-VGKC autoantibodies in patients with primary and secondary GINMD (Knowles *et al.* 2002; Viallard *et al.* 2005; Tornblom *et al.* 2007; Dhamija *et al.* 2008) takes on new relevance. Whilst not common, the detection of these potentially pathogenic antibodies in patients with GI dysmotility coupled with the knowledge that the human enteric nervous system possesses the antigenic targets could in

future help guide treatment, particularly in the direction of immunomodulation. The reason for GI involvement in a minority of individuals with anti-VGKC antibodies is unclear, but as discussed in relation to their detection in patients with Chagas' disease (Finding 4), it is likely that an accompanying event such as infection, or neoplasia (Viallard *et al.* 2005) is necessary. Further investigation of pathogenesis should include a comparative study of enteric K_V1 channels in disease.

Summary Box: Enteric K_V1 channel protein expression

Previous knowledge

- K_V1.1 identified in murine*, guinea pig and canine myenteric neurons and ICC
- K_V1.1 expressed by murine enteroglia*
- K_V1.2 expressed by murine myenteric neurons and enteroglia
- K_V1 subunits expressed by murine (K_V1.6), canine (1.2, 1.4, 1.5, 1.6) and human GI smooth muscle (1.2 and 1.5)

This study

- K_V1.1-1.6 expressed in epithelial cells and/or enteric neurons in all gut regions in humans
- Epithelial expression limited to gastric chief cells, surface enterocytes and enteroendocrine cells
- Each subunit exhibits a stereotypical subcellular distribution in enteric neurons
- K_V1.4 exhibited a unique punctate distribution in gastric and small bowel ganglia
- No expression evident in ICC or enteroglia
- Oesophageal exclusively expresses K_V1 channels: K_V1.5

*conflicting results regarding K_V1.1 distribution in murine myenteric plexus

Finding 6: K_V1.4 is specifically localised to synaptic and axonal locations in enteric ganglia

Of particular interest was the finding of discrete puncta of K_V1.4 immunoreactivity in ganglia neuropil. K_V1.4 containing channels are unique amongst K_V1 channels due to properties of fast-inactivation followed by a slow recovery from inactivation which enable the integration of brief depolarisations over tens of seconds (Pardo *et al.* 1992; Rasmusson *et al.* 1995). It is therefore of interest to determine the subcellular distribution of these channels in relation to neuronal functioning. In the central nervous system, particularly the hippocampus, K_V1.4 has been localised to axons and nerve terminals (Sheng *et al.* 1993; Cooper *et al.* 1998). We investigated K_V1.4 localisation to enteric synapses and axonal segments using double-labeling immunofluorescent staining for synaptophysin (a synaptic vesicle glycoprotein) and neurofilament (an intermediate filament which is a major component of the axonal cytoskeleton) respectively. In common with hippocampal findings we observed areas of K_V1.4 and synaptophysin co-localisation. We also saw occasional overlap of K_V1.4 and neurofilament. Taken together these findings demonstrate that in the human enteric nervous system K_V1.4 is located at some pre-synaptic termini and a subset of axons, consistent with the hippocampal findings (Cooper *et al.* 1998). The finding of K_V1.4 immunoreactivity at synaptic termini suggests that K_V1.4 may contribute to the process of neurotransmitter release (Sheng *et al.* 1993). Axonal K_V1 channels located at or near para-nodal regions are responsible for action potential repolarisation (Hodgkin *et al.* 1952; Stolinski *et al.* 1981; Chiu *et al.* 1982; Wang *et al.* 1993). As K_V1.4-containing channels inactivate quickly and are slow to recover, action potentials propagated along axons bearing K_V1.4 broaden (Bean 2007). A phenomenon known as activity-dependent spike broadening occurs during high frequency stimulation (Berry 1972; Carew *et al.* 1977) due to cumulative inactivation of these channels. When this occurs the number of Ca²⁺ ions entering the pre-synaptic terminal per action potential rises, increasing neurotransmitter release with consequent

post-synaptic effects (Ma *et al.* 1995; Geiger *et al.* 2000; Bischofberger *et al.* 2002). The finding of this channel in human enteric neurons has potentially important implications regarding the temporal coding of enteric neurotransmission and merits further investigation.

Summary box: Enteric neuronal K_v1.4 expression in humans

Previous knowledge

- K_v1.4 expression not previously investigated in enteric nervous system

This study

- K_v1.4 exhibits discrete punctate distribution in enteric ganglia of stomach and small bowel
- Located at synapses and axonal processes

4.3 CONCLUDING REMARKS

Despite increased knowledge regarding the pathophysiology of GINMD, the underlying aetiology of these disorders remains largely unknown. Due to phenotypic similarities between GINMD and certain neuromuscular disorders of the periphery which are known to occur as a result of pathogenic autoantibodies against ion channels, and the previous identification of anti-channel autoantibodies in small numbers of patients with GINMD, this thesis investigated ion channel expression in the gut and the presence of anti-channel autoantibodies in idiopathic and infective examples of GI dysfunction.

The headline findings are:

1. Humoral autoimmunity against currently recognised neuronal antigens is not commonly associated with chronic primary gastrointestinal neuromuscular diseases.
2. Circulating factors present in patients with Chagas' disease modulate colonic smooth muscle contractility through non-cholinergic / non-nitroergic-dependent pathways.
3. Humoral anti-neuronal autoimmunity, particularly in the form of anti-voltage-gated potassium channel antibodies, is present in some individuals with Chagas' disease
4. *Shaker*-related voltage-gated potassium channels are expressed by the human gastrointestinal neuromuscular apparatus
5. K_v1.4 is located in pre-synaptic termini and axons of a subset of enteric neurons

The results presented in this thesis demonstrate that the human enteric neuromusculature expresses some of the same ion channels that are targets for autoantibodies in the peripheral neuromusculature, but suggest that autoantibodies against these and other channels are not commonly detectable in chronic primary GINMDs. To what extent anti-channel antibodies are present in early GINMD remains to be elucidated but is an important question, as if present, early detection and immunomodulatory therapy could in theory prevent or limit deterioration in gut function. Certain phenotypes may increase the likelihood of finding anti-channel antibodies. Dhamija *et al.*, in their investigation of autoimmune gastrointestinal dysmotility conclude: 'Factors that raise the clinical suspicion of AGID were noted in several patients of this report: an antecedent event, subacute onset, evidence of multi-level GI dysmotility, co-existing organ-specific autoimmune disorder (including subtle autonomic/neurological abnormalities, such as postural orthostatism or somatic neuropathy) (Dhamija *et al.* 2008). A relevant antecedent event could provide an antigenic stimulus. This is clear in paraneoplastic

cases where a cross-reactive immune response to neural antigens expressed by a tumour leads to neuronal dysfunction. In idiopathic cases it is possible that an occult tumour may have been eliminated by immune surveillance mechanisms that subsequently generate a neurological syndrome (Irani *et al.* 2010) or even that a tumour may not be detectable until much later: anti-NMDAR encephalitis may precede by years the detection of an ovarian teratoma ((Iizuka *et al.* 2008). A preceding infection may also provoke an antibody-mediated autoimmune response as demonstrated in patients with Guillain Barré syndrome (Willison *et al.* 2002). Post-infectious IBS associated with altered cell-mediated immunity is now a commonly recognised phenotype (Gwee 2010) and it is interesting that the two patients in whom antibodies (anti-VGKC and – $\alpha 3$ nAChR) were detected in the study of Tornblom *et al.* had onset of disease after probable acute infectious enteritis. Our finding of anti-VGKC antibodies in patients with Chagas' disease lends further support to the hypothesis that these antibodies can often be a post-infectious phenomenon.

