Is Narcolepsy an Antibody-mediated Autoimmune Disease?

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Abstract

Narcolepsy is a lifelong sleep disorder of unknown cause. Patients experience excessive daytime sleepiness (EDS) in conjunction with the pathognomonic symptom of cataplexy, which is a sudden bilateral loss of motor tone triggered by positive emotion. Central to the pathology of narcolepsy-cataplexy is the loss of orexin-secreting neurons in the hypothalamus and the concomitant loss of orexin neuropeptide in the cerebrospinal fluid (CSF). The cause is thought to be autoimmune, mainly because of the tight association with HLA DQB1*0602. However, there is no direct evidence to support this hypothesis. Since 2010, a particular H1N1 vaccine has been implicated as a rare trigger of narcolepsy-cataplexy in children. Post-vaccination cases have a particularly abrupt onset, and may provide new insights into disease aetiology. This thesis explored the possibility of a humoral immune cause.

Tests for the presence of antibodies directed towards neuronal antigens were performed on 17 sera/CSF pairs from children with recent onset narcolepsy-cataplexy as well as unpaired samples and those from adults and controls. Methods included a cell-based candidate antigen approach, antibody-screening techniques using rat brain tissue and neuronal cultures, and testing for biomarkers of autoimmunity. There was no consistent evidence of autoantibodies to a specific antigen in a high proportion of patients, but immunoreactivity to neuronal antigens, including unidentified antigens on the surface of neurons in culture, was identified in some samples.

This large narcolepsy cohort included recent-onset and post-vaccination cases, patients in whom putative autoantibodies are most likely to be found. Immunoreactivity in some cases provides preliminary evidence for autoantibodies which needs to be followed up. Further strategies are required to investigate the aetiological mechanisms underpinning narcolepsy-cataplexy, including addressing the possibility of T cell-mediated damage.

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Abbreviations

AEC	3-amino-9-ethylcarbazole
ANA	antinuclear antibody
AQP4	aquaporin-4
ASO	antistreptolysin O
BBB	blood-brain barrier
BSA	bovine serum albumin
CASPR2	contactin associated protein-like 2
CBA	cell-based assay
(c)DNA	(complementary) deoxyribonucleic acid
CNS	central nervous system
CPM	counts per minute
CSF	cerebrospinal fluid
DAB	3,3'-Diaminobenzidine
DNA	deoxyribonucleic acid
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
EDS	excessive daytime sleepiness
E. coli	Escherichia coli
eGFP	enhanced green fluorescent protein
EMA	European Medicines Agency
FCS	foetal calf serum
GABA	gamma-aninobutyric acid
GAD65	glutamic acid decarboxylase 65
GFAP	glial fibrillary acidic protein
GluR	glutamate receptor
GWAS	genome-wide association study
HBSS	Hank's balanced salt solution
HC	healthy control
HEK cells	human embryonic kidney cells
HLA	human leukocyte antigen
HRP	horseradish peroxidase
ICSD	international classification of sleep disorders
I^{125}	iodine^{125}
I.F.	indirect immunofluorescence immunohistochemistry
ICV	intracerebroventricular
Ig	immunoglobulin

IGFβ	insulin-like growth factor beta
IRES	internal ribosome entry site
kDa	kilodaltons
LB	Luria Bertoni
LGI1	leucine-rich glioma inactivated 1
MCH	melanin concentrating hormone
MHC	major histocompatibility complex
MEM	minimal essential medium
MSLT	multiple sleep latency testing
NB	neurobasal
NDCN	Nuffield Department of Clinical Neurosciences
NMDAR	N-methyl D-aspartate receptor
NMO	neuromyleitis optica
O/N	overnight
PALP	pigeon acetone liver powder
PBS	phosphate buffered saline pH 7.2
PCR	polymerase chain reaction
PI	propidium iodide
PLL	poly-L-lysine
PTX	phosphate buffered saline with 1% tween
RCF	relative centrifugal force (xg)
REM	rapid eye movement
RIA	radioimmunoassay
RID	radioimmunodiffusion
RT	room temperature
SD	standard deviation
SEM	standard error of the mean
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
SDM	site-directed mutagenesis
TCR	T cell receptor
T_m	melting temperature
TRIB2	tribbles homologue 2
VGKC	voltage-gated potassium channel
WIMM	Weatherall Institute of Molecular Medicine

1. Introduction

Autoimmunity describes the aberrant targeting of self-structures by T cells or antibodies produced by B cells (Janeway & Travers, 2001). The essential feature of autoimmune disease is the expansion of self-reactive T cells and/or B cells not clonally deleted during development. Situations in which this may be triggered include infection, tissue injury and inflammation.

It is thought that polymorphisms in genes coding for fundamental proteins of the adaptive immune response underpin the molecular basis of much autoimmune disease susceptibility (Vyse, Todd, & Street, 1996). Human leukocyte antigen (HLA) loci code for the major histocompatibility complex (MHC) molecules responsible for antigen presentation in the immune system, and specific polymorphisms are associated with almost all autoimmune diseases. Encoded on the short arm of chromosome 6, HLA loci are both polygenic (an individual has several genes encoding different MHC molecules) and polymorphic (multiple alleles exist at each locus). This diversity enables the presentation of a wide repertoire of peptide fragments to the T cell receptor (TCR) of T cells, which mediate subsequent immune responses. However, specific HLA types may favour the presentation of particular self-antigens, perhaps accounting for the HLA associations seen with autoimmune diseases (Janeway and Travers 2001).

A tightly-linked HLA association has long been associated with narcolepsy (Honda et al. 1983), a finding that has lead many to hypothesise an autoimmune aetiology. Clinically described over 130 years ago (Westphal, 1877), narcolepsy is a lifelong, disabling condition of unknown aetiology, characterised by dysregulation of the sleep-wake cycle and inappropriate penetration of rapid eye movement (REM) sleep. Cataplexy, a sudden loss of motor tone triggered by emotion, is the only specific symptom of the condition, and in this thesis all references to narcolepsy specifically refer to narcolepsy with cataplexy. The absence of CSF hormone orexin is a highly sensitive marker of the condition (Mignot et al.

2002), and reflects the selective loss of orexin-producing neurons in the hypothalamus (Thannickal et al. 2000). These neurons play a pivotal role in sleep and energy homeostasis and their selective death, perhaps by autoimmune mechanisms, is thought to provide a pathological explanation for the condition.

In the past, the CNS has been largely overlooked as a potential target of antibody-mediated autoimmune attack. However, it is now understood that in certain circumstances (e.g. inflammation), disease-causing autoantibodies can cross the blood brain barrier (BBB) (Stolp & Dziegielewska, 2009). Several antibody mediated CNS autoimmune conditions have now been identified in patients with pathogenic autoantibodies against neuronal cell-surface channels (for a recent review, see Vincent et al. 2011). Sleep disturbance is reported as a feature in many of these conditions, a clinical correlate that further links possible antibody-mediated mechanisms with narcolepsy-cataplexy. Despite much research, however, no conclusive evidence of autoantibodies or other autoimmune mechanisms have been found in narcolepsy.

The aim of the work described here was to identify possible antibody-mediated immune mechanisms in narcolepsy.

1.1. Narcolepsy epidaemiology

Prevalence, age and sex

The worldwide prevalence of narcolepsy-cataplexy lies between 1:2000-1:4000, a figure reflected in Europe, North America, the Middle East and China (Longstreth et al. 2007). Higher prevalences are reported in Japan, but can perhaps be accounted for by the looser definitions of cataplexy and the self-report methodologies used in these studies (Honda et al. 1983). Meanwhile, lower prevalences (0.002%) are reported in Israel, a finding that correlates with the lower incidence of disease-associated HLA types found in Jewish populations (Wilner et al., 1988).

Some have also reported higher prevalence in men than women (Silber, Krahn, Olson, & Pankratz, 2002), but differences in the sex ratio appear to be slight. Although there is wide variation in age of onset, in general a bimodal distribution is observed with one large peak around 15 yrs., and a smaller peak around 36 yrs. (Dauvilliers et al. 2001). Approximately half of the patients have symptom onset prior to the age of 15. Overt onset of symptoms in early childhood however is very rare, with only 1.1% of narcoleptics suffering from cataplexy prior to the age of five (Aran et al., 2010). Earlier age of onset appears to be correlated with both a positive family history and frequency of cataplectic attacks (Dauvilliers et al., 2010).

Familial risk

Although narcolepsy-cataplexy has long been considered to be a familial disorder, first-degree relatives only have a 1-2% chance of developing the condition (reviewed by Mignot 1998). Nonetheless, this represents a 20-40 x increased risk over that of the general population. However, more than 20 monozygotic twin studies have been reported, and are discordant in the majority (65-80%) of cases (Mignot 1998). These features, together with an adolescent age of onset, make a multifactorial aetiology seem likely, with environmental factors acting on a background of genetic susceptibility. Genetic factors thought to underpin narcolepsy susceptibility, in particular the HLA association, will be discussed in **chapter 1.6**.

Environmental factors

Several environmental risk factors have been identified by retrospective studies. Historically cited associations include head trauma, change in sleep-wake habits and upper respiratory tract infections (reviewed by Mignot 2004). Life events prior to narcolepsy onset, such as stress and pregnancy, may be significant (Silber et al., 2002). A striking seasonal birth pattern has also consistently been reported in patients destined to develop narcolepsy, with a March peak and September trough in birth month (Dauvilliers et al. 2003; Dahmen & Tonn 2011; Picchioni et al. 2004). This latter factor perhaps suggests that *in-utero* exposures or neonatal infections also mediate an increased narcolepsy risk.

As previously mentioned, infections are commonly reported around narcolepsy onset. Foremost amongst these is streptococcal infection, an association supported by the temporal relation of antistreptolysin antibody titres to disease onset (Aran et al., 2009). Some speculate that molecular mimicry between host and pathogen could be responsible, such as that shown in post-streptococcal Sydenham chorea (Fontana et al., 2010). Alternatively, the association may be explained by the involvement of superantigens as triggers for T cell activation. The relationship with H1N1 vaccination is described below.

Rapid weight gain is a common feature close to disease onset (Kotagal, 2004) and Kok et al. 2003 found that patients with narcolepsy-cataplexy were almost three times more likely to be overweight than controls. It has been suggested that this may be accounted for by disturbances of the intrinsically-linked pathways of sleep and energy homeostasis (Vanitallie, 2006), although weight gain could also be explained by increased inactivity after narcolepsy onset. Similarly, although more than half of narcolepsy patients reportedly suffer from migraine (Dahmen et al. 2003), this may be accounted for by poor sleep or the treatments given rather than a central mechanism.

1.2. Association with H1N1 vaccination

Most recently, Swine fslu (H1N1) vaccination has been associated with abrupt-onset narcolepsy-cataplexy in paediatric age groups. To date, over 200 post-vaccination cases have been reported. Pandemrix® is the ASO3-conjugated (containing aquiline) split-virion vaccine at the centre of this controversy. Authorised by the European Medicines Agency (EMA) in September 2009, over 30 million people were vaccinated in Europe (European Medicines Agency, 2011). Worldwide, the vaccine was used in 47 countries, with particularly high coverage rates in Finland, Sweden, Norway and Ireland. A similar ASO-3 conjugated vaccine (Arepanrix®) was used in Canada. The first case of H1N1 in the UK was reported on 29th April 2009, and vaccination program announced in August (Health Protection Agency, 2009; World Health Organisation, 2009). 86% of all H1N1 vaccinations in the UK were with Pandemrix®, including around 20% of all children under the age of five (more than 650,000)

and 40% of at-risk individuals (Department of Health, 2010; MHRA, 2010). Although previously untested in paediatric age groups, Pandemrix® was chosen as the novel ASO3 adjuvant was thought to enhance H1N1 immunogenicity (Waddington et al., 2010).

Swedish and Finnish surveillance groups initially identified 20 cases of post-vaccination narcolepsy-cataplexy in children and adolescents in August 2010, prompting the suspension of this vaccination in these countries (Swedish Medical Products Agency 2010; Finnish National Institute of Health and Welfare 2010). An international, multicentre study ensued (Dauvilliers et al. 2010), reporting an additional 14 post vaccination cases between September 2009 and August 2010. An additional two cases of narcolepsy were reported following H1N1 infection itself. Researchers were struck by the abrupt onset of cataplexy within weeks of EDS onset; in classical narcolepsy, this latent period is often more than a year. Also of concern was the atypical age groups presenting, with two cases below the age of five and five cases post 38 years. High anti-streptococcal titres were found in 11 of the 14.

The European Medicines Agency (EMA) initiated a review of the reported association in August 2010. Upon conclusion in July 2011, the EMA recommended that Pandemrix® should be restricted to individuals over the age of 20 due to a 6-13 fold increase in narcolepsy incidence in younger age groups (European Medicines Agency, 2011). For other age groups, the report suggested that the vaccination only be used if seasonal trivalent vaccine was not available and if H1N1 vaccine was still required. However, this report also stressed the overall positive vaccine effects on balancing benefits against risks. Investigators proposed that the vaccine probably interacted with genetic or environmental factors and stressed the importance of continued research.

A recent Irish retrospective population-based cohort study identified an additional 18 post-vaccination narcolepsy-cataplexy cases. This reinforced conclusions of the prior studies regarding increased risk following vaccination (Irish National Narcolepsy Steering Comitee, 2012). This report also attempted to account for extensive narcolepsy-related media attention

in recent years as a source of potential bias. Researchers concluded that the association is unlikely to be explained by any bias and represents a true increase in paediatric age groups.

It remains to be seen whether the rate of paediatric narcolepsy will return to pre-swine flu levels now that Pandemix® vaccination is uncommon. Reports from several EU initiatives are now awaited, (e.g. the VAESCO study), which will hopefully address this question, as well as consider possible confounders.

1.3. Clinical features

With the release of the second edition of the International Classification of Sleep Disorders (ICSD) in 2005, traditionally-defined 'narcolepsy' is now separated into three separate entities: narcolepsy with cataplexy, narcolepsy without cataplexy and narcolepsy secondary to a medical condition (American Academy of Sleep Medicine, 2005). This was in recognition of the fact that cataplexy is a defining symptom of idiopathic narcolepsy and most likely reflects a common pathogenesis. Narcolepsy without cataplexy, diagnosed on the basis of excessive daytime sleepiness and multiple sleep latency testing, most likely reflects a mixture of pathologies. For this reason, only narcolepsy with cataplexy (narcolepsy-cataplexy) will be considered in this thesis, for which the diagnostic criteria are listed in **Table 1.1**.The sensitivity and specificity of diagnostic tests for narcolepsy are summarised in **Table 1.2**.

CSF orexin measurement is a sensitive and highly specific test, and is now routinely used as a surrogate for MSLT. Whereas CSF values of orexin in normal individuals are typically more that 200 pg/ml, the majority of narcolepsy-cataplexy patients have levels of <40pg/ml, well below the diagnostic threshold of 110 pg/ml. CSF orexin measurement is particularly useful in younger children who may not comply with MSLT (Aran et al., 2010).

Although HLA typing has previously been included in diagnostic criteria, the disease-associated HLA-DQB1*0602 polymorphism is also present in approximately one quarter of

Table 1.1. 1.2

the total population, limiting the diagnostic utility of the test. Other associated features, such as sleep paralysis, hypnogogic hallucinations and automatic behaviours close to disease onset are also not diagnostic due to lack of specificity (Billiard 2007).

Historically, there has been a diagnostic delay of several years between symptom onset and narcolepsy diagnosis (Morrish, 2004). This delay has reduced significantly in recent years, for which increased patient/clinician awareness and clear-cut international criteria have undoubtedly contributed. However, this alone does not account for the abrupt-onset post H1N1 vaccinated cases (Dauvilliers et al. 2010; Irish National Narcolepsy Steering Comitee 2012). It seems that this subgroup of patients have experienced an accelerated disease progression.

An Italian group recently reported a complex movement disorder around the time of disease onset in children (Plazzi et al. 2011). These included hypotonia as well as active motor disturbances such as perioral and dyskinetic-dystonic movements that did not meet the classical definition of cataplexy. These phenomena are apparently limited to disease onset and vanish later in the disease course, but are interesting because they resemble the movement disorders associated with NMDA receptor antibody encephalitis, a known antibody-mediated disease (Dalmau et al. 2008).