Autoimmune channelopathies are being increasingly identified, a pertinent example being the recent identification of autoantibodies binding aquaporin 4 (a water channel) in patients with neuromyelitis optica (Lennon *et al.* 2005), a severe inflammatory demyelinating disease of the optic nerves and spinal cord. This discovery has caused a dramatic paradigm shift in the identification and early treatment of this previously poorly understood condition. Further identification of autoimmune channelopathies may open new avenues in the study of gut dysfunction. For now careful selection of GINMD patients in whom to look for autoantibodies based on the conclusions of Dhamija *et al.* and following the findings of this study, will help to clarify whether or not AGID is a significant aetiological entity.

Appendices

1.0 CONFIRMATORY DOCUMENTS

		Royal Free Hospital & Medical School Local Research Ethics Committee Royal Free Hospital South House, Block A Pond Street London NW3 2QG Telephone: 0207 794 0581 Facsimile: 0207 794 0714
26 April 2006		
Dr Andrew W Hubball PhD Research Student Institute of Cell and Molecular Science, Centre for Adult and Paediatric Gastroenterology The Wingate Institute, 26 Ashfield Street, Whitechapel, London E1 2AJ		
Dear Dr Hubball		
Full title of study:	ENTERIC AUTOANTIBODIES IN THE PATHOGENESIS OF POST-INFECTIVE AND IDIOPATHIC GASTROINTESTINAL MOTILITY DISORDERS	
REC reference number:	06/Q0501/52	
The Research Ethics Committee reviewed the above application at the meeting held on 19 April 2006.		
Ethical opinion		
Dr Hubball attended. The committee asked for an amendment to page 3 of the information sheet "we would like to" rather than "we would need to". The researchers have now amended the information sheet and the application approved		
The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.		
Ethical review of research sites		
The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to complete Part C of the application form or to inform Local Research Ethics Committees (LRECs) about the research. The favourable opinion for the study applies to all sites involved in the research.		
Conditions of approval		
The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.		
Approved documents		
The documents reviewed and approved at the meeting were:		

06/Q0501/52

Page 2

Document	Version	Date
Application	5.1	24 March 2006
Investigator CV		20 February 2006
Protocol	7	20 February 2006
Covering Letter		24 March 2006
Letter from Sponsor		16 February 2006
Peer Review		
Compensation Arrangements		16 February 2006
Participant Information Sheet	1,2,3	20 February 2006
Participant Consent Form	1,2,3	20 February 2006
Letter from funder		

Research governance approval

You should arrange for the R&D Department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research at a NHS site must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0501/52

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Michael Pegg
Chair

Email: rosemary.brown@royalfree.nhs.uk

Enclosures:

*List of names and professions of members who were present at the meeting and those who submitted written comments
Standard approval conditions [SL-AC1 for CTIMPs, SL-AC2 for other studies]*

**Royal Free Hospital & Medical School
Research Ethics Committee**

Royal Free Hospital
South House, Block A
Pond Street
London
NW3 2QG

Tel:
Fax:

22 March 2007

Dr Andrew W Hubball
PhD Research Student
Centre for Adult and Paediatric Gastroenterology
The Wingate Institute, 26 Ashfield Street,
Whitechapel, London
E1 2AJ

Dear Dr Hubball

Study title: ENTERIC AUTOANTIBODIES IN THE PATHOGENESIS OF
POST-INFECTIVE AND IDIOPATHIC GASTROINTESTINAL
MOTILITY DISORDERS
REC reference: 06/Q0501/52
Amendment number: 06/Q0501/52/AM02 *2nd amendment*
Amendment date: 21 February 2007

The above amendment was reviewed at the meeting of the Sub-Committee of the REC held on 21 March 2007.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	9	21 February 2007
Participant Information Sheet	3.2	21 February 2007
Participant Information Sheet	2.2	21 February 2007
Participant Information Sheet	1.1	21 February 2007
Participant Consent Form	3.2	21 February 2007

Participant Consent Form	2.2	21 February 2007
Participant Consent Form	1.1	21 February 2007
Homerton Hospital R&D Approval		20 February 2007
Amended Protocol Extract		21 February 2007
Amended Patient Information Sheet Extracts		21 February 2007
Notice of Substantial Amendment (non-CTIMPs)		21 February 2007

Membership of the Committee

The members of the Committee who were present at the meeting are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0501/52:	Please quote this number on all correspondence
--------------	--

Yours sincerely

Thomas McQuillan
Committee Co-ordinator
 E-mail: Thomas.mcquillan@royalfree.nhs.uk

Enclosures List of names and professions of members who were present at the meeting and those who submitted written comments

Copy to: *Research and Development Dept. Barts and The London NHS Trust / QMUL*

Homerton University Hospital

NHS Foundation Trust

Research & Development Committee
Chair: Professor Kate Costeloe

Research & Development Office
Picton Suite, Homerton Row
London E9 6SR
020 8510 7544

Kate Costeloe
Director of Research & Development
Kate.costeloe@homerton.nhs.uk

Mr Charles Knowles
Department of Surgery
Homerton University Hospital NHS Foundation Trust
Homerton Row
London E9 6SR

20 February 2007

Dear Mr Knowles

Re: Enteric autoantibodies in the pathogenesis of post-infective and idiopathic gastrointestinal motility disorders

REC Ref Number: 06/Q05051/52

Thank you for sending confirmation of your approval from the Research Ethics Committee. I am happy to inform you that the Trust will indemnify against negligent harm that might occur during the course of this project. Should any untoward events occur it is **essential** that you contact the R&D Office immediately. If patients or staff are involved in an incident you must also contact the Risk Manager on 020 8510 7649.

Please note that all NHS and social care research is subject to the DoH *Research Governance Framework*. If you are unfamiliar with the standards contained in this document, you may obtain details from the Trust R&D Office or from the DoH website (www.dh.gov.uk).

As part of research governance, all investigators accessing individually identifiable personal information are required to comply with current data protection requirements. The Healthcare Records Department will not release patient notes for research purposes without the production of this letter and appropriate identification.

Please inform us if your project is amended and you need to re-submit it to the LREC/MREC or if your project terminates early/requires an extension. This is necessary to ensure that your indemnity cover is valid and also helps the office to maintain up-to-date records for submission to the National Research Register. Once your study has ended, you will be required to fill out a Project Outcomes form. A copy of any publications arising from the research should be sent to the R&D Office for use in the R&D Annual Report. Please be reminded that this hospital should be acknowledged in any publication.

If you believe that protectable intellectual property has arisen from your research, please contact the R&D Manager who will advise you on the proper course of action.

Please do not hesitate to contact me if you have any further questions.

Yours sincerely,

Professor Kate Costeloe
Director of Research & Development

The Homerton - The Hospital for Hackney

UNIVERSIDADE FEDERAL DO CEARÁ
DEPARTAMENTO DE MEDICINA CLÍNICA

CUSTOMS INVOICE

Date: 01 DE OUTUBRO DE 2008

HAWB: 608170

Shipper: Universidade Federal do Ceará –HUWC
Prof. Miguel Ângelo Nobre e Souza
Rua: Capital Francisco Pedro, 1290
Bairro: Rodolfo Teófilo
Fone: 55 85 3366.8101 – 3366.8170
Fortaleza – CE

Consignee: Mr C.H Knowles BChir PhD FRCS (Gen Surg)
Senior Lecturer and Hon Consultant Colorectal Surgeon,
Centre for Academic Surgery,
3rd Floor Alexandra Wing,
Royal London Hospital,
Whitechapel,
London E1 1BB.
0207 377 7449
07866 586766

Contents:

Non Hazardous Human Serum
Quantity: 980mL
Kept frozen,

Shipment is for therapeutic use.

Human material that was neither inoculated with nor exposed to infectious agents of agricultural concern, including zoonotic agents.

Human material containing no animal material and not of tissue culture origin.

Obtained directly from humans, not recombinant, not cultured.

No Commercial Value - For Customs Purposes Only \$ 10 USD

GROSS WEIGHT: 16Kg
NET WEIGHT: 980
NUMER OF PIECES: - 01

Signature and Job Title

Prof. Miguel Ângelo Nobre e Souza
Diretor de Ensino e Pesquisa
HUWC/UFC

WORLD COURIER DO BRASIL TRANSP. INTERNAC. LTDA. SÃO PAULO - SP: VIA ANHANGUERA S/N - KM 15 - GALPÃO 7 - PIRITUBA CEP 05112-000 - CENTRO LOGÍSTICO ANHANGUERA TEL: (11) 3622-1900 - FAX: (11) 3622-1919 RIO DE JANEIRO - RJ: PRAÇA TIRADENTES, 10 SL. 1302 - CEP 20060-070 TEL: (21) 2224-9106 - FAX: (21) 2221-2008		NON-NEGOTIABLE WAYBILL 0608170
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NAME: MIGUEL SOUZA/		TELEPHONE # 55 85 32236982		NAME: SENIOR LECTURER AND		TELEPHONE #	
COMPANY: UNIVERSIDADE FEDERAL DO CEARA		FLOOR/DEPT.		COMPANY: MR C H KNOWLES BCHIR PHD FRCS		FLOOR/DEPT.	
ADDRESS: CENTRO DE BIOMEDICINA 1 AND				ADDRESS: COL SURGEON CENTRE FOR ACAD SU			
CAMPUS DO PORANGABUSSU				3RD FLOOR ALEXANDRA WING RLH			
CY: FORTALEZA CE BRA		STATE/COUNTRY 60436-160		CITY: WITCHAPEL LONDON		STATE/COUNTRY E11 1BB LHD	
POST CODE				POST CODE			
SHIPMENT INFORMATION							
FULL DESCRIPTION OF CONTENTS: HUMAN SAMPLES/DRY ICE BIOLOGICAL SUBSTANCE CATEG B				SPECIAL HANDLING:			
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COUNTRY OF ORIGIN: BRA		DECLARED VALUE FOR CUSTOMS: U.S. \$		DECLARED VALUE FOR CARRIAGE: U.S. \$		DOES THIS SHIPMENT CONTAIN DANGEROUS GOODS? ☐ NO ☐ YES (Per attached shipper's declaration) (Shipper's declaration not required)	
CHECK IF SURCHARGE ELECTED: ☐		A RESPONSABILIDADE DA WORLD COURIER É LIMITADA. Ao propor esta remessa a World Courier, o remetente aceita as Condições descritas no verso deste Conhecimento de Embarque. A responsabilidade da World Courier por perda, dano ou demora nesta remessa, não excederá o valor declarado, valor depreciado, custo de reposição, custo de reparo da mesma ou US\$150, o que for menor. Tal quantia poderá ser aumentada, se o remetente especificar neste Conhecimento de Embarque, um valor declarado desta remessa superior a US\$150 e pagar a sobretaxa requerida. Esta remessa poderá também estar sujeita às regras a respeito de responsabilidade, estabelecidas na Convenção de Varsóvia e outros tratados internacionais e provisões. Sob nenhuma circunstância a World Courier será responsabilizada por danos ou perdas especiais, consequentes, indiretas ou incidentais. POR FAVOR, VEJA O VERSO PARA AS CONDIÇÕES DE FRETE.					
VISÃO: O transporte coberto por este conhecimento se rege também pelo código brasileiro de aeronáutica.		PRINT NAME OF SHIPPER OR SHIPPER'S AGENT: MIGUEL A. N. SOUZA		DATE: 1/10/08		PRINT NAME OF CONSIGNEE OR CONSIGNEE'S AGENT:	
SIGNATURE OF SHIPPER OR SHIPPER'S AGENT:		TIME: 10:30		SIGNATURE OF CONSIGNEE OR CONSIGNEE'S AGENT:		TIME:	

2.0 ANTI-NEURONAL IMMUNOFLOUORESCENT ASSAY

SOLUTIONS AND REAGENTS

Solutions and reagents were supplied by Instrumentation Laboratory (UK) Ltd., Warrington, Cheshire, UK (IL), VWR International, Leicestershire, UK (VWR) or Binding Site, Birmingham, UK (Binding Site).

Phosphate buffered saline (PBS) (VWR):

Mounting media (prepared weekly): 12.5ml PBS filtered through 0.2mm
37.5 glycerol (VWR)

SUBSTRATE

Monkey cerebellum, 5 well slides (IL)

INDIRECT-IMMUNOFLUORESCENT STAINING PROTOCOL

1. Apply 30µl of sera diluted 1:50 to slide well and incubate for 20 min RT
2. Apply ready-to-use positive controls (Hu/Ri and Yo) and incubate as above
3. Rinse off sera with PBS, and soak in PBS for 20 min
4. Dilute FITC-conjugated secondary antibody 1:40 in PBS and microcentrifuge at 13,000 rpm for 5 min.
5. Wipe around sections and apply secondary antibody for 20 min at RT
6. Rinse secondary antibody off with PBS and soak in PBS for 20 min
7. Blot off excess PBS and mount with mounting media
8. Store at 4°C until reading

3.0 RADIOIMMUNOPRECIPITATION ASSAYS

SOLUTIONS AND REAGENTS

Solutions and reagents were supplied by Sigma-Aldrich, Poole, Dorset, UK (not stated), Calbiochem-Novabiochem, San Diego, CA, USA (Calbiochem), Binding Site, Birmingham, UK (Binding Site), RSR, Cardiff, UK (RSR), New England Nuclear, Boston, MA, USA (New England) or Perkin Elmer, Waltham, MA, USA (Perkin Elmer)

3.1 PREPARATION OF CHANNEL EXTRACTS

Solubilisation buffer (SB), 1 litre:	25mM Tris-HCl	2.15g
(pH 7.4, stored at -20°C until use)	5mM HEPES	1.19g
	0.32M Sucrose	0.32g

Protease inhibitors:

Pepstatin A final concentration 1µM

Leupeptin	final concentration 2 μ M
Phenylmethanesulphonylfluoride (PMSF)	final concentration 0.1 μ M

Digitonin (50% pure) (Calbiochem)

2% Digitonin in solubilisation buffer for VGKC extraction only (DTX):

20mM Tris

100mM NaCl

5mM KCl

Natural pH $\cong$ 10.0, therefore add HCl to pH 7.12

1. To 2ml (will be 2.5 ml when 500 μ l membranes added), add digitonin 0.05g (50%) to give final concentration of 2% (active concentration: 1%).
2. Incubate in water bath at 37°C for 15 min.
3. Just before use, add protease inhibitor: PMSF: 5 μ l of 0.1M in propan-2-ol (final concentration 0.2mM).