Secondary causes of narcolepsy-cataplexy are widely reported. The most common causes include inherited disorders such as Niemann Pick disease type C, tumours such as craniopharingiomas, and head trauma (Nishino & Kanbayashi 2005). Other causes include multiple sclerosis, encephalitis, cerebral ischemia and neurodegeneration. Although CSF levels of orexin are usually deficient, these cases typically do not share the disease-associated HLA type, and are often in age groups atypical for narcolepsy. Unlike idiopathic cases, CSF orexin deficiency in some of these patients has been found to be reversible with improvement of the underlying neurological disorder and resultant hypothalamic disturbance (Nishino & Kanbayashi 2005). Narcoleptic symptoms and deficient levels of CSF orexin have

also been reported in several case reports of neurological autoimmune diseases including Guillian-Barré syndrome, neuromyelitis optica (Baba et al., 2009) and NMDA receptor encephalitis (Tsutsui et al., 2012). This will be discussed in greater detail in **chapter 1.7**.

There is no curative treatment for narcolepsy. Symptoms of EDS are commonly treated with psycostimulants such as methylphenidate, dexamfetamine and modafinil (Clarke, 2005). Cataplexy is often treated with sodium oxybate and the antidepressant venlafaxine. Given the putative autoimmune cause of narcolepsy-cataplexy, several have attempted treatment with intravenous immunoglobulins. In one case report by Dauvilliers et al. 2009, CSF orexin levels normalised following treatment, and the patient enjoyed a four month period of remission. Conversely, no benefit was reported in a similar case elsewhere (Knudsen et al., 2012), and benefits of this treatment remain unclear.

1.4. Pathophysiology

The orexins, also called hypocretins, are a pair of neuropeptides simultaneously discovered by two groups in 1998 (de Lecea et al. 1998; Sakurai et al. 1998). Named because of their observed role in stimulating feeding behaviour, Orexin A and B (hypocretin 1 and 2) are produced from the common precursor molecule prepro-orexin. They are released by a discrete group of neurons localised in the lateral hypothalamus, an area long thought to be associated with sleep disturbance and narcolepsy (Von-Economo 1930). With exception of the cerebellum, these orexin neurons have widespread projections throughout the brain, particularly densely adrenergic nuclei in the locus coerulus, as well as other areas thought to be involved in the regulation of sleep (Peyron et al. 1998). The actions of the orexins are mediated by two G protein receptors called orexin(/hypocretin) receptors 1 and 2 (Lin et al. 1999). The highest affinity interactions are between orexin A and orexin receptor 2, a linkage that is thought to be largely responsible for the biological effects of these neuropeptides (Sakurai et al. 1998). Therefore, future references made to ‘orexin’ in this thesis specifically refer to orexin A.

Shortly after the discovery of the orexins, evidence directly implicating their involvement in narcolepsy was discovered in animal studies. Narcolepsy-cataplexy has long been recognised in canines (Mitler et al. 1974), in which cataplexy is most often triggered by food. In contrast with human narcolepsy, which is generally sporadic, in Labradors and Doberman Pinschers the condition is transmitted as an autosomal recessive trait with 100% penetrance. By positional cloning methods, Lin et al. (1999) discovered that the familial canine form of narcolepsy was found to be caused by mutations in orexin receptor-2. Subsequently, prepro-orexin knock-out mice also suffered from a condition analogous to narcolepsy (Chemelli et al. 1999), further establishing the role of deficient orexin neurotransmission as an integral part of narcolepsy pathogenesis

Orexin deficiency in the CSF of human narcoleptic patients was discovered by Nishino et al. (2000); levels of the neuropeptide were undetectable in seven out of nine narcolepsy-cataplexy patients tested. Post-mortem studies of narcoleptic brains confirmed that the undetectable levels of CSF orexin were associated with a selective loss of 85-95% of the 70,000 orexin-producing neurons in humans (Peyron et al. 2000; Thannickal et al. 2000). Whilst levels of prepro-orexin RNA and other markers co-expressed in orexin neurons were absent or substantially reduced (Crocker et al., 2005), melanin-concentrating hormone (MCH) neurons distributed within the same region of the hypothalamus remained intact (Thannickal et al. 2000). Evidence of gliosis was also found in relation to orexin neuron axonal density (Thannickal et al. 2003). The effects of orexin are primarily mediated via excitatory projections to adrenergic cells in the brainstem (Peyron et al. 1998); the loss of orexin-producing cells is likely to result in a cholinergic/adrenergic imbalance, which could underlie the REM disturbances experienced in narcolepsy-cataplexy (Mignot 2011).

Together, these findings suggest that orexin neurons are selectively targeted and destroyed in human narcolepsy-cataplexy, resulting in CSF orexin deficiency and the symptoms of the condition. It is possible that narcolepsy pathogenesis follows a similar ‘hit and run’ model of

disease onset and progression as proposed for Type I diabetes (summarized in **Figure 1.1**); symptoms and clinical onset may only ensue after a 'critical mass' of orexin neurons are lost. This could partly explain why attempts to identify the cause of narcolepsy have thus far been unsuccessful. The causative agent may be only transiently present around the time of disease onset, in which case sampling at later time points could be unsuccessful.

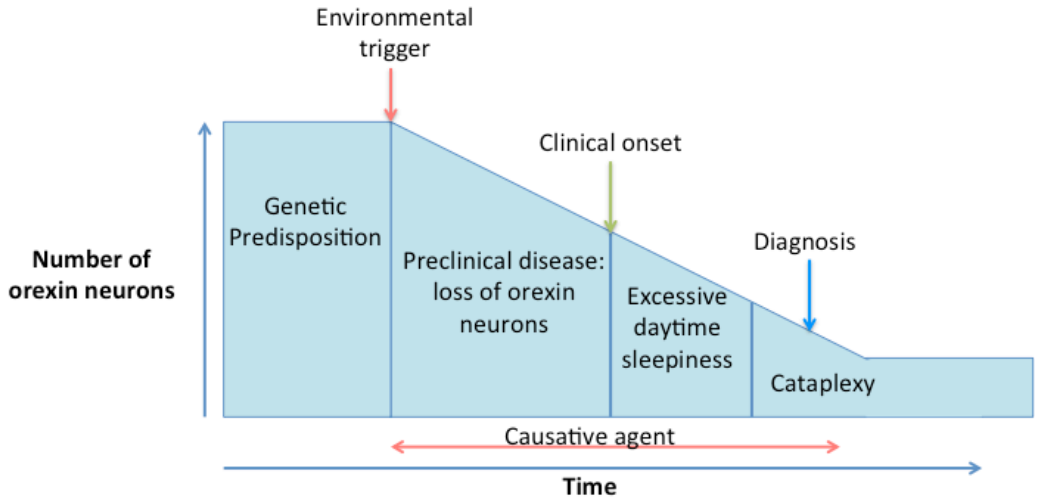


Figure 1.1: Model of narcolepsy-cataplexy disease progression (adapted from Longstreth et al., 2007)

1.5. Orexin neurobiology and MCH

Underpinning research into narcolepsy pathogenesis, a large amount of basic research has been performed to delineate the neurobiology of the orexin system and REM sleep. Orexin neurons are now understood to be stabilisers of wakefulness; whilst intracerebroventircular (ICV) administration of orexin A and B increases activity and decreases sleep in rats (Hagan & Leslie, 1999), optogenetic excitation of orexin neurons is sufficient to drive awakening (Adamantidis et al. 2007). Clearly, sleep and wakefulness must be regulated to occur at appropriate times in relation to internal and external environments. In this context, it is perhaps unsurprising that orexin has secondary functions involved in energy balance such as the regulation of feeding, and reward-seeking behaviour (reviewed by Sakurai & Mieda 2011).

Afferent signals to orexin neurons are from the amygdala and other hypothalamic nuclei (Yoshida, McCormack, España, Crocker, & Scammell, 2006). Although orexin neurons themselves are understood to be intrinsically active pacemakers (Eggermann et al., 2003), their firing rate is influenced by neurotransmitters such as glutamate, GABA, noradrenalin, glycine and serotonin in addition to several neuropeptides, including melanin concentrating hormone (MCH) , oxytocin, vasopressin, cholecystokinin and neurotensin (Sakurai & Mieda 2011; Karnani et al. 2011). Intriguingly, metabolic signals, including glucose, amino acids and thyrotropin releasing hormone, have also been identified as orexin regulators (Burdakov, Jensen, & Alexopoulos, 2006; Gonz, Horjales-araujo, Fugger, Broberger, & Burdakov, 2009; Karnani, Apergis-Schoute, et al., 2011) highlighting the role of these neurons as orchestrators of energy balance.

Orexin neurons project to almost all of the neuroaxis. As previously stated, the most significant projections are to monoaminergic nuclei in the brainstem including the locus coeruleus and raphe nuclei. (Peyron et al. 1998). These monoaminergic nuclei contribute to the Locus Coeruleus Noradrenergic System and Ascending Reticular Activating System (5-HT) respectively, accounting for the primary effects of orexin neurons in wakefulness.

Neurons containing the neuropeptide MCH have long been known to be co-distributed with orexin neurons in the posterior hypothalamus. However, unlike orexin neurons, MCH neurons are preserved in human narcolepsy (Thannickal et al. 2000). These neurons also densely project to the brainstem (Steininger, Kilduff, Behan, Benca, & Landry, 2004) and a growing body of evidence suggests that MCH may also play a crucial but opposite role in REM sleep regulation (see review by Torterolo et al. 2011); ICV injection of MCH results in a 200% increase in REM sleep, and MCH neurons fire exclusively in REM sleep (Verret et al., 2003). Orexin and MCH neurons could act in a reciprocal feedback mechanism in the regulation of REM sleep. It is therefore possible that a fall in orexin levels in narcolepsy-cataplexy could result in disrupted levels of MCH (Peyron et al. 2009). An elevation in MCH levels may account for some of the symptoms seen in the condition.

1.6. Genetic associations

The association between narcolepsy and HLA-DR2 was identified almost 30 years ago (Honda et al. 1983). More than 60 autoimmune diseases have been identified to date, almost all of which have HLA associations. Given that narcolepsy has one of the strongest such associations yet identified, an autoimmune pathogenesis is widely hypothesised. More recently, an even tighter association was found with the linked DQ alleles, most notably the DQB1*0602 variant (Mignot 1998); African-American narcolepsy-cataplexy patients are often DQB1*0602 positive but DR2 negative. The region has now been extensively mapped (Mignot et al. 2001), and the data suggests that DQB1*0602 itself is the main predisposing factor of narcolepsy, rather than an unknown gene in linkage disequilibrium (see review by Mignot 2011).

Coded on the short arm of chromosome 6, these DR and DQ alleles code for MHC class II molecules. These are responsible for the presentation of antigens to T cells by macrophages, dendritic cells and B cells, triggering immune responses. HLA genes are highly polymorphic and it is presumed that specific alleles code for subtle alterations in MHC peptide-binding pockets. This may favour the presentation of certain antigens, conferring evolutionary advantage. In the case of autoimmunity, the polymorphisms may also result in the preferential presentation of self-antigens, causing them to be seen as foreign and ultimately resulting in tissue destruction. Interestingly, whilst HLA DQB1*0602 confers susceptibility to both narcolepsy-cataplexy and multiple sclerosis (Fogdell, Hillert, Sachs, & Olerup, 1995), the polymorphism is protective of Type-1 diabetes (Siebold et al., 2004).

HLA associations in narcolepsy are estimated to account for a 2-3 x increased risk of narcolepsy-cataplexy (Mignot 1998). However, as previously mentioned, first-degree relatives share a 20-40 x increased risk; it is therefore probable that other genetic factors are also responsible. This search has accelerated in recent years thanks to novel techniques allowing genome-wide association studies (GWAS) for single nucleotide polymorphisms (SNP's). Most significantly, a second major locus for narcolepsy susceptibility has been

identified as the T cell receptor (TCR)-alpha gene (Hallmayer et al., 2009). This is the main receptor for HLA-peptide presentation and is responsible for the initiation of immune responses. No such polymorphism has been identified in any other disease. Thus, these polymorphisms could dictate a specific MHC II-peptide-TCR interaction, initiating an immune response that culminates in the destruction of orexin neurons.

Elsewhere, narcolepsy has been associated with additional SNP polymorphisms that result in the decreased expression of the purinergic receptor P2Y11 (Kornum et al., 2011). P2Y11 is a highly expressed regulatory receptor of cytotoxic lymphocytes, and is perhaps a further indication of aberrant immunity in narcolepsy (Sehgal & Mignot 2011).

1.7. Putative mechanisms of orexin neuron loss

Several pathogenic mechanisms of orexin neuronal loss have been proposed, encompassing autoantibody, T lymphocyte and T cell independent mechanisms (summarised by Fontana et al. 2010). These are listed below:

1) Autoreactive CD4+ T cells may become activated by presentation of self-antigens by MHC II- expressing dendritic cells, macrophages or microglia. H1N1 vaccination/ streptococcal infection could simply act as non-specific triggers, for example allowing CNS entry of lymphocytes and/or autoantibodies through increased blood-brain barrier permeability. An alternative mechanism of CD4+ cell activation may be molecular mimicry, akin to the situation thought to have occurred in post-streptococcal Sydenham's chorea (Church et al., 2002). Activated antigen presenting cells may stimulate B cells or produce neurotoxic factors. B cell responses may result in the production of autoantibodies that destroy orexin neurons. Factors released by antigen presenting cells themselves may be selectively neurotoxic to orexin neurons, including quinolinic acid, glutamate, TNF-alpha and oxygen free radicles, as proposed by Katsuki & Akaike 2004.

2) CD8+ T cells could kill orexin neurons directly (analogous to the likely situation in

Rasmussen's Encephalitis) or cause collateral neuronal degeneration (as in multiple sclerosis, where oligodendrocytes are the primary target) (Melzer, Meuth, & Wiendl, 2009). On one hand, direct CD8+ T cell killing seems unlikely, as MHC I molecules required for CD8+ mechanisms are expressed at low levels on neuronal soma (Goddard, Butts, & Shatz, 2007). On the other hand, CD8+ mediated attack on adjacent neuroglia could result in bystander killing of orexin neurons. However, as CD8+ antigen processing and presentation is via MHC class I molecules, this explanation would not account for the strong HLA DQB*0602 (MHC class II) association with narcolepsy.

3) Hypothalamic pathogens may directly activate T cells and antigen presenting cells via superantigen cross-linking of the TCR and MHCII molecules. Given the highly specific nature of orexin neuronal killing, this explanation seems less likely as superantigens usually stimulate a large number of T cells and result in polyclonal responses.

4) Non-immunological roles for MHCI have been demonstrated in the CNS (Goddard et al., 2007); it is conceivable that DQB1*0602-TCR interactions could cause narcolepsy independently of autoimmune mechanisms/ T cell activation.

1.8. Evidence for autoimmunity and candidate autoantigens

This is summarized in **Table 1.3.**

Despite over 30 years of research, no conclusive evidence for an autoimmune trigger of narcolepsy has been found. Biomarkers of inflammatory and autoimmune processes including ESR, CRP, oliglclonal bands, IgG index and autoimmune-associated antibodies have all been normal (Schuld et al. 2004; Rubin et al. 1988). Despite evidence of mild gliosis, the hallmarks of T cell-mediated autoimmunity have not been convincingly demonstrated; CD4+ T cells and macrophages were not found on post mortem, microglia were normally distributed and there was no increased expression of MHCII or cytokines (Peyron et al. 2000; Thannickal et al. 2000). The most obvious target of an autoimmune attack in narcolepsy is

Table 1.3

the orexin system itself. However, no likely pathogenic autoantibodies have been detected against orexin-producing neurons, the orexin peptides or it's receptors (Overeem et al. 2006; Tanaka et al. 2006; Knudsen et al. 2007).