Preparation of brain membranes

1. Remove tissue as soon as possible post-mortem, cut into small pieces and drop into liquid nitrogen. Store at -70°C until use.
2. Take 'x' gram of cerebellum, and make up with ice cold SB (with protease inhibitors added immediately before use) to 6-8 x volume of 'x'.
3. Homogenise using a Polytron Homogeniser (PT10-35, Kinematica GmbH, Littau, Switzerland), full speed 3 x 10 sec, resting on ice in-between.
4. Spin 1000rpm for 10 min
5. Harvest supernatant

6. Spin 13,000rpm for 10 min
7. Re-suspend pellet in a volume of SB equivalent to original brain tissue weight
8. Aliquot in 500µl amounts and store at -70°C until use

Voltage-gated potassium channel (VGKC) extract preparation

(made fresh for immediate use)

1M NaCl in SB: 5.84g NaCl in 100ml SB

1. Defrost aliquots of rabbit brain cortex-membrane preparation.
2. Add stepwise (2 x 250µl) to 2ml DTX (with fresh protease inhibitor), mix gently with pipette (1 : 5 dilution) (total volume 2.5ml: see above).
3. Incubate 37°C for 15 min.
4. Spin 13,000rpm for 15 min (bench-top Eppendorf centrifuge).
5. Harvest the supernatant (VGKC extract) and store on wet ice until use.

Voltage-gated calcium channel (VGCC) extract preparation

(made in batches for storage before subsequent use)

4% digitonin in SB:

Digitonin purchased is 50% pure. Therefore the solution is made up to 8%: 1.6g digitonin in 20ml SB, heated to 60°C, until dissolved and stored at 4°C before use. There is a lot of insoluble material remaining; before use in the assay an aliquot must be centrifuged: 13,000rpm for 5 min and clear supernatant removed for subsequent use.

1. Defrost aliquots of rabbit cerebellum membrane preparation

2. Add 700µl SB (with fresh protease inhibitors), vortex, spin 13,000rpm for 5min (bench-top Eppendorf centrifuge), discard supernatant.

3. Add 1 ml SB, vortex, spin 13,000rpm for 5 min, discard supernatant.

To the pellet (approx. 200µl), add:

100µl 1M NaCl in SB
200µl SB
500µl 4% digitonin in SB

(therefore the final solution will be 2% digitonin, 0.1M NaCl)

4. Rotate 1h, 4°C.

5. Spin 13,000rpm for 15 min

6. Remove supernatant, aliquot and store at -70°C as source of VGCCs.

Ganglionic $\alpha 3$ nAChR extract preparation

1. Grow neuroblastoma cells IMR32 to confluence (5% CO₂, 37 °C).

2. Trypsinise cells from three flasks and spin 1000rpm for 5 min

3. Combine the pellets and re-spin 13,000rpm for 5 min

4. Remove the supernatant and freeze at -80°C until use

Detergents

Triton X100 (t-octylphenoxypolyethoxyethanol)

Radiolabelled toxins and ligands

The toxins are received as lyophilised powder and are reconstituted with distilled water at a concentration of 0.1µCi/10µl, aliquotted into 10µCi amounts and

stored 70°C until use.

Potassium channels

^{125}I - α -dendrotoxin (^{125}I - α -DnTx) (Perkin Elmer),

Calcium channels

^{125}I - ω -conotoxin MVIIC (^{125}I - ω -CmTx), 5 μCi , S.A. > 2000 Ci/mmol (New England)

Ganglionic AChR- α -3 channels

^{125}I -Epibatidine (^{125}I -Epi) (Perkin Elmer)

Glutamic acid decarboxylase tracer

^{125}I -recombinant GAD₆₅ radioimmunoassay kit (RSR)

3.2 RADIOIMMUNOPRECIPITATION ASSAYS

Sheep anti-human IgG (Binding Site)

Washing buffer (PTX): 0.1% Triton X100 in 20mM phosphate buffer
(pH 7.4, stored at 4°C)

VGKC assay

Radio-labelling using ^{125}I - α -dendrotoxin (^{125}I - α -DNTX)

1. Defrost 1 μCi of ^{125}I -DNTX and add 3.5-4ml of VGKC extract
2. Count on automatic gamma counter (Canberra Packard Cobra II auto-gamma, Meriden ®, CT, USA) and adjust to 50,000cpm / 50 μl
3. Incubate at 4°C until use

4. Calculate total amount of labelled extract required ($50\mu\text{l} \times \text{number of samples}$),
and mix gently with pipette:
 $x \mu\text{l}$ VGKC extract
 $x \mu\text{l}$ PTX
 $x / 10$ ^{125}I - α -DnTx
5. Transfer to Eppendorfs, and microcentrifuge 13,000rpm for 5 min before use to
remove particulate matter.

Anti-VGKC radioimmunoprecipitation assay

1. Dilute $10\mu\text{l}$ patient sera 1:10 with PTX, vortex, spin 13,000rpm for 5 min.
2. Add $50\mu\text{l}$ of supernatant from each sample of diluted serum to $50\mu\text{l}$ of labelled
extract, vortex, and incubate overnight at 4°C .
3. Add $50\mu\text{l}$ sheep anti-human IgG to each sample (not microcentrifuged)
4. Incubate 1h at room temp.
5. Add $500\mu\text{l}$ PTX, vortex, spin at 13,000rpm for 5 min
6. Remove supernatant by suction, wash pellet twice in $500\mu\text{l}$ PTX.

[N.B if large number of samples, perform steps 12 and 13 stepwise in sets to prevent
time dependent dissociation of ^{125}I -DNTX-complexes]

7. Remove excess liquid. Count pellet on automatic gamma counter (Canberra
Packard Cobra II auto-gamma, Meriden ®, CT, USA).

VGCC assay

Radio-labelling using ^{125}I - ω -conotoxin MVIIC (^{125}I - ω -CmTx)

Incubation buffer (IB) pH 7.4, stored at 4°C :
 $500\mu\text{l}$ digitonin 4%
 17.5 ml dH₂O
 2 ml 0.2M phosphate

1. Defrost extracts on wet ice
2. In a 7ml bijou, add 25µl IB to 5µl cerebellar extract, i.e. up to a total of 30µl per sample, multiplied by the total number of samples to be tested in assay.
e.g. cerebellar extract for 50 samples = 250µl extract + 1.25ml IB
3. Defrost 1.0µCi aliquots of ^{125}I - ω -conotoxin, make up each aliquot with 500µl IB, spin 13,000rpm for 5 min.
4. Add 5 fmol of ^{125}I - ω -conotoxin per sample, usually 6-10 µl / sample: see Appendix 3.3 for calculation.
5. Make up total volume / sample to 50µl with IB. i.e. 30µl + 6-10µl (above) + 20-(6-10)µl. This is the labelled extract preparation.
6. Incubate 2h at 4°C on wet ice.

Anti-VGCC radioimmunoprecipitation assay

1. Dilute 10µl test sera diluted 1:10 with IB, vortex, spin 13,000rpm for 5 min.
2. Add 25µl of supernatant from each sample of diluted serum (equivalent to 2.5ul serum) to 50µl of labelled extract, vortex, and incubate overnight at 4°C.
3. Add 25µl sheep anti-human IgG to each sample (not microfuged)
4. Incubate 1h at 4°C.
5. Add 1ml PTX, vortex, spin at 13,000rpm for 5 min
6. Remove supernatant by suction, wash pellet twice in 1ml PTX.
[N.B if large number of samples, perform steps 12 and 13 stepwise in sets to prevent time dependent dissociation of ^{125}I - ω -conotoxin-complexes].
7. Remove excess liquid. Count pellet on automatic gamma counter (Canberra Packard Cobra II auto-gamma, Meriden ®, CT, USA).
8. Calculate final binding of labelled extract in pM / l using algorithm (Appendix 3.3)

Ganglionic $\alpha 3$ -AChR assay

Radio-labelling using 125 I-Epibatidine

1. Defrost IMR32 pellets and wash once in 0.05M Tris pH 7.2
2. Spin 13,000rpm for 5 min and remove the supernatant
3. Re-suspend the pellet in 400 μ l 0.05M Tris pH7.2 and add 400 μ l 4% Triton X in 0.05M Tris pH7.2 and 10 μ l PMFS
4. Vortex the pellet until homogenous and rotate at 4°C for 1 h
5. Spin 13,000rpm for 15 min and remove the supernatant
6. Determine the amount of extract required by titration by setting out 0, 5, 10, 20, 30 μ l made up to 50 μ l with PTX
7. Defrost 1.0 μ Ci aliquots of 125 I-Epibatidine, make up each aliquot with 500 μ l PTX, spin 13,000rpm for 5 min
8. Aliquot 10 μ l and count on automatic gamma counter (Canberra Packard Cobra II auto-gamma, Meriden ®, CT, USA).
9. Add 50,000cpm to each titration tube and incubate for 2h at 4°C

Anti- $\alpha 3$ nAChR radioimmunoprecipitation assay

1. Dilute 10 μ l patient sera 1:10 with PTX, vortex, centrifuge 13,000rpm for 5 min.
2. Add 50 μ l of supernatant from each sample of diluted serum to 50 μ l of labelled extract, vortex, and incubate overnight at 4°C.
3. Add 50 μ l sheep anti-human IgG to each sample (not microcentrifuged)
4. Incubate 1h at room temp.
5. Add 500 μ l PTX, vortex, spin at 13,000rpm for 5 min
6. Remove supernatant by suction, wash pellet twice in 500 μ l PTX.
7. Remove excess liquid. Count pellet on automatic gamma counter (Canberra Packard Cobra II auto-gamma, Meriden ®, CT, USA).

Glutamic acid decarboxylase assay using ^{125}I -GAD 65

1. Dilute 10 μl patient sera 1 / 10 with PTX, vortex, spin 13,000rpm for 5 min.
2. Add 50 μl of supernatant from each sample of diluted serum to 50 μl ^{125}I -labelled human recombinant GAD 65 tracer, vortex, and incubate overnight at 4°C.
3. Add 50 μl sheep anti-human IgG to each sample (not microcentrifuged)
4. Incubate 1h at room temp.
5. Add 500 μl PTX, vortex, spin at 13,000rpm for 5 min
6. Remove supernatant by suction, wash pellet twice in 500 μl PTX.

[N.B if large number of samples, perform steps 12 and 13 stepwise in sets to prevent time dependent dissociation of complexes of ^{125}I -labelled human recombinant GAD 65 tracer]

7. Remove excess liquid. Count pellet on automatic gamma counter (Canberra Packard Cobra II auto-gamma, Meriden ®, CT, USA).

3.3 *CONVERSION OF OBSERVED COUNTS TO CONCENTRATIONS*

Final concentrations of antibody are expressed as pmol / litre of serum (i.e. pM).

VGKC and AChR assays

Conversion factor = 0.1 (not isotope date dependent). Therefore final concentration = (observed cpm – mean control cpm) .0.1

VGCC assays

Conversion is dependent on isotope decay. The conversion factor (κ) may be calculated from a further variable (λ) which is date dependent, and taken from the decay table (below), and the volume of serum used (2.5 μ l for all assays), where:

$$\kappa = \lambda \text{ (fmol cpm)} / 2.5\mu\text{l}$$

The final concentrations of antibody are then calculated, where:

$$[\text{ab}] = (\text{observed cpm} - \text{mean control cpm}) \cdot \kappa$$

e.g. on day -15

$$\kappa = 0.000243 \text{ (fmol cpm}^{-1}\text{)} / 2.5\mu\text{l}$$

$$\kappa = 0.243 \text{ (pmol cpm}^{-1}\text{)} / 2.5 \text{ l}$$

$$\kappa = 0.0972 \text{ (pmol cpm}^{-1} \text{l}^{-1}\text{)}$$

$$\text{and: } [\text{ab}] = (\text{observed cpm} - \text{mean control cpm}) \cdot 0.0972$$

Total and non-specific binding curves (perform this every 2 months):

Calculate a quantitative precipitation curve for known strongly positive (LEMS) and known negative (healthy control) sera. Total binding is calculated by using increasing serum concentrations in the presence of labelled toxin only, and non-specific binding, by repeating the assay at each serum concentration in the presence of excess unlabelled toxin. The specific binding is calculated at each point by subtracting the non-specific binding from the total binding.

Calculation of volume equivalent of 5fmol ¹²⁵I- ω -conotoxin

Count 10 μ l of diluted ¹²⁵I- ω -conotoxin (cpm).

Find “expected count” using decay table (see below) and knowledge of the time period since preparation by the manufacturer. N.B “minus” days are a nuance of the decay curve and manufacture.

Calculate the volume (x) equal to 5fmol by using the equation:

$$\text{actual cpm} / 10 \mu\text{l} = \text{expected cpm} / x$$

i.e. on day – 23, if the observed count for 10 μ l is 37,000 cpm, then:

$$37,000 / 10 = 21,960 / x$$

and $x = 6 \mu\text{l}$ (1 s.f)

GAD assay

The GAD kit contains a calibration kit containing sera with known levels of GAD antibodies (0-300 international units (IU) /ml). All test samples were assessed in an assay containing GAD standards.