Based on this lack of evidence for autoimmune processes, it is speculated that the immune-mediated destruction of orexin neurons may be self-limiting. The relatively immune-privileged status of the brain and limited epitope spreading may explain difficulties in identifying immune abnormalities (Mignot 2011). Furthermore, patient sampling in these studies was not necessarily performed close to disease onset; as illustrated in **Figure 1.1**, the causative T cell or antibody may only be transiently present in the early phase of the disease. Sampling several years after diagnosis, investigators may have missed time points where the causative agent was still present.

Sleep disturbance is a common symptom in patients with autoimmune diseases of the CNS such as anti-VGKC and anti-NMDAR autoimmune encephalitis (Vincent et al., 2011). At the cell surface, these receptors play crucial roles in synaptic transmission and plasticity. Resulting syndromes partly resemble pharmacological and genetic models of receptor disruption, implying that the autoantibodies directly mediate pathological effects (Dalmau et al. 2011; Lalic et al. 2011).

Several studies have reported the co-occurrence of such conditions with orexin-deficient narcolepsy-cataplexy (Overeem et al. 2004; Baba et al. 2009; Tsutsui et al. 2012). One large study assessed CSF orexin levels in 228 neurological disease patients and 64 healthy controls (Mignot et al. 2002). Whilst orexin levels were normal (>200 pg/ml) in all controls, of the neurological disease patients, four were orexin-deficient (<110 pg/ml), and 30 had intermediate levels (110-200 pg/ml). All four orexin-deficient patients had autoimmune conditions, three with Guillain-Barré and one later found to have Hashimoto's thyroiditis.

Anti-NMDA receptor autoimmune encephalitis was first identified in 2007 by Dalmau and collegues, and NMDAR antibodies were recently found in three orexin-deficient narcoleptic patients who also had psychiatric disturbances (Tsutsui et al., 2012). Another case report identified orexin-deficient narcolepsy in a child with encephalitis lethargica (Gandhi, Agrawal, Notghi, & Wassmer, 2012), a condition in which NMDAR antibodies (Dale et al. 2009). Further parallels can be drawn between paediatric narcolepsy and encephalitis lethargica; as mentioned previously, movement disorders have been reported close to narcolepsy-cataplexy onset in children (Plazzi et al. 2011).

Narcolepsy has also been reported as an initial manifestation of aquaporin-4 (AQP4) positive neuromyelitus optica (Baba et al., 2009). Separately, of four AQP4 positive patients exhibiting excessive daytime sleepiness, two met the diagnostic threshold for narcolepsy with CSF orexin levels 90-110pg/ml, and the other two had intermediate levels between 110pg/ml and 200pg/ml (Kanbayashi et al. 2012). As AQP4 is known to be highly expressed in periventricular hypothalamic regions (Nielsen et al., 1997), antibodies for AQP4 may be responsible for generalised disruption that could account depressed yet not absent levels of orexin in these patients.

Although the narcolepsy in the majority of these cases is likely to be secondary to other neurological disturbances, reactivity to these autoantigens has not been systematically investigated in idiopathic cases of narcolepsy-cataplexy; Katsuki & Akaike, 2004 report that NMDA neurotransmitter is another factor selectively neurotoxic to orexin neurons; stimulation of this receptor by functional autoantibodies could account for some cases of orexin neuronal degeneration. Elsewhere, LGI1 antibodies, a common autoimmune cause of limbic encephalitis, appear to colocalise with orexin neurons (Irani et al., 2012).

In 2010, a Czech group reported that a subgroup of narcoleptic patients had antibodies directed against tribbles homologue 2 (TRIB2) (Cvetkovic-lopes et al., 2010). This autoantigen has been previously reported in autoimmune uveitis (Zhang et al. 2005). This

finding, subsequently confirmed by two other groups (Kawashima et al., 2010; Toyoda et al., 2010), is now regarded to be the strongest evidence of humoral immunity in narcolepsy to date. Most significantly, titres were reported to be highest at disease onset. From these exciting findings, these authors inferred that TRIB2 antibodies might be the causative agent of narcolepsy. Although the presence of TRIB2 autoantibodies provides evidence of an inflammatory process in at least some narcolepsy patients, as reviewed by Lim & Scammell, 2010, a more likely scenario is that these antibodies are generated as a consequence rather than a cause of orexin cell death. Firstly, TRIB2 antibodies have been identified in only 21-26% of narcolepsy/cataplexy patients tested compared to 2-4% of controls, indicating that they are neither necessary nor sufficient to cause disease. Secondly, TRIB2 is a ubiquitous protein kinase inhibitor (Kiss-Toth et al., 2004), and is likely to be expressed widely in the CNS. On this basis, it would be difficult to account for the very specific orexin neuronal death seen in narcolepsy patients. Finally, and perhaps most significantly, akin to GAD65, TRIB2 is a cytoplasmic protein. Without prior cell destruction, this antigen would not be visible to immune cells or accessible to antibodies. Indeed, of 16 recent onset post-H1N1 cases reported by Dauvilliers et al. 2010, none were TRIB2 positive.

Other plausible candidate autoantigens on orexin neurons remain uninvestigated, including $GABA_A$ subunits. Local GABAergic interneurons appear to be distributed in the lateral hypothalamic area, and evidence suggests that orexin neurons but not MCH neurons are subject to increased GABA-mediated control during sleep (Alam et al., 2005; O. K. Hassani, Henny, Lee, & Jones, 2010). Both $GABA_A$ and $GABA_B$ receptors are expressed on orexin neurons (Xie et al., 2006). In particular, the $GABA_A$ receptor α3 subunit was found to colocalise strongly with orexin neurons (Bäckberg, Ultenius, Fritschy, & Meister, 2004). Katsuki & Akaike 2005 report that $GABA_A$ stimulation blocks neurotoxic effects on orexin neurons mediated by quinolinic acid. Quinolinic acid is an excitotoxin produced by activated microglia (Espey & Chernyshev, 1997), and GABAergic inhibitory control may be a decisive factor in protecting orexin neurons against such insults. As a hypothesis, the autoantibody targeting of $GABA_A$ receptor subunits could abrogate these protective effects, ultimately

leading to the destruction of orexin neurons in the context of wider brain inflammation and microglial activation.

Autoantigens to be investigated in this thesis are summarized in **Tables 1.4 and 1.5**. Also included are the predicted immunofluorescence and western blot appearances of these antibodies as discussed in **chapter 5**.

1.9. Approach and aims of this MSc thesis

Despite indications from genetic studies, an autoimmune cause for narcolepsy-cataplexy has yet to be proven or disproven. One of the main limitations of prior studies has been the sampling of patients at time points much later than disease onset. Interest in the area has been stimulated by the reports of narcolepsy-cataplexy following H1N1 vaccination. The most striking feature of these patients is the abruptness and severity of disease onset; samples from these patients could provide unique insights into the pathogenic mechanisms of the condition.

The overall aim of the work described was to investigate potential antibody-mediated mechanisms of narcolepsy. The study was conducted in a laboratory with experience of antibody-mediated neurological diseases, and patient sera and CSF was available from narcoleptic patients at the time of diagnosis, the earliest feasible time point for testing. Serum/CSF pairs were also obtained from clinically validated post-vaccination Swedish and Finnish cohorts. These samples were examined with immunological techniques.

The main aims therefore were:

1) To look for biomarkers of narcolepsy and antibody-mediated immunity in sera/CSF. These may include markers of immune dysfunction or other markers of pathology.
2) To test for immunoreactivity against known and candidate autoantigens expressed on the surface of live cells.
3) To screen for other evidence of autoantibodies.

Table 1.4

Table 1.5

2. Materials and methods

The solutions and commercial antibodies used in this thesis are summarised in **Appendix 1** and **2** respectively.

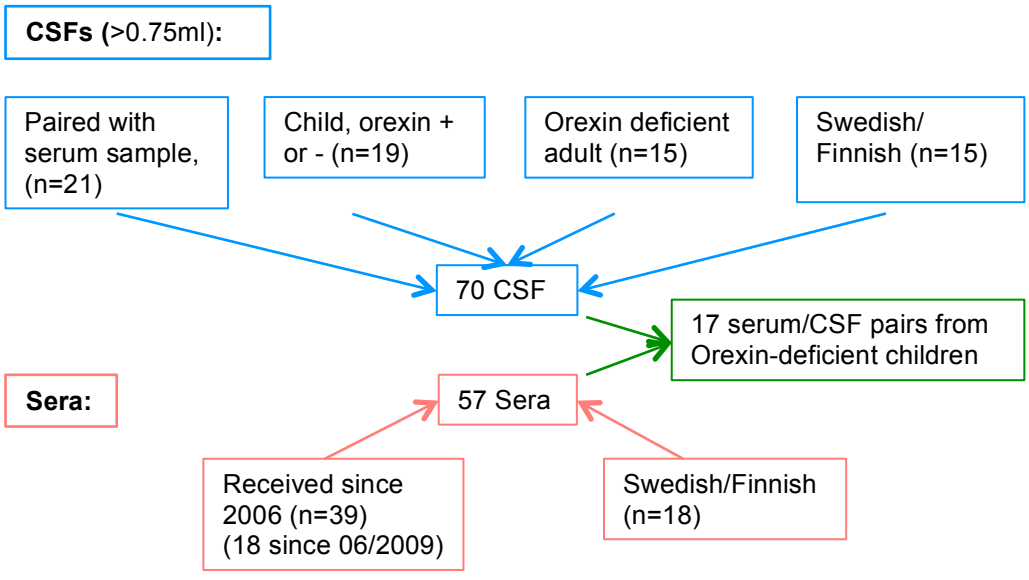
All reagents were purchased from Sigma-Aldrich® unless otherwise specified.

2.1. Sera/CSF samples

2.1.1. Narcolepsy cohort

The Nuffield Department of Clinical Neurosciences (NDCN) has provided a diagnostic CSF Orexin A testing service since 2006. As of the 1st July 2012, CSF orexin measurements had been quantified on 264 CSF samples with a commercially available radioimmunoassay (RIA) kit (Phoenix ®, see section 3.2 for method). The data from these prior tests is analysed in chapter 3.

In total, 70 CSF and 57 sera met inclusion criteria for the core narcolepsy cohort, including 25 serum/CSF pairs, 17 of which were from orexin-deficient children. These criteria and numbers of samples are summarised in **Figure 2.1** (below).



All samples had been obtained since June 2009 unless specifically stated. Of these, only samples with more than 0.75ml volume remaining were included, so that sufficient tests could be performed. The June 2009 cut-off corresponds to the declaration of H1N1 pandemic by the World Health Organization, (WHO; Chan, 2009). Of these samples, all CSF samples with a serum pair were included (n=21). Of the CSF samples for which there was no serum pair, only samples from children (both orexin-normal and deficient, n=19) or orexin-deficient adults (n=15) were included, eliminating a large group of adult orexin-normal CSF samples. 39 serum samples had been received at the NDCN since 2006 alongside CSF samples sent for orexin testing, and all were investigated.

A further 17 sera and 15 CSF, including 14 serum/CSF pairs were generously provided by Swedish and Finnish collaborators (Dr.s N. Darin, T. Hallböök, M. Partinen and A. Huutoniemi). These were all from clinically defined narcolepsy-cataplexy patients who received Pandemrix® vaccination. Particular emphasis was placed on these samples, as they were all taken from recent onset post-vaccination cases of the condition.

An additional five known positive control sera were included in the serum cohort prior to coding and randomisation. Unbinding sample identity occurred in March 2012, after all cell-based assay and initial immunohistochemical screening had been completed. Following decoding, sample orders were randomised prior to experimentation to maintain blinding. For the purposes of this thesis, the narcolepsy serum and CSF samples have been numerically ordered on the basis of date received at the NDCN (Numbered 1 to 57 and 1 to 70 respectively).

2.1.2. Other cohorts

In parallel studies, 23 sera/CSF pairs were available in the lab from patients with a variety of other autoimmune diseases, and used for CSF orexin and Total IgG quantification. In addition, 100 paired sera were obtained from healthy control children, taken before and after H1N1 vaccination (Waddington et al., 2010). These samples were kindly provided by the

Oxford Vaccine Group (Dr. M. Snape, Prof. A Pollard). IgG quantification with these samples was performed blinded; sample order (pre vs. post), age and vaccine given were revealed subsequently.

2.1.3. Healthy serum controls

With the appropriate consent, a total of 15 healthy serum controls were used throughout these experiments including five children and 10 adults. CSF health controls were not available; CSF extraction is much more invasive and has greater associated risks, and is therefore not routinely performed on healthy individuals.

2.2. Serum and CSF biomarkers in patients and controls

2.2.1. CSF orexin A

CSF orexin A was measured using a commercially available ^{125}I competitive radioimmunoassay (Phoenix®). CSF/ standard solutions (100 µl) provided were diluted in 100 µl of the RIA buffer supplied. A further 100 µl of rabbit-anti orexin antibody was added, and solutions incubated for 24 hr at 4^{o}C. On the second day, 100 µl of ^{125}I-Orexin A was added (8000-10000 cpm/ tube). Following a second 24 hr. incubation at 4^{o}C, 100 µl of provided secondary antibody (goat anti rabbit IgG) and 100 µl of normal rabbit serum to act as carrier were added, and incubated at room temperature (RT) for 90 min. Finally, samples were centrifuged at 1700 g for 20 min at 4^{o}C. The supernatant was then removed by aspiration and pellet washed twice in 1% Triton/PBS. Radioactivity of the pellets was measured (Wallac Wizard gamma counter), and orexin A concentrations were determined against a known standard curve (10-12,800 pg/ml, see results chapter 4.1). This method is summarised in **Figure 2.2.**

The lower detection limit of the assay was 40 pg/ml, below which all results were reported to be ‘undetectable’ and ascribed a value of 10pg/ml for indexing purposes. As per international criteria, CSF orexin A levels below 110 pg/ml were considered deficient, 110-200 pg/ml intermediate, and more than 200 pg/ml normal (American Academy of Sleep Medicine, 2005).

Figure 2.2

Any sample found to have undetectable levels of orexin was repeated in a different assay to confirm the results.

2.2.2. CSF MCH

The MCH assay (Phoenix®) followed an identical principal to the orexin A assay, using the same volumes and incubation durations, but with the substitution of rabbit anti-MCH and I^{125}-MCH. The normal human CSF level of MCH are commonly around 100-140 pg/ml (Peyron et al., 2011), but the standard curve of the unmodified MCH assay ranges from 100-128,000 pg/ml. Therefore, in order to interpolate results off the linear part of the standard curve, it was necessary for all CSF samples to be "spiked" with 400 pg/ml of MCH to increase their levels. This was achieved by adding 12.5 µl of 3.2 µg/ml standard peptide to each 100 µl sample. MCH levels were then ascertained by subtracting 400 from the observed MCH concentration (pg/ml).

2.2.3. Quantification of total IgG in sera and CSF by western blot

Rather than using a more conventional immunodiffusion-based method, western blot was found to be a sensitive method for measuring the very low levels found in CSF paralleled with serum levels (after dilution 1:300). SDS Gel electrophoresis of sera and CSF was performed using a XCell SureLock™ Mini-Cell Electrophoresis System with NOVEX® Bis-Tris 4-12% pre-cast 15 well mini gels in accordance with manufacturer's instructions. Following centrifugation (13000 RCF, 5 minutes), sera/IgG standard peptide were diluted 1/300 in PBS. To reduce pipetting errors of this dilution, two serial dilution steps were used for sera; CSF was used undiluted. 9.75 µl of diluted serum/standard or neat CSF was then made up to a total volume of 15 µl with NuPAGE® sample reducing agent (10%) and LDS sample buffer (25%). These samples were then heated to 95^0C for 5 min and loaded onto the gel. SeeBlue® Plus2 Pre-stained Standard molecular weight marker was loaded into lane 1 of each gel. 10 µl of each boiled sample was loaded into the remaining wells. Electrophoresis was performed at constant 200 volts (expected current 190 mA-140 mA start-finish) until the tracking dye bromophenol blue reached the bottom of the gel. Western

blot of the gel was performed immediately using an Invitrogen® XCell II™ Blot Module as per manufacturer's instructions, and proteins allowed to transfer (30 V for 30 min 50 V for 30 min, RT). In order to assess the success of electrophoresis and subsequent transfer, blots were rinsed with distilled water and reversibly stained with Ponceau S (0.1% (w/v) Ponceau S in 5% acetic acid) and scanned between two sheets of clean acetate film. Nitrocellulose blots were blocked in baths containing 10 mls of 3% milk (diluted in PBS/1% Tween, 1hr, RT) prior to incubation with rabbit anti-human IgG-HRP secondary antibody (Dako®, diluted 1/2000 in 3% milk/PBS, 4^0C, Overnight). After 3 PBS/Tween (1%) washes, brown colour was developed for 30 seconds by peroxide staining with 3,3'-diaminobenzidine (50mg DAB-HCl, 20ml PBS, 20µl 3% H_2O_2), and the colour changing reaction halted by dH_2O immersion. Following drying and scanning of the blots, densitometric analysis of the 50kDa IgG heavy chain was performed using ImageJ (Rasband, 1997) using the "Analyze → Gels" toolbar.

2.2.4. Oligoclonal bands

Oligoclonal bands were kindly tested at the University College of London Institute of Neurology, Queen's Square by Dr. V Worthington.

2.3. Candidate antigen approaches

2.3.1. The cell-based assay (CBA)

The CBA enables the detection of antibodies in patients' sera binding to proteins transiently expressed on human embryonic kidney (HEK) cells. Following transfection with plasmids coding for the antigen of interest, live cells are stained to ensure that only antibodies to extracellular epitopes are detected; fixative is only applied after the first incubation with patient serum. **Figure 2.3** illustrates the overall method using the eGFP-tagged CASPR-2 antigen as a representative example.

Amplification and purification of DNA plasmids

DNA plasmids containing human (c)DNA of NMDAR, LGI1, CASPR2 or CONTACTIN2 are routinely used in the Oxford lab and had been developed prior to the start of this thesis

Figure 2.3

(Irani, Bera, et al., 2010; Irani, Alexander, et al., 2010). Specific information on these plasmids is documented in **Appendix 3**.

On Day one of the DNA amplification process, JM109 competent cells were transformed. All stages involving live *E.coli* cells were performed under a roaring Bunsen flame to prevent contamination. Firstly, 50 µl of cells were mixed with 50 ng of DNA diluted in nuclease-free water (Promega®) for 20 minutes on ice. Controls containing no DNA were also included. A heat-shock step was then performed (1 min, 42^0C), which is thought to partially permeabilise the live *E.coli* and enabling the DNA to enter. Following a further 5 min on ice, 950 µl of LB media was added to the 50 µl cell solution and incubated (40 min, 37^0C) with shaking (200 rpm). Following centrifugation (1300 RCF, x5 min), pellets were re-suspended in LB media (250 µl) and spread onto an LB Agar plate, supplemented with 50 µg/ml of antibiotic (Ampicillin or Kanomycin); the inclusion of antibiotics in the culture medium and antibiotic resistance genes as part of the plasmids enabled the selective culture of successfully transformed cells. Inverted petri dishes were then incubated overnight at 37^0C.

On the morning of Day 2 plates were inspected; successful transformation would be indicated by the presence of numerous colonies on plates containing the intended plasmid and the absence of colonies on control plates. Initial cultures were prepared by selecting a single colony from the LB Agar plates to inoculate 15 ml of antibiotic-containing LB media. Following incubation (5-7 hr), initial cultures were poured into conical flasks containing 600 ml of antibiotic-supplemented LB media (600ml) and incubated overnight (37^0C, 200 rpm) in an upright shaker (Certomat®).

On Day 3, DNA was isolated from the 600ml of transformed JM109 cells using a PureYield™ Plasmid Maxiprep System (Promega®) as per manufacturer's instructions. DNA concentration was determined by spectrophotometry (Shimadzu®). Absorbance readings were taken in triplicate at 260 nm, and the mean recorded. Typically, 2-2.5 mg of DNA was eluted per 600 ml flask.

Culture and preparation of HEK cells

HEK 293 cells were routinely cultured using HEK cell culture media (DMEM supplemented with 10% FCS and 1% antibiotic-antimycotic solution) in disposable 175 cm^3 plastic flasks at 37^0C, 5% CO_2, and passaged every 4 days using trypsin (GIBCO®, 0.5% diluted in PBS) to dislodge cells prior to centrifugation (1300 RCF, 5 min, RT). Pellets were re-suspended in 10ml of HEK culture medium and counted on a haemocytometer using Trypan Blue (0.08%) to stain dead cells to exclude in the counting.

Typically, the cell-based assay (CBA) was performed over four days. On the 1st day, 3-4 borosilicate glass coverslips (13 mm, VWR®) were placed in 6 well plates and coated with poly-L-lysine (PLL, 0.1 mg/ml, 30 min, RT). PLL was aspirated, coverslips were allowed to dry, and $4x10^5$ HEK cells were seeded-out per well and grown overnight.

Transfection of HEK cells

24 hrs after seeding-out, cells were transiently transfected with DNA plasmids containing the gene or the candidate antigen of interest. In short, a total of 3 µg of DNA was diluted in 1.25 µl of 20% glucose and vigorously added to 1.5 µl of polyethylenimine (9 mM, PEI) per well of a six well plate. Following overnight incubation (37^0C), the media was replaced, and cells cultured for a further 24 hrs.

Indirect immunofluorescence

Coverslips were transferred to individual wells of a 24 well plate, containing 250 µl of suitably diluted serum/plasma/commercial antibodies in DMEM supplemented with HEPES buffer (4.6 mg/ml) and BSA (0.5 mg/ml), and incubated on a rocker (1 hr, RT). For NMDAR, LGI1, and Contactin-2 constructs, sera were screened at 1:20 dilution. For CASPR2, sera were screened at 1:100. All CSF were screened at 1:2 dilutions.

Coverslips were washed 3 times in DMEM/HEPES and fixed in formaldehyde (3%, TAAB®). After a further three washes, secondary antibodies (usually Alexa Fluor™ 568 anti-human

IgG) were incubated in the dark (45 min, RT). Finally, cells were washed in DMEM/HEPES (x3) and PBS (x3) and the coverslips were mounted onto glass microscope slides (VWR®) using mounting medium (Dako®) containing a 1/1000 dilution of DAPI (4',6' -diamidino-2-phenlindoledichloride, 1:1000).

Antibody binding to candidate antigens was observed using a fluorescence microscope (Leica DM 2500™, fitted with a Qimaging Rolera XR™ camera). Successful transfection and antibody binding were confirmed by the visualised of green and red fluorescence respectively. Subjective thresholds of positivity were established by comparison with positive and negative controls. **Table 2.1** (below) describes the scoring system adopted as a subjective assessment of the frequency and intensity of serum binding to the cells of each coverslip (adapted from Leite et al., 2008).

Score	Description
0	No labelling
0.5	Very weak/ questionable labelling, less than the low positive control
1	Weak labelling, similar to the low positive control, serum colocalisation with eGFP tagged constructs, considered low positive.
2	Moderate labelling of some (20-50%) of transfected cells. Precise colocalisation where constructs eGFP tagged
3	Moderate-strong labelling of 50-80% transfected cells. Precise colocalisation if constructs were eGFP tagged.
4	Strong labelling of all transfected cells. Near-perfect colocalisation

Also, intermediate scores were possible; for example a score of 1.5 was given when observed binding was stronger than the low positive control (CBA score 1), but did not satisfy criteria to be scored a 2. Any result with observed positivity (0.5 or above) was reassessed by repeat assay. All samples were checked by blinded colleagues and retested on HEK cells transfected with a different construct to exclude non-specific binding to the cells. As with all visual comparisons made in this thesis, all images were photographed under the same conditions, and no specific feature within any image/comparison was enhanced, obscured, moved, removed or introduced unless explicitly stated.

2.3.2. Molecular genetics

All steps in the establishment of the $GABA_AR\alpha3$ CBA were performed under the guidance of Dr. Patrick Waters and Dr. Mark Woodhall. Using the Nucleotide resource of the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/nucleotide/), the sequence for human gamma-aminobutyric acid (GABA) A receptor alpha 3 was located (GABRA3, Accession number BC028629.1). This 1901 base pair (bp) sequence contains the coding sequence for GABRA3 between position 240 and 1718. Upon comparison to the reference human genome, a single nucleotide mutation was identified in this coding sequence; a T→C missense mutation at position 778 resulting in Leucine→Proline amino acid change. The I.M.A.G.E (Integrated Molecular Analysis of Genomes and their Expression) full-length cDNA clone (4716 bp, sequence verified, purified plasmid) was obtained from Source BioScience GenomeCube®. This bacterial clone contains GABRA3 within the Multiple Cloning Site (MCS) of the pBluescriptR vector (**Appendix 3**). The individual methodologies used are listed here; an in-depth protocol is described in **chapter 4.2**.

DNA primer design

Primers were designed for DNA sequencing and site-directed mutagenesis. To ensure optimal annealing and elongation, forward and reverse primer pairs were designed to be >22 bp, have 40-60% G/C content (with most G/C in 5' and central regions and A/T at the 3' end) and to have as similar melting temperatures (T_m) as possible. The sequence of these primers is listed below.

PCR sequencing primers: ("**atg**"/"**cta**" are complementary to start/stop sequences)
Forward: gatcctcgaccacc**atg**ataatcacacaaacaagtcactg
Reverse: catcggatcc**cta**ctgtttgcggatcatgc

Mutagenic primers: ("**t**"/**"a**" are complentrary to the mutated base)
Forward: ggacaacggaaccctcc**t**ctatacaatgaggttaac
Reverse: cttaacctcattgtatagg**a**gagggttccgttgtcc

DNA sequencing by PCR

Polymerase Chain Reaction (PCR) DNA sequencing was then performed by the *in-house* WIMM DNA sequencing service. This was performed upon first receiving the plasmid from Source BioScience GenomeCube® and after each subsequent ligation reaction to confirm the correct sequence of the insert. In addition to specifically designed GABRA3 sequencing primers that read between the start and stop sequences (**see Figure 2.4**), M13 forward and reverse commercial primers were used to read into the insert from the adjacent multiple cloning site.

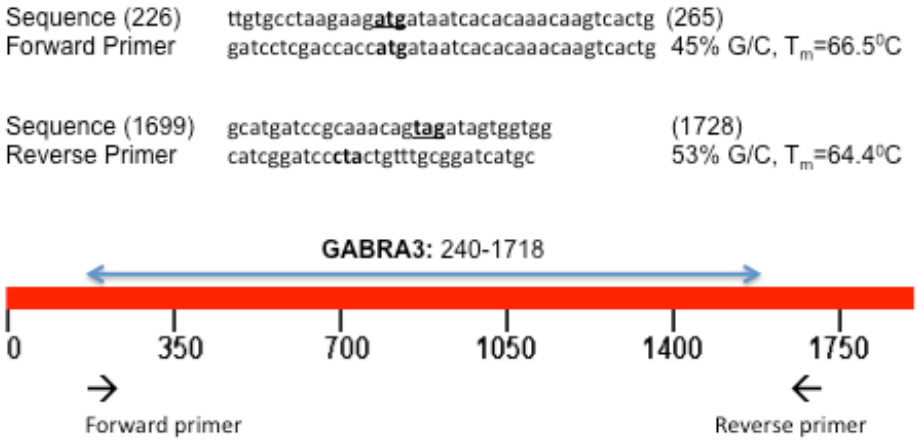


Figure 2.4: PCR sequencing primers

Amplification of GABRA3 insert by PCR

The Polymerase Chain Reaction technique (PCR) was used to amplify the DNA insert. All PCR reactions were performed using an Applied Biosciences® Veriti™ Thermal Cycler. PCR amplification of GABRA3 was achieved with sequencing primers (**Figure 2.4**) and PfuUltra™ II polymerase (Agilent®) adopting the following reaction protocol:

- 1 Cycle: 95^{0}C for 3 minutes (denaturing)
- 32 cycles: 95^{0}C 1 minute, 50-70^{0}C 1 minute (anneal), 72^{0}C 30 seconds (extension with DNA polymerase)
- 1 Cycle: 72^{0}C 5 minutes (final extension)

Although the annealing temperature is usually chosen to be 3-5^{0}C above the T_m of the primers (72^{0}C) a range of annealing temperatures was tested (50-70^{0}C). As the resultant

PCR products were blunt-ended, a-tailing was necessary prior to subsequent ligation reactions (performed according to pGEM®-TEasy manufacturer's instructions).

For the mutant strand synthesis step of Site Directed Mutagenesis (see below), the PCR protocol with aforementioned mutagenic primers was dictated by manufacturer's instructions (QuickChange® Lightening Site-Directed Mutagenesis Kit).

Amplification of plasmids by Miniprep and Maxiprep

Small scale amplification of whole plasmids was achieved by overnight incubation of transformed JM109 cells in 10 ml of LB media followed by Miniprep according to QIAprep™ Miniprep kit (QIAGEN®) manufacturer's instructions. Typically, this would achieve a DNA yield of 20 µg, adequate for most intermediate molecular cloning steps.

Large scale DNA amplification was achieved by Maxiprep as described in **chapter 2.2.1**.

Restriction digests

Restriction digests were necessary to excise the GABRA3 insert at several stages. The New England BioLabs® NEBcutter online tool (Vincze, 2003) was used to find enzymes with unique restriction sites within the Multiple Cloning Site of each vector but not within the GABRA3 insert itself. XhoI1 and BamH1 were found to be suitable candidates for all vectors, and were therefore used in all restriction digests. 'Double digests' were performed according to manufacturer's instructions (5 units of enzyme per µg of DNA, incubated for 2 hr, 37^0C).

Agarose gel electrophoresis and gel extraction

Agarose gel electrophoresis was used to separate and visualise DNA fragments. These were prepared by dissolving agarose (1-2.5% w/v depending on anticipated DNA size) in TBE (0.9 M Tris Base, 0.9 M Boric acid, 10 µm EDTA in dH_2O). The melted agarose/TBE was poured in Thermo Scientific® Easycast chambers™, 0.5 µg/ml of ethidium bromide was added, and gels allowed to set in a fume hood to avoid ethidium bromide inhalation. Samples were

prepared by the addition of 10X loading buffer (0.42% w/v bromophenol blue, 0.42%w/v xylene cyanol FF, 25% w/v Ficoll). Samples were then loaded alongside 5 µl of HyperLadder™ 1 (Bioline®), a marker that enabled estimation of DNA size and quantity (**Appendix 3**). Gels were run submerged in TBE for 45-120 minutes 90-120 V. DNA bands were visualised and photographed under low energy UV light.

For gel extraction, DNA fragments of interest separated by gel electrophoresis were excised from the gel on a UV transluminator. DNA was isolated from the cut gel with a QIAquick™ gel extraction kit (QIAGEN®) as per manufacturer's instructions.

Ligation reactions

Ligations were performed in a 10 µl final volume using 1:1, 3:1, 1:3 cut-vector pGEM®-TEasy, pIRES2-DsRed2 or pIRES2-AcGFP1):cut-insert (GABRA3) molar ratios. The amount of insert to add was calculated relative to 50 ng of vector using the following equation:
ng insert= ((ng of vector x (size of insert / size of vector)) / (vector : insert ratio)

T4 DNA ligase (1 µl) and 1 µl 10X buffer (New England BioLabs®) were added and incubated for 1 hr at RT. JM109 cells were then transformed with the ligation mixtures prior to selective overnight culture and miniprep purification as previously described.

Site-directed mutagenesis (SDM)

Mutagenic primers were specially designed for the SDM of the insert in pGEM-T Easy (**Appendix 3**). This was performed with a QuickChange® Lightening Site-Directed Mutagenesis Kit as per manufacturer's instructions. Mutant strand synthesis with mutagenic primers was achieved by PCR. Methylated parent strand DNA was then removed by Dpn1 enzymatic digestion.