¹²⁵I- ω -conotoxin decay table

	S.A.(Ci/mmol)	1 cpm-fmol	5 fmol-cpm	DAYS		S.A.(Ci/mmol)	1 cpm-fmol	5 fmol-cpm
-29	2572	0.000219	22839	21	21	1567	0.000359	13915
-28	2556	0.000220	22697	22	22	1548	0.000364	13746
-27	2539	0.000222	22546	23	23	1530	0.000368	13586
-26	2522	0.000223	22395	24	24	1512	0.000372	13427
-25	2505	0.000225	22244	25	25	1495	0.000377	13276
-24	2488	0.000226	22093	26	26	1478	0.000381	13125
-23	2473	0.000228	21960	27	27	1461	0.000385	12974
-22	2458	0.000229	21827	28	28	1444	0.000390	12823
-21	2433	0.000231	21605	29	29	1428	0.000394	12681
-20	2414	0.000233	21436	30	30	1412	0.000399	12539
-19	2396	0.000235	21276	31	31	1395	0.000404	12388
-18	2378	0.000237	21117	32	32	1378	0.000409	12237
-17	2359	0.000239	20948	33	33	1362	0.000413	12095
-16	2340	0.000241	20779	34	34	1346	0.000418	11952
-15	2320	0.000243	20602	35	35	1331	0.000423	11819
-14	2300	0.000245	20424	36	36	1316	0.000428	11686
-13	2280	0.000247	20246	37	37	1301	0.000433	11553
-12	2260	0.000249	20069	38	38	1286	0.000438	11420
-11	2240	0.000251	19891	39	39	1271	0.000443	11286
-10	2220	0.000254	19714	40	40	1256	0.000448	11153
-9	2199	0.000256	19527	41	41	1242	0.000453	11029
-8	2178	0.000259	19341	42	42	1228	0.000459	10905
-7	2156	0.000261	19145	43	43	1214	0.000464	10780
-6	2134	0.000264	18950	44	44	1200	0.000469	10656
-5	2112	0.000267	18755	45	45	1186	0.000475	10532
-4	2090	0.000269	18559	46	46	1172	0.000480	10407
-3	2068	0.000272	18364	47	47	1158	0.000486	10283
-2	2046	0.000275	18168	48	48	1144	0.000492	10159
-1	2023	0.000278	17964	49	49	1131	0.000498	10043
0	2000	0.000281	17760	50	50	1118	0.000504	9928
1	1977	0.000285	17556	51	51	1105	0.000510	9812
2	1954	0.000288	17352	52	52	1092	0.000516	9697
3	1932	0.000291	17156	53	53	1080	0.000521	9590
4	1910	0.000295	16961	54	54	1068	0.000527	9484
5	1888	0.000298	16765	55	55	1055	0.000534	9368
6	1866	0.000302	16570	56	56	1042	0.000540	9253
7	1844	0.000305	16375	57	57	1030	0.000547	9146
8	1822	0.000309	16179	58	58	1018	0.000553	9040
9	1801	0.000313	15993	59	59	1007	0.000559	8942
10	1780	0.000316	15806	60	60	996	0.000565	8844
11	1760	0.000320	15629	61	61	984	0.000572	8738
12	1740	0.000324	15451	62	62	972	0.000579	8631
13	1720	0.000327	15274	63	63	961	0.000586	8534
14	1700	0.000331	15096	64	64	950	0.000593	8436
15	1680	0.000335	14918	65	65	939	0.000600	8338
16	1660	0.000339	14741	66	66	928	0.000607	8241
17	1641	0.000343	14572	67	67	918	0.000613	8152
18	1622	0.000347	14403	68	68	908	0.000620	8063
19	1604	0.000351	14244	69	69	897	0.000628	7965
20	1586	0.000355	14084	70	70	886	0.000636	7868

4.0 CELL-BASED ASSAY

4.1 BACTERIAL CULTURE

All media components were purchased from Oxoid Ltd., Hampshire, UK

Bacteria: Escherichia coli, strain DH5 α

Lysogeny broth (LB): 10 g Tryptone

5 g Yeasty extract

5 g NaCl

1 L dH₂O

Transformation buffer-I (TF-I):/L 12 g RbCl

30ml 1M (pH7.5) K acetate

1.5 g calcium chloride dihydrate

150 g glycerol

Make up TF-I to a final volume of 950 ml, adjust to pH 6.4 with 0.2 M acetic acid, and divide into 95 ml aliquots. Autoclave, allow to cool, and then add 5 ml of 1 M manganese chloride tetrahydrate.

Transformation buffer-II (TF-II): /L 1.2g RbCl

11g calcium chloride dihydrate

150g glycerol

Make up TF-II to a final volume of 980 ml and divide into 98 ml aliquots. Autoclave, allow to cool, and then add 0.5 M 3-[N-morpholino]propanesulfonic acid.

Culture growth and collection

1. Set up overnight pre-culture(s) by inoculating 2.5ml LB in 15 ml test tube
2. In 250 ml conical flask, inoculate 50 ml LB with 0.5 ml pre-culture
3. Measure optical density (OD) at 650 nm of culture every hour using a Unicam Helios β UV spectrophotometer (Unicam, Cambridge, UK)
4. Plot log OD_{650nm} vs. time
5. When OD is predicted to rise above 0.1, remove 7 ml and place in ice cold bottle
6. Measure OD of 1 ml and transfer exactly 0.5 OD units (0.5/OD observed) into fresh chilled tube
7. Centrifuge 13,000 rpm, 4°C for 5 min and remove supernatant by aspiration
8. Continue to collect 0.5OD units every hour until growth is complete

OD = 0.5-1.0	remove 2.5 ml, use 1 ml for OD measurement and rest to take sample
1.0-2.0	remove 1 ml and mix with 1 ml ice cold saline, use 0.9 ml for OD measurement and rest to take sample
2.0-3.0	remove 1 ml and mix with 2 ml ice cold saline, use 1 ml for OD measurement and rest to take sample
3.0-4.0	remove 1 ml and mix with 3 ml ice cold saline, use 1 ml for OD measurement and rest to take sample

Preparation of competent cells

Use sterile technique throughout

1. Inoculate 2.5 ml of LB and grow overnight at 37°C with moderate agitation (250 rpm)

2. Inoculate 50 ml LB with 0.5 ml of overnight culture
3. Grow at 37 °C, 250 rpm until OD is 0.4-0.5
4. Centrifuge 4000 rpm for 15 min at 4°C
5. Remove supernatant and re-suspend in 4 ml of TF-I
6. Place on ice for 15 min
7. Centrifuge 2,500 rpm for 15 min at 4°C
8. Resuspend in 2 ml TF-2
9. Keep cells on ice until use, or aliquot into 0.2 ml and store at -80°C

4.2 BACTERIAL TRANSFORMATION

Plasmid: cDNA encoding full length muscarinic 2 human cholinergic receptor (GenBank NM_001006630) cloned in- frame using the restriction sites EcoRI-XhoI in the polylinker region of pcDNA3 (Invitrogen Ltd., Paisley, UK). Provided by Dr Carl Nelson, University of Leicester, UK.

1. Add various dilutions of plasmid DNA (1-50 ng in 4 µl) to 0.2 ml aliquots of competent cells
2. Mix gently and incubate on ice for 40 min
3. Heat shock at 42 °C for 1 min
4. Place on ice for 5 min

5. Add 0.7 ml of pre-warmed LB and incubate at 37 °C for 30 -60 min
6. Centrifuge 13,000 rpm for 5 min and remove 0.7 ml of supernatant
7. Resuspend cells in remainder of supernatant and plate out onto plates containing L agar and ampicillin
8. Allow to dry, cover and incubate overnight at 37 °C

4.3 DNA ISOLATION AND SEQUENCING

DNA purification

Miniprep: for plasmid yields of less than 10 µg

QIAprep® Spin Miniprep kit (Qiagen Ltd., West Sussex, UK)

1. Centrifuge overnight culture at 4,000 rpm, 4 °C for 5 min
2. Re-suspend pelleted cells in 250 µl Buffer P1 (in kit) and transfer to a microcentrifuge tube
3. Add 250 µl Buffer P2 (in kit) and gently invert the tube 4–6 times to mix
4. continue inverting the tube until the solution becomes viscous and slightly clear.
Do not allow the lysis reaction to proceed for more than 5 min
5. Add 350 µl Buffer N3 (in kit) and invert the tube immediately but gently 4–6 times
6. Centrifuge for 10 min at 13,000 rpm
7. Remove supernatant and apply to the QIAprep spin column (in kit) by decanting or pipetting
8. Centrifuge for 30–60 s. Discard the flow-through
9. (Optional): Wash the QIAprep spin column by adding 0.5 ml Buffer PB (in kit) and centrifuging for 30–60 s. Discard the flow-through.

10. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s

Maxiprep: for plasmid yields greater than 10 µg

Purelink™ HiPure Plasmid (Maxi) Filter Purification Kit (Invitrogen Ltd., Paisley, UK)

1. Place the Maxi Column on the PureLink™ Nucleic Acid Purification Rack (in kit)
2. Apply 30 mL EQ1 buffer (in kit) directly into the Filtration Cartridge, which is inserted into the Maxi Column
3. Allow the solution in the HiPure Filter Maxi Column to drain by gravity flow
4. Prepare the cell lysate (see below) while the HiPure Filter Maxi Column is equilibrating.
5. For high copy number plasmids, use 100– 200 mL of an overnight LB culture per sample.
6. For low copy number plasmids, harvest 250– 500 mL of an overnight LB culture per sample.
7. Harvest the cells by centrifuging the overnight LB culture at 4,000 rpm for 10 minutes. Remove supernatant
8. Add 10 mL R3 buffer (in kit) with RNase A to the pellet and resuspend the cells until homogeneous
9. Add 10 mL L7 buffer (in kit) and mix gently by inverting the capped tube until the lysate mixture is thoroughly homogeneous. Do not vortex.
10. Incubate at room temperature for 5 minutes
11. Add 10 mL N3 buffer (in kit) and mix immediately by inverting the tube until the mixture is thoroughly homogeneous. Do not vortex.
12. Transfer the precipitated lysate including all the precipitated material into the equilibrated HiPure Filter Maxi Column

13. Let the lysate run through the filter by gravity flow until the flow stops (10– 15 minutes) or becomes very slow (<1 drop per 10 seconds)
14. Discard the flow through
15. Optional: The final DNA yield may be increased by washing the residual bacterial lysate in the HiPure Filter Maxi Column with 10 mL W8 buffer (in kit).
16. Let the buffer flow through the HiPure Filter Maxi Column by gravity flow until the flow stops or dripping becomes very slow
17. Immediately after the HiPure Filter Maxi Column has stopped dripping, remove the inner Filtration Cartridge from the column and discard.
18. Wash the Maxi column with 50 mL of W8 buffer. Allow the solution in the column to drain by gravity flow and discard the flow-through.

Eluting DNA

1. Place a sterile 50-mL centrifuge tube (elution tube) under the HiPure Filter Maxi column.
2. Add 15 mL E4 buffer (in kit) to the Maxi column and allow the solution to drain by gravity
3. Discard the HiPure Filter Maxi column.

DNA precipitation

1. Add 10.5 mL isopropanol to the DNA to the elution tube and mix well.
2. Centrifuge the tube at 10,000 rpm for 30 min at 4°C
3. Remove and discard the supernatant
4. Add 5 mL 70% ethanol to resuspend the DNA pellet.
5. Centrifuge the tube at 10,000 rpm for 5 min at 4°C
6. Remove and discard the supernatant.
7. Air-dry the pellet for 10 min
8. Resuspend the DNA pellet in 500 μ L TE Buffer (in kit)

9. For low copy number plasmids, use 200 μ L TE
10. Transfer the supernatant (DNA sample) into a fresh tube.
11. Store the purified DNA at -20°C , or store at 4°C and proceed to desired downstream application.

DNA digestion

Restrictions enzymes (New England BioLabs UK Ltd., Hertfordshire, UK):

EcoR1. Recognition site: 5'...G[↓]AATTC...3'
 3'...CTTAA[↓]G...5'

NEBuffer EcoR1 (supplied with enzyme):

100 mM Tris-HCl

50 mM NaCl

10 mM MgCl₂

0.025 % Triton X-100

Reaction conditions:

Incubate 5 units of enzyme per μg of DNA for 1 h at 37°C

Xho1. Recognition site: 5'...C[↓]TCGAG...3'
 3'...GAGCT[↓]C...5'

NEBuffer 4 (suuplied with enzyme):

20 mM Tris-acetate

50 mM potassium acetate

10 mM magnesium acetate

1 mM dithiothreitol

Reaction conditions:

Incubate 5 units of enzyme supplemented with 100 µg/ml bovine serum albumin for 1 h at 37 °C

DNA sequencing

Agarose gel electrophoresis

5x TBE buffer: 54 g Tris
 27.5 g Borate (orthoboric acid)
 20 ml 0.5 M EDTA (pH8)
 980 ml dH₂O

Mix Tris, Borate and dH₂O, then add EDTA. Make to final volume of 1 L.

0.5 M EDTA (pH8): 18.6 g EDTA
 175 ml dH₂O
 2 g NaOH

Increase final volume to 200 ml, check pH and autoclave.

1% agarose gel: 1g agarose
 10ml 5x TBE
 90 ml H₂O

Melt in microwave, cool under tap and add ethidium bromide to final concentration of 25 µg/ml.

6x gel loading buffer: 2.5 ml bromophenol blue
 2.5 ml xylene cyanol FF
 4.0 ml Ficoll in H₂O

Store at room temperature

4.4 MAMMALIAN CELL CULTURE

Tissue culture plastic-wear, all from Appleton Woods Ltd. Birmingham, UK:

75cm² Corning tissue culture flasks

6 and 24-well plates

Media and solutions:

Dulbecco's Modified Eagles Medium (DMEM) containing 4500 mg/l glucose, L-glutamine and sodium pyruvate (Sigma-Aldrich, Poole, Dorset, UK)

Antibiotic – antimycotic solution, containing / ml, in 0.85 saline: 10,000 units of penicillin, 10,000 µg streptomycin, 25 µg amphotericin (Invitrogen Ltd., Paisley, UK)

DMEM+ab: DMEM containing antibiotic –antimycotic solution

DMEM wash: DMEM containing 4.6 g/l HEPES

DMEM diluent: DMEM containing 4.6 g/l HEPES and 1 % BSA

Poly-L lysine (Sigma-Aldrich)

Poly-L lysine solution: 1 ml poly-lysine
 100 ml PBS

Trypsin-EDTA solution (Invitrogen)

Harvesting and counting of HEK cells

1. Aspirate off medium
2. Wash cells with 5 ml PBS and aspirate off
3. Detach cells by adding 1-2 ml of trypsin and incubating at 37 °C for 1 min
4. Resuspend cells in 10 ml of DMEM+ab
5. Transfer to 50 ml Universal tube and take off 10 µl to count using a haemocytometer
6. Centrifuge for 6 min at 1000 rpm

Seeding of HEK cells

1. In a flow cabinet, place 3 coverslips / well into a six well tray
2. Add 2 ml of poly-L lysine solution into each well
3. Leave for 20 min
4. Aspirate off poly-L lysine solution and allow to dry for 2 h
5. Make a 12 ml cell suspension with DMEM+ab+FCS containing 1.8×10^6 cells
6. Add 2 ml of cell suspension to each well and incubate overnight
7. Transfect the following day

HEK cell transfection

1. Make up transfection solution according to number of wells:

Mix in two separate tubes (per well):

4 µg DNA

250 – (x µl DNA) ab-free DMEM: incubate for 5 min

10 µl lipofectamine

240 µl ab-free DMEM

2. Mix the contents of the two tubes and incubate for 15 min RT
3. Change medium of wells to ab-free DMEM (2 ml / well) and add 500 µl of transfection solution to each well
4. Incubate overnight at 37 °C
5. Change medium to ab+DMEM
6. Incubate overnight at 37 °C
7. The following day cells are ready for immunostaining