Western blot of HEK cell lysate

Following HEK cell transfection of pIRES2-AcGFP1(corrected GABRA3), expression of the $GABA_A$ α-3 protein was confirmed by western blot of cell lysate. Cells were scraped from cultured flasks of transfected/untransfected HEK cells and pelleted by centrifugation (1300 RCF, 5 min). Cells were counted and re-suspended to a density of $5x10^6$ cells/ml in culture medium. Appropriate amounts of NuPAGE® sample reducing agent (10X) and LDS sample buffer (4X) were added, cells mixtures heated (10 min, 95^0C) and 10µl of resultant cell lysate were then loaded onto protein gels. SDS-PAGE gel electrophoresis and western blot was performed as descried in **chapter 2.2.3**. Nitrocellulose with blotted cell lysate was then incubated with an anti-$GABA_A$ α-3 commercial antibody (4^0C, O/N). Subsequent wash steps, incubation with an appropriate secondary antibody and DAB staining were performed as described in **chapter 2.2.3**.

2.3.3. Radioimmunoassays for candidate antigens

Potassium channel immunoprecipitation assays were performed by colleagues using an *in-house* ^{125}I-α-dendrotoxin labelling method as described previously (Hart et al., 1997). Serum autoantibodies for GAD were screened using a method adapted from a commercially available radioimmunoassay kit (RSR Ltd.). In short, patient sera/controls were diluted 1:10 in 0.2%Triton-X (w/v) in PBS (0.2 M, pH 7.2, PTX) and incubated with ^{125}I-GAD_{65} (overnight, 4^0C). Sheep anti-human IgG was added (1hr, RT) and the immunoprecipited complexes were collected by centrifugation (14,000 RCF, 5 min, RT). Pellets were washed twice in PTX and radioactivity counted as previously described.

2.4. Screening approaches

2.4.1. Immunohistochemistry

Rat brain collection and preparation

Sprague Dawley rats were decapitated and their brains perfused with PBS or 4% formaldehyde solution. Brains were removed by careful dissection and snap frozen by immersion in isopentane cooled in liquid nitrogen. Neonatal rats (P0-P8) were not perfused but the whole heads were snap frozen. Frozen brains/heads were wrapped in foil and stored

at -80^{O}C. Frozen tissue was mounted with Tissue-tek® embedding medium and sections (12 µm) cut on a cryostat ((ThermoScientific Cryotome® FSE). Section location was identified by comparison with a rat brain atlas (Paxinos & Watson, 2007). Cut sections were transferred onto X-tra® adhesive slides, dried overnight and stored at -20^{0}C wrapped in aluminium foil.

Indirect immunostaining

Slides were allowed to reach room temperature and hydrophobic wells created around each section using a Dako® delimiting pen and left to dry. All subsequent procedures were carried out at RT. Sections from PBS perfused brains were fixed by immersion in acetone and then washed in PBS. Peroxidase inhibition was achieved by immersion in a bath containing 6 ml H_2O_2, 60 ml MeOH, and 140 ml H_2O (5 min). Following additional PBS washes (x3), sections were blocked in FCS (10% in PBS, 40 min) in a humidity chamber. Sections were incubated in primary antibodies (1:100- 1:500, in 1% FCS/PBS) for 1 hr and washed 3 times in PBS prior to incubation with horseradish peroxidase (HRP)-conjugated secondary antibody solution were added (45 min). 3-Amino-9-ethylcarbazole (AEC) red staining was achieved using an AEC staining kit as per manufacturers instructions. Colour changing reactions were stopped by immersion in deionized water. For double staining experiments, following additional washes and blocking steps, the second primary antibody was incubated as previously described. This time, a biotin-conjugated secondary antibody was applied for 45 minutes. Following another series of washes, avadin-biotin-complex was prepared and applied as per manufacturers instructions. End-point colour was developed using brown 3,3'-iaminobenzidine DAB . Slides were left to dry and then mounted as previously described using Faramount aqueous mounting medium (Dako®). Stained slides were viewed and photographed using a light Microscope (Zeiss Axoplan 2™ fitted with Qimaging MicroPublisher™ 5.0).

Indirect immunofluorescence (I.F.)

Slides were thawed and fixed as described above. Additionally, sections were blocked for non-specific secondary antibody binding using an appropriate reagent (e.g. FCS/ Normal

Goat Serum/ Normal donkey serum, 10% in PBS), before addition of primary antibody (1:100-1:1000 in the respective blocking solution) for either 1hr, RT or overnight (16 hours) at 4^0C in a humidity chamber. Slides were washed (3x PBS, 1min) and then incubated in the appropriate secondary fluorchrome-conjugated antibody (1:500-1:2000 in 1%FCS/PBS 45 min, RT in the dark). Slides were washed (3x PBS, 1 min), air-dried (10 min) and mounted with DAPI- supplemented florescence mounting media and visualised as previously described.

Screening of patient sera/CSF binding to coronal sections was assessed in the hypothalamus, hippocampus, cortex white matter and basal ganglia. Neuronal binding was scored using a subjective scoring system (0=non, 1=weak, 2=moderate, 3=strong) in each brain area and appearance of binding (nuclear/cytoplasmic) noted.

Double staining experiments, where two antibodies were visualised in different colours, was achieved by performing serial immunofluorescence incubations; following blocking, primary and secondary incubation steps for the first antibody, these steps were repeated for the second antibody.

In later experiments, some sera were preadsorbed using commercially available pigeon acetone liver powder (PALP). Diluted serum was added to liver powder (10 µg/100 µl final dilution) and mixed for 24 hr, 4^oC on a rotating mixer. Insoluble material was removed by two centrifugation steps (13,000 RCF, 15 min, RT). The preadsorbed supernatant was applied to rat brain sections and stained as previously described. By comparing the staining with adjacent unpreadsorbed samples, the effects of PALP preadsorption were discerned.

2.4.2. Western blot of brain extracts

Brain extract preparation

Following decapitation, brains from Sprague Dawley rats were immediately removed and immersed in ice-cold Hank's balanced salt solution (HBSS). The hypothalamic and other

brain regions were carefully dissected and weighed. Brain areas were homogenized (Polytron™, Kinematica®) in 6 ml of Solublisation Buffer (**see Appendix 3**) per gram of tissue, centrifuged (13,000 RCF, 15 minutes), and reconstituted at equivalent concentrations.

Gel electrophoresis and western blot transfer

Protein concentration of brain homogenate was first determined using a Thermo Scientific® Pierce BCA Protein Assay Kit as per manufacturer's instructions. Gel electrophoresis was performed using a XCell SureLock™ Mini-Cell Electrophoresis System with NOVEX® Bis-Tris 4-12% pre-cast 2D mini gels in accordance with manufacturer's instructions. Following centrifugation (13000 RCF, 10 minutes) brain homogenates (250 µg) were resuspended in NuPAGE® sample reducing agent (10X) and LDS sample buffer (4X), made up to a total volume of 300µl in nuclease-free water and heated to 95^0C for 5 min and loaded onto the gel. SeeBulue® Plus2 Pre-stained Standard molecular weight marker was loaded alongside. Electrophoresis was performed at constant 200 volts (expected current 190 mA-140 mA start-finish) until the loading dye bromophenol blue reached the bottom of the gel. Western blot was performed as described in **chapter 2.2.3**. In addition to the reversible Ponceau S staining of the blotted nitrocellulose as described previously, gels were stained with Coomassie Blue stain (0.2% w/v Coomassie Blue R-250, 10% v/v acetic acid, 30% v/v methanol) for 30 minutes and protein bands visualised following 1 hour destain (10% v/v acetic acid, 30% v/v methanol) on a tilting platform. Both gels and blots were scanned between two sheets of clean acetate film.

Serum screening

Using the Ponceau S stain to delineate the location of transferred protein, numbered strips of each protein extract blot were cut, inserted into Falcon tubes and blocked in milk powder (3% w/v in PBS, 1 hr, RT). Each falcon tube contained same-numbered strips from each brain region. Strips were then washed in PBS/1%Tween (3x, 5 min) prior to the addition of patient serum (1:250 in PBS, 1 hr) before a further wash (3x PBS, 20 min). The secondary antibody layer was then incubated (Rabbit anti-human IgG-HRP, 1:1000, 1 hr) prior to subsequent

washing steps (3x PBS/1%Tween, 20 min). Brown colour was developed by peroxide staining with DAB (50 mg DAB-HCl, 20 ml PBS, 20 µl 3% H_2O_2). After 120 seconds, the colour changing reaction was stopped by H_2O immersion. The original arrangement of numbered strips was re-assembled and dried prior to scanning. Densitometric analysis of band intensity was performed using ImageJ (Rasband, 1997) using the "Analyze → Gels" toolbar.

2.5. Neuronal cultures

2.5.1. Dissociated primary cultures (Adapted from Fath et. al, 2009)

Following sacrifice of newborn rats (P0), brains were removed and immediately immersed in ice cold antibiotic-supplemented Hanks's Buffered Saline Solution (Gibco®, HBSS). The bilateral hippocampi were located and removed following midline sagittal bisection of the brain and involution of the cortex. Using a magnifying glass and fine forceps, fimbrai and remaining meninges were removed. Hypothalamic dissection was achieved by placing brains on their dorsum and dissecting around the outline of the visible hypothalamic bulge. The dissected material was dispersed (0.5% trypsin in HBSS, 20 min, $37^{O}C$), and the tissue re-suspended in complete MEM (1X MEM alpha, Gibco® supplemented with 10% FCS and 1% antibiotic antimycotic solution). The tissue was triturated by gently aspirating and tissue 10 times and then centrifuged (1000 RCF, 10 min) before being re-suspended in complete MEM. Dissociated cells were plated-out ($0.5x10^6$ cells/ml) and left for 2 hr at 37^0C before the media was replaced with complete neurobasal medium (Neurobasal media 1X, GIBCO® supplemented with 1% antibiotic antimycotic solution, 0.5 mM L-Glutamate and 1% B27 supplement). Media change with complete neurobasal medium was performed every 4 days.

After 12 days in culture, antibody-binding studies to neurons were performed with patient serum (1:100 in complete neurobasal medium/ 1% BSA). Primary cultures on coverslips were transferred into serum solutions and incubated (1hr, RT). Coverslips were washed 3 times (complete NB/1%BSA) prior to fixation with 3% formaldehyde. After 3 additional PBS washes, coverslips were then incubated with Alexa Fluor® Goat anti-Human 488 secondary

antibodies (45 min, RT, dark). After further PBS washes (3x, 1 min), cells were permeabilised in Triton (0.3% in PBS, 15 min) prior to incubation with anti-MAP-2 commercial antibodies (DAKO, 1:1000 in PBS, 45 min, dark). Following PBS washes, secondary antibody was added (Alexa Fluor® Goat anti Mouse 568 1:1000, 30 min). After final PBS washes, coverslips were mounted with DAPI supplemented fluorescence mounting media and visualised as before.

Binding of patient IgG to neurons to patient IgG was only positive if MAP-2 staining was present, showing the neurons to have been healthy. Any observed binding was scored subjectively on a scale of 0-3 (none, weak, moderate, intense) in relation to a panel of healthy and positive controls

2.5.2. Organotypic slice cultures

Sectioning and culture

Rats (P8-9) were decapitated and the brains removed and immediately immersed in ice-cold antibiotic supplemented HBSS (Gibco®). The brainstem was removed and brains mounted on pre-cooled vibratome chucks with cyanoacrylate-based glue (Locktight® Super Glule Liguid), the rostral aspect facing upwards. Coronal sections of live tissue were obtained using a Leica® VT1000 S vibrating blade microtome, with the frequency and speed set to 10/10 and 2.5/10 respectively. Moving in a caudal direction, the appearance of the hippocampus and third ventricle were used as gross landmarks to identify the area of interest; 6-9 consecutive coronal brain slices (300µm) were collected. Slices were immediately divided along a median sagittal plane and transferred into adjacent wells of a 6 well plate containing Millicell®-CM Low Height Culture Plate Inserts and 1 ml of ice-cold HBSS. Cultures were then moved into a sterile environment and washed with antibiotic-supplemented HBSS prior to transferal into sterile 6 well plates containing pre-warmed organotypic slice culture media (50% 1X MEM, 25% HBSS 1X, 25% normal horse serum (heat inactivated for 30 min, 56^0C), supplemented with antibiotic antimycotic solution (1%), L-Glutamate (2 mM), glucose (6mg/ml), and HEPES (2.3 mg/ml) (Sigma®). Culture medium

was replaced every 2 days and slices maintained for 7-10 days at 35^0C, 5% CO_2. Over this time, cultures became embedded in respective culture inserts.

Incubation with drugs/serum

After 7 days (for experiments involving a 72 hour post-incubation) or 10 days (for experiments without post-incubation), drugs/serum were added to culture medium and incubated for a 24 hrs. Drugs tested on the sections included NMDA at dilutions ranging from 10-1000 µM. Serum was tested at 1:100 dilution. Additionally, the nuclear exclusion dye Propidium Iodide (PI) was used as a means of quantifying neuronal death.
Binary comparisons of equivalent test versus control slices were possible as whole sections had been bisected and halves cultured in adjacent wells. For each experimental condition, test drug/serum would be applied to one half and the other cultured normally as a control. Later analysis would look for any differences between test and control.

Staining protocol for organotypic cultures (adapted: Gogilla et al, 2006)
Cultures were either immediately fixed or alternatively transferred into normal culture medium for 72 hrs prior to fixation. To fix sections, 1ml of cold formaldehyde solution (4% in PBS) was added above and below insert membranes for 5 min and washed similarly in PBS. Inserts were then immersed in Methanol (20%) in PBS (5min) prior to another PBS wash step. Permeabilisation of fixed cultures was performed overnight at 4^0C by adding 1 ml of 0.5%Triton / PBS above and below culture membranes. The permeabilisation solution was then replaced with BSA blocking solution (20% in PBS, 4hr, RT).
The sections and attached insert membrane were excised from insert housings, rapidly transferred into primary antibody solutions (200 µl, diluted in 5% BSA/PBS) and incubated overnight at 4^0C. All subsequent steps were performed on 24 well plate lids with hydrophobic wells drawn with a Dako® pen to prevent solutions form mixing. Slices were then transferred between 3 x 20 minute 5%BSA/PBS washes on a tilting platform and incubated in secondary antibody solutions (4 hr, RT, dark). Following a final 3 PBS washes, slices with attached membrane insert were mounted on glass slides with DAPI-supplemented florescent

mounting media. Slides were stored at 4^0C prior to visualisation and photography.

Confocal microscopy to detect of orexin neuronal killing

Organotypic slices were visualised and photographed using a confocal microscope (Zeiss® LSM 710™) by taking serial transverse images at 5μm intervals throughout the depth of the slices, known as 'stacks'. To enable quantitative comparison of Orexin/MCH neurons in test/control halves, a standard method of photographing and counting was adopted. For each half, two stacks were taken using oil immersion and the 40x objective in fields immediately superior-medial and superior-lateral to the fornix, covering an area where most of the these neurons were found. Neurons were counted using the ImageJ Cell Counter plugin; to prevent double counting of neurons spanning more than one section of the stack, neurons were only counted in sections where the nucleus was visible.

3. Serum and CSF biomarkers in patients and controls

3.1. Analysis of available data on narcolepsy samples

Prior to performing any tests, available clinical details were first analysed and compared with existing data. **Figure 3.1** displays the frequency distribution of mean orexin measurements carried out at the NDCN since 2006. Of 264 samples tested, 36% (96) had levels below 110 pg/ml, consistent with a diagnosis of narcolepsy with cataplexy (Mignot et al., 2002). Of orexin-deficient samples, almost all had undetectable CSF levels of the neuropeptide, below the assay's detectability threshold of 40 pg/ml. Of samples with levels greater than 110 pg/ml, a Gaussian distribution was observed, with a mean value of 300 pg/ml. This group most likely represents a mixture of other sleep-related pathologies, with orexin levels approaching the mean values reported in healthy control populations of 320-360 pg/ml (Mignot et al., 2002).