Immunofluorescent staining of transfected HEK cells

1. Transfer coverslips to a new 24 – well plate containing / well 250 µl PBS
2. Wash with PBS x 2 aspirating each time
3. Fix cells with 4 % formaldehyde for 10 min
4. Aspirate off formaldehyde and wash with PBS x 3
5. Permeabilise cells to be stained with anti-M2 antibody by adding 250 µl Triton X for 10 min
6. Aspirate off Triton X and wash with DMEM wash x 3
7. Dilute each serum sample, 1:20 in DMEM diluent
8. Dilute anti- M₂ primary antibody 1:200 in DMEM diluent
9. Add 250 µl of serum or anti-M₂ antibody solution / per well
10. Incubate for 1 h at 37 °C
11. Remove by aspiration
12. Wash coverslips with 250 µl DMEM wash x2, then PBS x1, pipetting and aspirating each time
13. Aspirate off formaldehyde and wash 3 times with PBS

14. Permeabilise cells
15. Add 250 µl of secondary, fluorescently conjugated anti-human antibody (Diluted 1:700 in DMEM diluent) to each well and incubate for 45 min in the dark at RT
16. Aspirate off secondary antibody and wash with DMEM wash x 3 and PBS x 2
17. Transfer coverslips to microscope slides, mounting 3 – 4 per slide using fluorescent mounting medium containing DAPI, 1:1000
18. Store at 4 °C before reading

5.0 IMMUNOHISTOCHEMISTRY

5.1 SOLUTIONS AND REAGENTS

All solution and reagents were supplied by VWR International, Lutterworth, Leicestershire, UK (not stated), Dako, Ely, Cambridgeshire, UK (Dako), Bio-Genex Laboratories, San Ramon, CA, USA (Bio-Genex), or Sigma Aldrich, Poole, Dorset, UK (Sigma). R.T.U Vectastain Universal Elite ABC kit was supplied by Vector Laboratories Inc., Burlingame, CA, USA (Vector) (Cat. No. PK-7200).

IMS = 100% industrial methylated spirits.

Endogenous peroxidase blocking solution	6ml H ₂ O ₂ 194ml dH ₂ O
Acid Alcohol	300ml dH ₂ O 700ml IMS 10ml conc. HCl (added to 990ml of the above)
Gill's Haematoxylin	1L dH ₂ O 625ml ethylene glycol,

	10g haematoxylin
	1g sodium iodate
	176g aluminium sulphate
	50ml glacial acetic acid
	825ml dH ₂ O
1% Eosin	1g eosin Y

100ml dH₂O

Immunohistochemistry

Endogenous peroxidase block (3%)	3ml 30% H ₂ O ₂ 97ml 100% methanol
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REAL™ Antibody diluent (DAKO)

Wash buffer (DAKO)	0.05mol/L Tris HCl 0.15mol/L NaCl 0.05% Tween 20
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Vectastain ABC Elite reagent (Vector)

Diaminobenzidine tetrachloride (DAB) solution (Bio-Genex)	1ml substrate buffer 1 drop DAB chromagen
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Phosphate buffered saline (PBS)

Mounting medium for immunohistochemistry: Canada balsam

Fluorescence mounting medium (Dako)

Antigen retrieval

Citrate buffer solution (Vector) Citrate buffer stock 100ml
dH₂O 900ml

5.2 *FIXATION AND PROCESSING OF TISSUE*

1. Fix tissue for at least 24 h in formal saline and embed in paraffin wax.
2. Cut 3 µm thick sections and float in a warm water bath
3. Pick up sections on negatively charged microscope slides (VWR International, Leicestershire, UK) and allow water to drain off
4. Dry sections overnight at 37 °C

5.3 *HAEMATOXYLIN AND EOSIN STAINING*

1. Dewax sections in xylene (2x 2 min)
2. Remove xylene with IMS (2x 2 min)
3. Wash in running tap water 5 min
4. Stain in Gill's haematoxylin for 20 min
5. Wash in running tap water 5 min
6. Differentiate by dipping in 1 % acid alcohol
7. Wash in running tap water 5 min
8. Stain in eosin for 3 min
9. Dip in tap water and blot
10. Dehydrate rapidly in IMS (2x 2 min)
11. Clear in xylene (2x 2 min)

12. Mount in Canada balsam

5.4 *INDIRECT-IMMUNOHISTOCHEMICAL STAINING PROTOCOL*

1. Dewax sections in xylene (2x 2 min)
2. Remove xylene with IMS (2x 2 min)
3. Wash in running tap water 5 min
4. Antigen retrieval (see below)
5. Wash in running tap water 5 min
6. Incubate sections in endogenous peroxidase blocking solution for 15 min
7. Wash in running tap water for 5 min
8. Soak in wash buffer (WB) 2 min
9. Dry carefully around sections and encircle with ImmEdge pen (Vector Labs)
10. Apply blocking serum for 20min:
 25 % normal horse serum (in kit)
11. Tip off blocking serum.
12. Apply primary antibody at the appropriate dilution in antibody diluent for 60 min RT
 (antibody diluent only to negative control sections)
13. Rinse sections with WB and soak in WB 2x 5 min
14. Wipe around sections and apply biotinylated secondary antibody diluted 1:250 in
 wash buffer for 30 min at RT
15. Apply Vectastain Elite ABC reagent (from kit) for 30 min RT
16. Rinse sections with WB and soak in WB 2x 4 min
17. Make up DAB solution and apply for 5 min (examine positive control slide for
 progress of development)
18. Wash in running tap water for 5 min
19. Counterstain in Gill's Haematoxylin for 2 min
20. Wash in running tap water for 5 min

21. Differentiate by dipping two to three times in 1% acid alcohol
22. Wash in running tap water for 5 min
23. Dehydrate in IMS
24. Clear in xylene
25. Mount in Canada balsam

5.5 *INDIRECT-IMMUNOFLUORESCENT STAINING PROTOCOL*

1. Dewax sections in xylene (2x 2 min)
2. Rehydrate with IMS (2x 2 min)
3. Wash in running tap water 5 min
4. Antigen retrieval (see below)
5. Wash in running tap water 5 min
6. Soak in PBS 2x 5 min
7. Dry carefully around sections and encircle with ImmEdge pen (Vector Labs)
8. Apply serum-free protein-block for 10min:
9. Tip off blocking serum.
10. Apply both primary antibodies in single solution at the appropriate dilution in PBS for 60 min RT (antibody PBS only to negative control sections)
11. Soak slides in PBS 2x 5 min
12. Wipe around sections and apply Alexa-fluor™ secondary antibodies diluted in PBS (1:200), in a single dilution (finally 1:100) in PBS for 45 min at RT
13. Soak slides in PBS 2x 5 min
14. Mount in fluorescence mounting medium containing DAPI diluted 1:1000
15. Store at 4°C until viewing

Antigen retrieval

1. Place sections into plastic staining rack leaving a gap between the slides
2. Place rack into a large glass staining trough containing exactly 1000ml of the antigen retrieval buffer
3. Cover the trough with cling film and make several holes in the cling film
4. Place the trough in the microwave and run at full power for 25 minutes
5. With care, remove trough from microwave
6. Carefully remove cling film
7. Leave sections to stand in the hot buffer for 5 minutes
8. Return to original technique

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