An increase in the number of children with orexin deficiency has been noticed at the NDCN since 2009, a timeframe that corresponds with H1N1 emergence and vaccination (European Medicines Agency, 2011). In combination with Scandinavian, French and Canadian reports of sudden-onset post-vaccination H1N1 (Dauvilliers et al., 2010; Swedish Medical Products Agency, 2010), this observation provided the impetus for this investigation. **Figure 3.2** compares the ages of patients found to be orexin-deficient tested pre and post June 2009. The June 2009 cut-off corresponds the declaration of pandemic flu by the World Health Organisation (Chan, 2009). Comparison of the mean values of these groups by a non-parametric unpaired T test (Mann-Whitney U) did not show significant differences. However, contingency table analysis of children (<18)/adults (≥18) and pre/post June 2009 reveals a significant increase in the number of children who tested orexin-deficient.

The Swedish and Finnish cohorts were examined, the clinical details of which are summarised in **Table 3.1**. All of these samples were HLA DQB*0602 positive and from clinically validated cases of narcolepsy-cataplexy (N. Darin, personal communication). Orexin levels were quantified on all 14 CSF samples received, and were undetectable in all but one

Fig 3.1 and 3.2

sample. The mean delay from H1N1 vaccination to symptom onset in six Finnish samples was 3.6±3.3 months (mean±SD). The mean delay from symptom onset to serum/CSF sampling in the 11 Swedish samples was 12.2±3.8 months. No further information was available at the time of writing.

	Sample Number	Sex	CSF orexin (pg/ml)	Age at time of sampling	Vaccination	Symptom onset	Delay H1N1 vaccination-symptom onset (days)
Finnish:	45	M	10	18	13/12/2005	14/01/2006	32
	46	M	10	24	14/12/2005	?	?
	47	F	NO CSF	12	07/12/2005	14/10/2006	311
	48	F	NO CSF	9	23/11/2005	06/02/2006	75
	49	F	NO CSF	13	30/11/2005	14/02/2006	76
	50	F	10	18	08/12/2005	19/02/2006	73
	51	M	10	17	14/01/2006	14/04/2006	90
	Sample Number	**Sex**	**CSF orexin (pg/ml)**	**Age at time of sampling**	**Symptom onset**	**Date of sampling**	**Delay symptom onset-sampling (months)**
Swedish:	34	F	10	16	30/06/2006	31/07/2007	13
	35	F	10	15	30/04/2006	31/05/2007	13
	36	M	10	4	31/01/2006	31/05/2007	16
	37	M	52	9	31/01/2006	30/11/2006	10
	38	F	290	9	31/12/2005	30/06/2007	18
	39	F	10	8	31/10/2005	31/05/2006	7
	40	F	No CSF	8	31/10/2005	30/06/2006	8
	41	M	10	12	30/11/2005	31/01/2007	14
	42	M	10	12	31/03/2006	31/03/2007	12
	43	M	10	16	30/09/2006	30/04/2007	7
	44	M	10	14	31/01/2006	31/05/2007	16

Table 3.1: Available data on Swedish and Finnish post-vaccination samples
These were kindly donated by Drs N.Darin, T. Hallböök, M. Partinen and A.Huutoniemi

The inclusion criteria for the main narcolepsy cohort are summarised in **chapter 2.1.1**. As previously described, of particular interest are samples from children, especially those provided by Swedish and Finnish collaborators who are clinically-validated cases of narcolepsy-cataplexy. As data on CSF orexin levels was available on all samples in the narcolepsy cohort, samples were grouped according to age and orexin status for all subsequent analyses. Thus adults and children were grouped as orexin-deficient (<110 pg/ml) or orexin-normal (>110 pg/ml).

3.2. Total IgG

One might expect IgG levels in sera/CSF to be elevated in orexin-deficient samples as a non-specific marker of on-going autoimmune processes. Measurement of total IgG was achieved by DAB staining western blots of serum/CSF IgG and densitometric analysis of the 50 kDa IgG heavy chain in each sample (**Figure 3.3, a)**. For each blot, the 50 kDa band

Fig 3.3

densities were calculated relative to the mean density of a duplicate 11.25 mg/ml standard **(b)**. Total IgG was then interpolated off a standard curve (**c**). This method was found to give reproducible results over a range of IgG dilutions (**d**).

3.2.1. Paired sera/CSF

Total IgG was quantified in 38 serum/CSF pairs. These samples included 17 orexin-deficient patients, 12 orexin-normal patients, and eight disease controls. Representative examples of these western blots are shown in **Figure 3.4**; from inspection of these alone, some of the orexin-deficient sera appeared to have low serum IgG compared to CSF IgG levels. The results of all measurements are given in **Appendix 4**, but this analysis will focus on the paediatric data. **Figure 3.5** compares values only in orexin-deficient children to those in orexin-normal children. There was no difference in mean total IgG in the sera (Unpaired T test) (**a**) and a modest correlation between serum IgG values and age (**b**). Of the children tested, the mean serum total IgG was 5.0±3.4 mg/ml. One orexin-deficient sample (44, a Swedish post-vaccination case) had a level more than two standard deviations (SD) above this mean. CSF Total IgG levels were significantly higher in orexin-deficient compared to orexin-normal children (**c**). Of all the children tested, mean CSF total IgG was 0.026±0.011 mg/ml. Two orexin-deficient samples (41 and 42 also Swedish post-vaccination cases) had levels more than two standard deviations above this mean. Serum/CSF IgG ratios were also calculated (**d**). Ratios were significantly lower in orexin-deficient children, reflecting the elevated CSF relative to the serum IgG.

Total IgG reference ranges are given in **Figure 3.5, e** (adapted from Meulenbroek and Zeijlemaker, 2000 Thompson, 2005). 4/16 orexin-deficient children and 2/7 orexin-normal children had serum IgG levels below the lower limit of their age-related reference range, whereas all CSF measurements were lower than the reported CSF reference maximum of 0.06 mg/ml. 12/16 orexin-deficient children and 3/7 orexin-normal children had serum/CSF IgG ratios above the upper reference value for healthy adults, although the paediatric reference ranges are not known (Thompson, 2005).

Fig 3.4

Fig 3.5

Additionally, all of the paediatric serum/CSF pairs were sent to the University College of London Institute of Neurology at Queen's Square for oligoclonal band testing. The presence of oligoclonal bands in the CSF but not the serum is another marker of intrathecal synthesis of specific antibodies in other neurological autoimmune conditions such as multiple sclerosis. No bands were found in the narcolepsy samples.

3.2.2. Paediatric healthy controls pre and post H1N1 vaccination

Paediatric H1N1 vaccination with the adjuvanted Pandemrix® was found to be more immunogenic and reactogenic, and result in higher H1N1 seroconversion rates than the unadjuvanted Celvapan® vaccination (Waddington et al., 2010). One possibility was that children that had been vaccinated with Pandemrix® might have developed higher total IgG levels. If this were the case, it could indicate an increased likelihood of developing autoimmunity.

Therefore, total IgG was quantified in 86 children who's samples were taken one week pre and two weeks post vaccination. 42/86 children received Pandemrix®, and 44/86 received the Celvapan®. Total IgG levels were slightly but significantly elevated following Pandemrix® but not Celvapan® vaccination (**Figure 3.6, a**). However, comparison of the two vaccines did not reveal significant differences (**a and b**).

3.3. CSF orexin A in neurological autoimmune disease patients

Several cases of secondary narcolepsy-cataplexy have been reported in patients with autoimmune disorders (Mignot et al., 2002; Overeem et al., 2004); narcolepsy has even been reported as a presenting symptom of neuromyelitis optica (NMO) (Baba et al., 2009). For comparison with CSF orexin levels in the narcolepsy cohort and to investigate a possible link between orexin deficiency and autoimmunity, levels were quantified in neurological autoimmune disease patients (n=23). In the absence of healthy control CSF, the autoimmune samples were matched with an equal number of orexin-normal CSFs selected from the NDCN cohort by random number generation.

Fig 3.6

Figure 3.7 displays the standard curve used to interpolate results for this analysis. Using Graphpad® Prism 4, a sigmoid dose response curve was fitted to standard dilutions with known concentrations of orexin. Counts per minute of each sample were then used to interpolate the amount of orexin present. CSF orexin levels in the autoimmune disease patients and orexin-normal controls are compared in **Figure 3.8**. Orexin measurements in the autoimmune cohort were consistently lower than those in the orexin-normal control group, with a difference between the mean values of 121.7 pg/ml. Orexin levels between 110 pg/ml and 200 pg/ml are generally considered to be intermediate (Serra, Montagna, Mignot, Lugaresi, & Plazzi, 2008). Intermediate CSF orexin levels were found in 11/23 autoimmune samples versus 1/23 controls. However, unlike idiopathic narcolepsy-cataplexy, levels were not deficient (<110 pg/ml) or undetectable (<40 pg/ml).

Table 3.2 groups these CSF orexin levels according to the autoantibodies detected in sera and CSF of these patients. Lower CSF orexin levels did not appear to be correlated with a particular autoimmune condition. The lowest orexin measurement in the autoimmune cohort was 114.3 pg/ml in a patient who was negative for VGKC, NMDA and Glycine antibodies in both serum and CSF. This level almost meets the diagnostic threshold for narcolepsy with cataplexy (110 pg/ml).

3.4. CSF MCH in narcoleptic patients

Orexin and MCH neurons are co-distributed in the hypothalamus, and orexin and MCH may share reciprocal roles in sleep regulation (Torterolo et al., 2011). It was possible that low orexin levels in narcolepsy-cataplexy patients would be associated with a disturbance of neurosecretory MCH neurons. Therefore, CSF MCH levels were investigated as a potential biomarker in narcolepsy.

As with CSF orexin, quantification was achieved by a competitive radioimmunoassay. Unlike the orexin assay, it was not possible to accurately interpolate normal CSF MCH levels with the commercially available kit as provided, as the standard curve was not optimised for such

Fig 3.7, 3.8, Table 3.2

low levels. As described in **chapter 2.2.2**, the standard protocol was modified by spiking samples with 400 pg/ml of MCH peptide; values could then be interpolated in the measurable range (**Figure 3.9**). To assess the reproducibility of this method, one sample was repeated four times. On average, samples deviated from the mean by 13.4% (mean and standard deviation = 247.5 ± 40.6 pg/ml). Much of this error may be accounted for by the inaccuracies incurred when spiking the samples.

MCH levels were quantified in 36 samples (**Figure 3.10)**. MCH levels did not correlate with prior orexin measurements. Comparison of groups by unpaired-T test revealed no significant differences. However, the four highest MCH measurements were all found in orexin-deficient children; two of these samples had MCH levels more than two standard deviations from the overall mean value (174.5± 39.4pg/ml). Observed levels were higher than that previously reported using a similar methodology (Peyron et al., 2011: 118± 20 pg/ml). Unfortunately, low sample volumes and insufficient kit reagent meant that these findings could not be repeated.

Results from serum/CSF biomarkers for children with paired serum/CSF samples investigated are summarised in **Table 3.3**. Adult data is included in **Appendix 6**.

Fig 3.9, 3.10

Table 3.3

3.5. Summary and discussion

The availability of serum/CSF pairs for some narcolepsy samples, in particular Swedish and Finish post-vaccination children made it possible to look at serum and CSF total IgG and the ratio between them; it was thought that that total IgG might be elevated in the sera and CSF of orexin-deficient patients as a marker of autoimmune processes. Although serum levels were similar between orexin-normal and orexin-deficient children (both lower than adult levels), the CSF levels were higher in the orexin-deficient children. These findings are perhaps evidence of on-going intrathecal synthesis of specific IgG antibodies, although oligoclonal band testing on these samples performed in a different lab was negative.

To investigate immune responses to the disease-associated Pandemrix® vaccination itself, a case-control study of total IgG in serum from healthy control children was performed. There was no significant difference in post-vaccination IgG levels in children vaccinated with Pandemrix® compared to a unadjuvanted alternative, providing no evidence to suggest that Pandemix® in particular led to heightened immune responses in these children.

In addition to diagnostic CSF orexin quantification routinely carried at the NDCN, levels were also quantified in a cohort of autoimmune disease patients. These were found to be significantly depressed compared to controls, although none met the diagnostic threshold for narcolepsy. This supports the hypothesis that the orexin system and/or CSF flow are disrupted secondarily to these conditions. However, the presence of depressed rather than deficient levels indicates that the processes underlying this change is likely to differ from the selective orexin neuronal loss seen in idiopathic narcolepsy-cataplexy.

Additionally, CSF MCH levels were quantified in some of the narcolepsy samples. Although the experimental error was relatively large, the highest MCH levels were consistently found in paediatric post-vaccination narcolepsy-cataplexy samples. Although CSF MCH levels did not appear to be an informative biomarker, MCH disinhibition may be a feature close to disease onset.

3.5.1. Cohort discussion

The NDCN cohort is primarily composed of CSF samples sent for diagnostic orexin testing. Sampling from this cohort had several advantages. The need for invasive sample collection further than that already performed for diagnostic purposes was mitigated, and samples were taken from the earliest feasible disease time point. The necessity for early sampling is consistently stressed as a major limitation of previous studies investigating autoimmune hypotheses in narcolepsy, which retrospectively collected samples from patients with a prior narcolepsy diagnosis (see review by Fontana et al., 2010). An additional benefit of sampling from this cohort was the availability of CSFs and some sera from patients with normal levels of orexin; these represent an important internal control group.

A major disadvantage of this approach, however, is that clinicians commonly only send enough CSF for diagnostic orexin measurement, and little excess is available for experimentation. Therefore, not all subsequent experiments were performed on all samples, reducing power. Moreover, paired serum samples were rare. Meanwhile, we were fortunate enough to obtain an adequate volume for Swedish and Finnish post-vaccination samples; these were arguably the most important samples to be investigated, as all were from clinically defined post-vaccination paediatric cases. The mean delay of 109.5 days from vaccination to symptom onset is compatible with autoimmune hypotheses. In a condition where diagnostic delay periods of several years are seen, samples taken several months after symptom onset can still be considered close to disease onset.

In this study, CSF orexin A deficiency has been used as a surrogate marker of narcolepsy with cataplexy. Given the high sensitivity of this test, and in the absence of available clinical details for the majority of samples, this was a reasonable assumption to make. However, some patients with clinically-defined narcolepsy with cataplexy do not have deficient orexin levels; for instance, Swedish sample 38, with orexin levels of 290 pg/ml, is a reported to be a definite case of post-vaccination narcolepsy-cataplexy (N. Darin, personal communication).

Although CSF orexin deficiency is one of the diagnostic criteria for narcolepsy, it is neither necessary nor sufficient to diagnose the condition.

Also included in the 'orexin-normal' group are patients with intermediate orexin levels (i.e. 110-200 pg/ml). These patients could feasibly represent narcolepsy-cataplexy patients in whom not all orexin neurons have yet been destroyed. Immunologically speaking, narcolepsy-cataplexy patients with intermediate orexin levels may be the most interesting, as the autoimmune processes destroying orexin neurons may still be on going.

3.5.2. Total IgG

Methods considered to quantify total levels of IgG included radioimmunodiffusion (RID), the standard technique used to measure total serum IgG. However, in previous work, RID was not sensitive enough to detect CSF levels of IgG reliably, and for comparison of serum and CSFs, an in-house method was chosen. Moreover, quantification by densitometry of western blots was a method that required a very small CSF sample volume. The reliability of the method was confirmed by repeat assay of nine samples spanning a range of IgG concentrations, giving consistent results.

Although the elevated CSF IgG levels in orexin-deficient children was statistically significant, the clinical relevance of this result is unclear; no CSF total IgG and serum/CSF ratio reference ranges were available for paediatric age groups. Of particular note, of the two CSF that had levels more than two standard deviations above the mean CSF IgG for all children tested (both known post-vaccination cases), patient 42 also had elevated MCH levels. However, CSF Total IgG and CSF MCH measurements in these groups were not otherwise correlated.

There are several possible explanations for relatively raised CSF IgG seen in these patients. Firstly, it may be evidence of CNS inflammation; it is well understood that in some situations, the blood brain barrier can become permeable to serum proteins and immunoregulatory cells

(Kermode et al., 1990). In such an event, IgG may leak into the CNS compartment. Secondly, increased CNS IgG may reflect intrathecal synthesis of specific IgG's. In order to establish if CNS IgG leak or intrathecal synthesis had occurred, it would have been necessary to quantify serum and CSF levels of albumin in addition to IgG. This could have been achieved using a similar western blot methodology. From this, the IgG index for each patient could have been calculated, as this is the ratio between serum/CSF IgG: serum/CSF albumin. However, the absence of detectable oligoclonal bands in these samples provides evidence against the clonal expansion of CNS B cells and intrathecal immunoglobulin synthesis, although oligoclonal bands don't necessarily correlate with the presence of specific intrathecal antibodies (A. Vincent, unpublished observations).

As one might expect, the overall highest levels of sera and CSF IgG to be found were in patients with neurological autoimmune diseases (**Appendix 4**). Curiously, two of the orexin-normal adults also had CSF total IgG levels well above the 0.06 mg/ml reference maximum; although these samples had normal levels of orexin, the elevated CSF IgG may indicate that other autoimmune disease processes are responsible for the symptoms that resulted in narcolepsy investigation.

3.5.3. CSF orexin in neurological autoimmune disease samples

Although CSF orexin levels in neurological autoimmune disease patients were significantly lower than matched controls, levels did not correlate with the presence of a particular autoantibody. Also, unlike cases of idiopathic narcolepsy-cataplexy, CSF orexin measurements tended to be intermediate (110-200 pg/ml, found in 11/23 samples tested) rather than undetectable (<40 pg/ml). Nonetheless, this finding provides evidence that links autoimmunity with disturbance of the orexin system. However, the depressed yet detectable orexin levels in these patients may point to a separate aetiology to idiopathic narcolepsy-cataplexy. Furthermore, CSF orexin levels in these patients can improve with time (Mignot, 2011), suggesting that an acute suppression of orexin release or disruption to CSF flow is responsible, rather than orexin neuron death. Although repeat measurements were not

performed, the results of the CSF orexin levels in the orexin-deficient controls that were re-tested were very similar to levels found previously.

3.5.4. CSF MCH in narcoleptic samples

It was hypothesised that CSF MCH and orexin levels would be associated close to disease onset, identifying MCH as a relevant biomarker of disease. No such association was found in a mixed cohort of orexin-deficient and orexin-normal adults and children. However, the four highest MCH measurements were all found in post-vaccination samples received from the Swedish and Finnish collaborators. Two of these had values of more than two standard deviations above the population mean. Although comparisons of the group means did not yield significant results, the presence of these outliers suggests that MCH levels were elevated in some of these children close to narcolepsy-cataplexy onset, but it is unlikely that this would throw any light on disease mechanisms or provide a useful biomarker.

The primary limitation of these experiments was the error introduced by intentionally spiking samples with MCH peptide. In the aforementioned study of MCH measurements (Peyron et al., 2011), a similar RIA method had been used. However, investigators had sufficient sample volume to 4x concentrate test CSF (400 µl into 100 µl), so that measurements could be interpolated off the standard curve without further manipulation. Although a similar approach would have been preferable and was attempted in this study, the low sample volumes available meant that the intentional spiking approach was chosen. The final MCH levels found by this method were greater than those reported previously: 174±39.4 pg/ml in this study compared to 118± 20 pg/ml pg/ml found in controls previously (mean±standard deviation). Although the reason for these differences cannot be explained, it is possible that the intentional spiking of samples introduced a consistent procedural error into all subsequent CSF MCH measurements.

Not only was the study reported here larger than the one previously reported by Peyron et al. in 2011, it also represents the first attempt to quantify MCH in narcoleptic patients close to

disease onset. MCH is increasingly seen as an important functional link in REM sleep regulation, (Peyron et al., 2009; Torterolo et al., 2011). In contrast with orexin neurons, MCH neurons maximally fire during REM sleep (Hassani & Lee, 2009) and intracerebral injection of MCH increases REM sleep. These seemingly antagonistic roles for the pair of neuropeptides have led some to hypothesise that a disinhibition of MCH neurons in narcolepsy-cataplexy patients may augment REM manifestations. Seemingly contradictory to this hypothesis, one recent study (Peyron et al., 2011) reported slightly but significantly depressed levels of MCH in central hypersomnia patients compared to controls. However, akin to the findings of this study, the highest overall MCH measurements reported were also from narcolepsy-cataplexy patients. Therefore, although MCH and orexin were not associated as hypothesised, these findings do not rule out the possibility of MCH disinhibition close to disease onset, which may contribute to the symptoms.

4. Candidate antigen approaches

The NDCN laboratory has extensive experience of candidate antigen approaches to screen cohorts of probable neurological autoimmune disease patients for serum/CSF immunoreactivity (see review by Vincent et al. 2011). In particular, a live cell-based assay (CBA) has been developed, with which samples can be tested against cell surface antigens of interest (Waters et al. 2008; Leite et al. 2008; Irani, Bera, et al. 2010; Irani, Alexander, et al. 2010). Comparison of candidate antigen approaches in a recent multicentre study of AQP4 antibodies found the CBA to be the most sensitive and specific method (Waters et al. 2012).

This study set out to test narcoleptic sera/CSF for antibodies directed against NMDAR, VGKC and VGKC-associated LGI1, CASPR2 and Contactin2, all neuronal surface receptors or related proteins that are accepted as important targets in CNS autoimmune diseases. Although the hippocampus appears to be the main target of autoimmune attack in these conditions, hypothalamic dysfunction and REM sleep disturbances are common (Vincent et al. 2011; Iranzo et al. 2006), and several reports have linked these autoantigens to narcolepsy in individual patients (see **chapter 1.7**), establishing the rationale for screening narcolepsy samples for reactivity to these autoantigens. GAD65, an intracellular antigen central to GABA metabolism, was also assayed; GAD65 antibodies have been associated with several neurological autoimmune conditions including paediatric encephalitis (Haberlandt, Bast, & Ebner, 2011).

Additionally, the CBA enabled the development and testing of novel candidate antigens to which the putative autoantibodies in narcolepsy-cataplexy may be directed. GABAa receptor subunits are hypothesised here to be potential candidates that have not previously been tested. Prior studies have highlighted these receptors as important inhibitors of orexin neuron function during sleep (Alam et al., 2005). α subunits have been found to be selectively expressed on orexin neurons (Cvetkovic-lopes et al., 2010) and α3 was found to colocalise with orexin by immunofluorescence (Bäckberg et al., 2004).

4.1. The cell-based assay: NMDAR, CASPR2, LGI1, Contactin2

Sera/CSF immunoreactivity to candidate antigens was tested on transfected HEK cells. Binding of specific patient antibodies was visualised by immunofluorescence. Positivity was scored on a subjective scale by comparison to healthy and positive controls included in every assay (**Figure 4.1)**. Binding was scored subjectively from 0 (no binding) to 4 (very strong binding) with increments of 0.5 and a positivity threshold of 1.

The DNA inserts for CASPR2 and LGI1 were eGFP tagged; successful transfection resulted in cytosolic eGFP expression of the transmembrane constructs and serum binding colocalised with eGFP labelling (**Figure 4.1, a**). In this system, one might expect that serum binding would be proportional to eGFP expression, i.e. the brightest eGFP-expressing cells would also have the most intense serum binding. However, this was not the case: sera positive for a particular antigen consistently bound brightest to HEK cells weakly expressing eGFP, perhaps because overexpression of eGFP may be toxic to HEK cells (Liu, Jan, Chou, Chen, & Ke, 1999). DNA inserts for NMDAR (co-transfected NR1 and NR2b subunits) and Contactin2 were untagged, and eGFP was co-transfected. For these untagged antigens, eGFP expression was mainly to confirm efficient transfection and serum binding/eGFP colocalisation was less relevant for assessing positivity (**Figure 4.1, b**).

Screening

Table 4.1 documents the number of sera and CSFs screened on each of the candidate antigens. The five known positive control sera were correctly identified (see **Appendix 6**), and have been excluded from subsequent analyses. 67 narcolepsy/healthy control sera were tested at 1:20 dilutions for NMDAR, Contactin2 and LGI1 assays and 1:100 dilutions for CASPR2. Overall, there were 15 serum CBA low positive results (CBA score 1-2) found in 13 sera, including one healthy control child and one healthy control adult (both scored 1 on Contactin2). Two CASPR2-Contactin2 double positives were identified (serum 10, an orexin-normal adult, and serum 38, the Swedish post-vaccination narcolepsy child who had normal orexin levels). No CSF positivity was detected on any of the 70 samples diluted 1:1.

Fig 4.1

Table 4.1 Fig 4.2

Figure 4.2 demonstrates the inter-assay repeatability of blinded CBA scores for all patient serum samples declared as positive. Any observed positivity was independently scored by a blinded colleague and repeated on at least two other assays. After a minimum of three repeats, the modal CBA score was taken to be the final score. For the 13 sera with positivity, the modal inter-assay variability was 0.5 subjective CBA points. A consistent pattern of CBA positivity was not observed more frequently in orexin-deficient children for any of the constructs tested (**Figure 4.3**). Indeed, the group most frequently positive was orexin-normal adults, which may include samples with other autoimmune diseases. A curious observation was that orexin-deficient children consistently had higher rates of sub-threshold positivity (i.e. scored 0.5, signifying low level binding observed on few cells). 6/9 of the orexin-deficient samples with this 'sub-threshold' scored 0.5 on more than one antigen.

End point titrations

End-point titrations were performed on all low positive sera. Serial dilutions of each positive serum sample were simultaneously assayed and scored (**Figure 4.4**). Without exception, antibodies were found to be low titre; the highest dilution at which any positivity was observed was 1/200 for CASPR2 in Serum 10 and 38, both patients who had normal levels of CSF orexin. All other sera were negative at 1/100 dilution.

Preadsorption experiments

Finally, to confirm binding specificity of the 13 CBA positives from the cohort, sera were preadsorbed against their respective candidate antigens. This was achieved by pre-incubating the sera with transfected HEK cells. As a control, identical serum dilutions were also added to untransfected, 'blank' HEK cells. Cell-based assays were then repeated for the preadsorbed/blank-preadsorbed sera (**Figure 4.5**). In all cases, serum binding was largely abolished by preadsorption with the respective antigen, confirming specificity. In 4 cases, very low-level serum scoring was still observed after preadsorption (e.g. Patient 10 in **Figure 4.5**). The low-level binding most likely represents incomplete preadsorption.

Fig 4.3

Fig 4.4 and 4.5

4.2. $GABA_A$ receptor α3

Previous studies have shown $GABA_A$ receptor α subunit expression to be highly enriched in orexin neurons (Cvetkovic-lopes et al., 2010). These receptors have been shown to be important in the regulation of orexin neurons (Alam et al., 2005) Additionally, the α3 subunit has been shown to be expressed in orexin as well as MCH neurons (Bäckberg et al., 2004). Immunofluorescence immunohistochemistry (I.F.) double staining of orexin and an anti-α3 was performed and convincing colocalisation demonstrated (**Figure 4.6)**. Therefore, $GABA_A$ receptor α3 subunit was selected as a candidate antigen of interest for which a CBA assay was to be designed and tested.

Molecular genetics

Steps to establish a $GABA_A$ α3 CBA were performed under the guidance of Dr. Patrick Waters and Dr. Mark Woodhall. Individual molecular cloning methodologies are described in **chapter 2.3.3.** The overall protocol for steps undertaken is summarised in **Figure 4.7.**

After obtaining the (c)DNA construct for pBluescript(GABRAα-3), the sequence was checked by Polymerase Chain Reaction (PCR) sequencing. Initial attempts made to directly clone the insert into the mammalian expression vectors pIRES2-AcGFP1 and pIRES2-DsRed ultimately proved unsuccessful. As an alternative approach, the insert was first cloned into pGEM-T Easy vector as an intermediate vector. Site directed mutagenesis was then performed to correct a missense mutation within the gene prior to final cloning into the pIRES2 mammalian expression vectors and HEK cell transfection.

Figure 4.8 describes the steps undertaken to clone the GABRAα3 insert into pGEM-T Easy vector. In the first step, the insert was amplified by PCR with specifically designed primers at a range of annealing temperatures. An annealing temperature of 70^0C gave the clearest band at 1.4kDa, which was gel extracted for subsequent cloning (**Figure 4.8, a**).

Following a-Tailing, the 70^0C insert was ligated into pGEM-T Easy. JM109 *E.coli* cells were then transformed and cultured overnight on ampicillin-supplemented plates. To maximize

Fig 4.6, 4.7, 4.8

chances of selecting a colony with the correctly ligated insert, 10 colonies were randomly selected and cultured. DNA was extracted from each of the amplified colonies by miniprep, cut with XhoI1 and BamH1 restriction enzymes, and electrophoresed on a 1% agarose gel. Colony 4 was found to have the correctly ligated insert, as bands were present at 3kb and 1.4kb, as anticipated, corresponding to pGEM-T Easy and GABRAα3 respectively (**Figure 4.8, b**).

Mutagenic primers (**Chapter 2.3.2)** were specially designed for the site directed mutagenesis of the insert in pGEM-T Easy (**Appendix 3**). Mutant strand synthesis was performed using these primers, prior to digestion of the methylated parental DNA template by the enzyme Dpn1. Following transformation and amplification in JM109 cells, the corrected DNA sequence was confirmed by PCR (**Figure 4.9, a**). The mutated cytosine at position 778 had been successfully replaced with thymine. When expressed, this would result in an amino acid change from proline to leucine as in the native peptide. After an additional miniprep amplification and digest with Xho1 and BamH1, the corrected insert was finally ligated into the mammalian pIRES2 vectors. The presence of bands at the expected sizes was confirmed by gel electrophoresis of digested fragments (**b**) prior to maxiprep amplification and final sequencing.

HEK cell transfection and cohort screening

Functioning $GABA_A$ receptors exist as pentamers. Many $GABA_A$ subunits exist, however receptors most commonly consist of 2x α, 2x β, and 1x γ subunits. A $GABA_A$ receptor CBA had previously been established in the lab consisting of α1-GFP, β3, γ2 subunits co-transfected in equal proportions. This has been used to screen 62 patients with idiopathic epilepsies. Only one patient serum was previously identified to be positive on this CBA. The pcDNA™3.1 constructs α1-GFP, β3, γ2 were obtained and the correct sequences confirmed by PCR. CBA positivity in the one previously identified sample was then replicated (**Figure 4.10)**, confirming that conformational $GABA_A$ receptors had been formed.

Fig 4.9

Fig 4.10, 4.11

To create a $GABA_A$ receptor CBA including the α3 subunit, the newly cloned and corrected pIRES2-(GABRAα-3) was substituted for the α1 subunit used above. To confirm expression of the α3 subunit, two flasks of HEK cells were co-transfected with α3, β3 and γ2 or α1, β3 and γ2 respectively, and cell lysates run on western blots. Probing with an anti-α3 commercial antibody, a 50-60 kDa band was observed only with α3-transfected lysate, confirming expression (**Figure 4.11, a and b**). Cell surface expression of the α3 subunit was confirmed by strong binding of the commercial antibody on the CBA (**Figure 4.11, c**). All 57 sera were screened on the $GABA_A$ receptor α-3, β3, γ2 CBA, and no positives were found.

4.3. Radioimmunological candidate antigen assays: Potassium channel and GAD65

Sera were also screened for antibodies to the Voltage-gated Potassium Channel (VGKC) and Glutamic Acid Decarboxylase (GAD). These candidate antigens are tested by radioimmunoassay.

VGKC antibody testing was conducted by Dr. Bethan Lang. Of the 57 sera, one low positive sample was identified: serum 50. This was an orexin-deficient child from the Finnish post-vaccination cohort who also scored a one on the CASPR2 CBA.

GAD65 autoantibodies have been reported to be associated with Type 1 diabetes, stiff person syndrome, cerebellar ataxia and other neurological conditions including epilepsy, limbic encephalitis and myasthenia gravis (Graus, Saiz, & Dalmau, 2010). As an intracellular antigen, anti-GAD65 antibodies are likely to be a marker rather than a target of autoimmune attack. Antibody testing was personally conducted on all samples and revealed no positive results.

The results of the paediatric sera are summarised in **Table 4.2**. Results of all adult sera are available in **Appendix 6**.

Table 4.2

4.4. Summary and discussion

The CBA and RIA were used to test narcoleptic patient sera against putative autoantigens. No immunoreactivity was observed in test CSFs. Low-titre specific serum binding was observed in 15 CBA and one RIA test, however this was not specific to orexin-deficient patients or a particular candidate autoantigen. Two Scandinavian post-vaccination samples did produce interesting results; one Swedish sample (38) was double positive for the VGKC associated proteins CASPR2 and Contactin2, and one Finnish sample (50)was double positive for CASPR2 and VGKC. $GABA_AR\alpha3$ was successfully modified and expressed on the surface of HEK cells, although no serum positives were identified. According to these experiments, it is unlikely that NMDAR, VGKC, CASPR2, Contactin2, LGI1, GAD65 and $GABA_AR\alpha3$ are autoantigens of relevance in the majority narcolepsy-cataplexy patients. However, the low level positivity observed in some patients may be evidence of immunological epiphenomena.

Appropriateness of candidate antigens tested

The rationale for testing each of the candidate antigens has been previously described. NMDAR, VGKC complex and VGKC complex-related proteins (LGI1, CASPR2, Contactin2) are autoantigens of pathogenic importance in CNS autoimmune diseases in which hypothalamic and sleep disturbance often feature (Vincent et al. 2011). Of these known targets, NMDAR was arguably the most likely candidate in narcolepsy-cataplexy; NMDAR antibodies have been identified in orexin-deficient cases (Tsutsui et al., 2012), and narcolepsy-cataplexy shares phenotypic similarities with NMDAR-related disorders (Dale et al., 2009; Gandhi et al., 2012). GABAa receptor α3 subunits are expressed on orexin neurons where they help regulate the orexin system (Alam et al., 2005; Bäckberg et al., 2004; Hassani et al., 2010).

Other antigens that could have been tested include AQP4, TRIB2 and Insulin-like growth factor binding protein 3 (IGFB3). Narcolepsy has been reported as an initial manifestation of AQP4-related NMO (Baba et al., 2009), and some patients with NMO also have orexin-

deficiency (Kanbayashi et al. 2012). However, in the work reported in this thesis (see **chapter 3.4.3**), intermediate but not deficient orexin-levels were found in 3/8 AQP4+ NMO patients. Furthermore, Unlike NMDAR and VGKC, AQP4 is not expressed on neurons (Nielsen et al., 1997). In this context, disturbances to the orexin system are likely to be secondary to a more generalised hypothalamic dysfunction rather than the selective neuronal targeting seen in idiopathic narcolepsy. Additionally, when present, AQP4 antibodies usually produce a clear binding pattern on rat brain sections (Lennon & Kryzer 2005). Since these tests were also performed (see **chapter 5**), AQP4 was not tested as a candidate antigen.

Although antibodies against tribbles homologue 2 (TRIB2) are reported in narcolepsy (Cvetkovic-lopes et al., 2010; Kawashima et al., 2010; Toyoda et al., 2010), as previously discussed (**chapter 1.8**), this is unlikely to represent a pathogenic autoantigen. This antigen is not expressed at the cell surface, and would be ordinarily invisible to the immune system (Vincent et al., 2011; Dale & Brilot 2010). Furthermore, TRIB2 antibodies against this ubiquitous cytosolic kinase inhibitor have only been reported in a minority of narcolepsy patients. In a situation akin to intracellular paraneoplastic autoantibodies or GAD65, TRIB2 autoantibodies most likely represent a marker of cellular destruction in these patients. For these reasons, TRIB2 was not investigated as a candidate autoantigen.

Finally, a recent study identified insulin-like growth factor binding protein 3 (IGFB3) to be expressed in orexin neurons (Honda et al. 2009). In the same study, this secreted protein was found to be involved in sleep regulation and down-regulated in narcolepsy. Using a western blot of IGFB3-expressing cell extracts to screen narcoleptic sera and CSF, no positives were identified. Although IGFB3 seems an unlikely autoantigen, the patient samples examined were not specifically taken close to disease onset and screening samples by western blot may have missed antibodies directed against conformational epitopes. A cell-based assay of IGFB3 could be performed on our patients to rule out this possibility.

Method advantages and limitations

One criticism of the CBA in comparison to equivalent candidate antigen approaches is that CBA measurements are subjective. However, alternative approaches such as flow cytometry are generally more costly and time-consuming. Comparatively, the CBA is a sensitive and efficient way to identify positive binders in cohort samples, after which more objectively quantitative methods may follow; in particular, FACS analysis of the IgG binding is both highly sensitive and specific (Waters et al. 2012). As no consistent patterns or high titre binding was found, no such quantification was deemed necessary.

Interpretation of the low titre positivity found in this study is limited by a lack of data on large cohorts of healthy controls, especially children. In addition, as subjective scoring is used, the experience of the observer is clearly an important factor, even when positivity was checked by a blinded colleague. As previously described, rates of 'sub-threshold' scores of 0.5 were higher in orexin-deficient children compared to controls, and sera commonly scored 0.5 on multiple constructs. If there was non-specific binding to HEK cells, one would expect similar binding in all four candidate antigens tested, yet this was not usually the case. Therefore, whether this binding reflects elevated autoimmunity is not yet clear.

Curiously, $GABA_A$ α3 was the only candidate antigen for which no positivity whatsoever was detected. Furthermore, this was also the only candidate antigen not yet known to be associated with autoimmune disease. This study suggests that individuals, including healthy controls, may commonly have low titre autoantibodies to the potentially pathogenic autoantigens tested. It is feasible that such autoantibodies may only become pathogenic following a triggering event that stimulates a rise in the antibodies and allows access to the antigen. In the brain, such an event could include inflammation that causes the blood-brain barrier to become permeabilise, perhaps indicated by the relatively raised CSF IgG levels found in orexin-deficient children.

5. Screening approaches

5.1. Indirect immunofluorescence of frozen brain sections

Immunohistochemistry (IHC) of rat brain tissue has proved a useful method in the recognition of several CNS autoimmune diseases, including aquaporin-4 antibodies in neuromyelitis optica, LGI1 antibodies in limbic encephalitis, CASPR2 antibodies in Morvan's syndrome, and NMDAR antibody encephalitis (Vincent et al. 2011). The use of immunofluorescence (I.F.) over traditional colorimetric staining techniques is widely considered to be more sensitive and specific (Hughes et al. 2008). Compared to antigen specific assays, I.F. can be used as a screening tool that requires no preconceived ideas of what candidate antigens might be. As such, it represents a good initial step in a search for novel autoantibodies.

Coronal distribution of orexin neurons in adult rats

Orexin neurons are located in a small and discrete area of the rat lateral hypothalamus. Studies were first carried-out to identify the distribution of these neurons before screening serum samples on orexin containing sections (**Figure 5.1**). For this, adult rat brain was coronally sectioned (12 um), every fourth section stained with an orexin A commercial antibody, and all neurons on one side of the 3rd ventricle counted. In one such brain, twenty-three consecutive sections contained orexin A immunoreactive cell bodies over a distance of 1.3 mm (-2.3 to -3.6 mm relative to the bregma) (**Figure 5.1**). This distribution is similar to that reported in the literature (Nambu et al., 1999). Orexin neurons were generally distributed around the fornix and posterior-lateral hypothalamic area. Orexin neurons also extended into the dorsomedial nucleus at -3 mm. By comparison, co-distributed MCH neurons, were found to be much more numerous (with a peak incidence of 200 neurons per section at -3mm) and had a wider distribution of -1.5 to -4 mm relative to the bregma.

Orexin and MCH double-staining: neuronal development in the postnatal rat and morphology

In order to study the co-distribution of Orexin and MCH neurons, various double-staining

Figure 5.1

methodologies were attempted and optimised. Initially, a green florescent secondary antibody was used to stain orexin neurons, with MCH in red. However, for reasons that remain unclear, the green secondary antibody was found to give a high background level of staining, even though MCH neurons were visible in red. This issue was overcome by using different secondary antibodies, switching to red for orexin and green for MCH (**Figure 5.2, a**). Double staining by indirect immunofluorescence (I.F.) was then compared to that by HRP-based immunostaining, (also shown in **Figure 5.2, b**). Although very low levels of background were found with the HRP-based immunostaining method, staining orexin neurons in red (AEC) and MCH neurons in brown (DAB) meant that it was often hard to differentiate one cell type from the other. Therefore, I.F. was used for all subsequent staining experiments.

The development of orexin and MCH immunoreactivity in the postnatal rat was studied in order to determine the optimal age at which brains should be harvested for neuronal cultures (see **chapter 6**). 12 µm sections were taken in postnatal day (P) 2, 3, 8 and 16 rats and double stained with orexin and MCH. Whereas MCH immunoreactive neurons were clearly visible at P2, orexin immunoreactivity did not clearly emerge until P8 (**Figure 5.3, a**)**.** By P16, orexin and MCH cell body immunoreactivity and size had reached levels similar to those seen in adults.

Orexin/MCH double staining was also used to assess the sizes of the cell bodies. By photographing the neurons at 400x magnification in sections from three adult rats, the sizes of orexin and MCH cell bodies and nuclei were quantified (**Figure 5.3b**). Both neurons were medium sized (mean diameter 27.8 um and 25.0 um respectively) with one or two primary dendrites. These measurements are similar to those reported previously (22.6 um for orexin and 24.8 um for MCH, Steininger et al. 2004).

Initial screen of sera and CSF

Prior to screening sera by immunofluorescence (I.F.), each step of the staining protocol was

Figure 5.2 5.3

first optimised. These included fixation conditions, the use of different wash solutions, choice of protein blocking agent, serum dilution, serum incubation conditions and secondary antibody choice. The final conditions selected are highlighted in **Figure 5.4** (**a**). These conditions lead to the clearest differences between healthy control and positive control serum binding (**b**). The anticipated patterns of I.F. immunoreactivity against candidate autoantigens and positive controls have been summarised in **Tables 1.4 and 1.5**. Unstained sections were also inspected under the microscope to exclude the possibility of autofluorescence. Additionally, secondary antibody only controls were tested to exclude the possibility of non-specific secondary antibody binding.

Sections containing orexin neurons were first identified by staining with an anti-orexin A commercial antibody. 55/57 narcolepsy serum samples,15 healthy controls and 5 positive controls were then diluted 1/100 in PBS, and screened by I.F (**Table 5.1**). Different brain areas present on each section were systematically inspected at low power (50x), including hypothalamus, thalamus, hippocampus, cortex basal ganglia and white matter. Slides were then viewed at higher power (400x). Patient binding for each brain area was given a subjective score (0-3) on the basis of binding intensity (see methods). Additionally, note was taken of the nature of the binding observed at high power (i.e. nuclear: ‘n’ or cytoplasmic: ‘c’).

Positivity was often most easily observed in the hippocampus, where the granule cell layer tended to be brighter than surrounding tissue and adjacent white matter. Of the positive neurological autoimmune controls included in the randomised narcolepsy cohort, 3/5 were correctly identified as having hippocampal binding patterns, although it was not possible to determine what candidate antigens these patterns corresponded to. **Figure 5.5** shows representative examples of the binding observed in the narcolepsy samples and the scores given. Several of the narcolepsy sera appeared to have generalised binding patterns, i.e. not specific to one brain area. For instance, a patient that appeared ‘2n’ (signifying moderate nuclear binding) in the hippocampus would also typically score ‘2n’ in the hypothalamus. A curious hippocampal pattern was observed in serum 10, a female adult with normal levels of

Figure 5.4

Table 5.1 and Fig 5.5

orexin, who was positive for both CASPR2 and Contactin2 autoantibodies. The pattern observed was in the stratum moleculare and stratum radiatum. Similar patterns were not observed with any known narcolepsy sera.

Figure 5.6 summarises the results of the initial serum screen using the subjective hypothalamus scores. The binding observed did not appear to discriminate patient groups on the basis of age or orexin status. Once again, the most frequent 'positive' group were adults with normal levels of orexin. Interestingly, whereas cytoplasmic binding patterns were observed in orexin-deficient children (3/24), orexin-normal children (2/8) and orexin-normal adults (2/17), no cytoplasmic binding was observed in healthy controls. 2/5 of the neurological autoimmune positive controls had cytoplasmic hypothalamic binding in addition to that already mentioned in the hippocampus; indeed, the strongest overall binder (score 3c) was a control for whom the target antigen is not know.

I.F. screening of all 70 CSFs was carried out at 1:1 dilution. This had to be done in two separate experiments, due to the number of samples and rat brains required. Despite clear hippocampal binding with the CSF of an NMDA positive patient, no binding was observed in any of the narcolepsy samples (**Figure 5.7**).

Colocalisation and preadsorption studies

Having identified a few immunoreactive sera (above), colocalisation studies were performed to assess the specificity of patient binding for orexin cell bodies. Unlike the double-staining experiments performed previously with orexin A and MCH commercial antibodies, both primary antibody layers (anti-orexin A commercial and patient serum) could not be incubated at the same time, as background binding was too high. Respective primary and secondary antibodies were therefore incubated serially, using positive and healthy controls to assess the effectiveness of the optimisations (**Figure 5.8 (a)**). Single-labelled controls were also tested to exclude bleed-through (not shown). **Figure 5.8 (b)** shows representative examples of serum binding. More in-depth results are provided alongside the findings of preadsorption

Fig 5.6 and 5.7

Fig 5.8

experiments (**Figure 5.10**); typically subjective scores did not differ greatly from those given in the initial I.F. screen. Although scores were given on the basis of binding to orexin neurons, all other surrounding neurons appeared to have equally bright immunoreactivity.

Due to the lack of cell-specific serum binding observed, attempts were made to eliminate non-neuronal antibodies from the sera. This has commonly been achieved by incubating sera with acetone liver powder, usually derived from rodents (McLaughlin, Wood, Saito, & Barber, 1974). As this was no longer in production, pigeon acetone liver powder (PALP) was used as a surrogate. Unpreadsorbed vs. preadsorbed serum from each positive control were compared in adjacent sections (**Figure 5.9**). This method made binding patterns of positive control sera clearer by eliminating background signal, and it was hoped that this method would unmask any 'true' neuronal binding in the narcolepsy sera.

In total, 18 narcolepsy sera were preadsorbed with PALP prior to orexin double staining. These included six orexin-deficient children, three orexin-normal children, one orexin-deficient adult and seven orexin-normal adults who were all identified as positive (score >1) on either the initial I.F. screen or CBAs. In addition, all 15 healthy controls were included. By comparing non-preadsorbed vs. preadsorbed sera for each particular serum in adjacent sections, binding to orexin neurons was subjectively scored. Whereas PALP preadsorption did not reduce orexin neuronal binding in a VGKC + patient, binding with narcolepsy serum was reduced in most cases (**Figure 5.10, a**). This pattern is reflected in the subjective scores given for each serum sample tested (**b** and **c)**; whereas six orexin deficient children had cytoplasmic binding without preadsorption, only one was positive after preadsorption. Interestingly, of the seven orexin-normal adults positive without preadsorption (4x nuclear, 3x cytoplasmic), five were still positive after preadsorption (three cytoplasmic, two nuclear). These findings do not provide evidence for a consistent pattern of orexin neuronal binding in orexin-deficient children.

Fig 5.9

Fig 5.10 and 5.11

Following PALP preadsorption, intriguing binding patterns became apparent elsewhere in the coronal brain sections. Several sera clearly bound to the apical aspect ependymal cells in the third ventricle (**Figure 5.11 a**). Meanwhile other sera showed a blood vessel pattern of binding (**b**). Of particular note, serum 38, the Swedish post-vaccination child with normal orexin levels, showed a clear blood vessel binding pattern in the cortex that appeared similar to aquaporin-4 (**Figure 5.9, b**). However, AQP4 CBA testing by a colleague did not reveal positivity. Additionally, one NMDAR positive sera clearly bound to nucleoli (**b**). Most importantly, however, these binding patterns did not discriminate patient samples by age or orexin status, and were also observed in positive and negative controls.

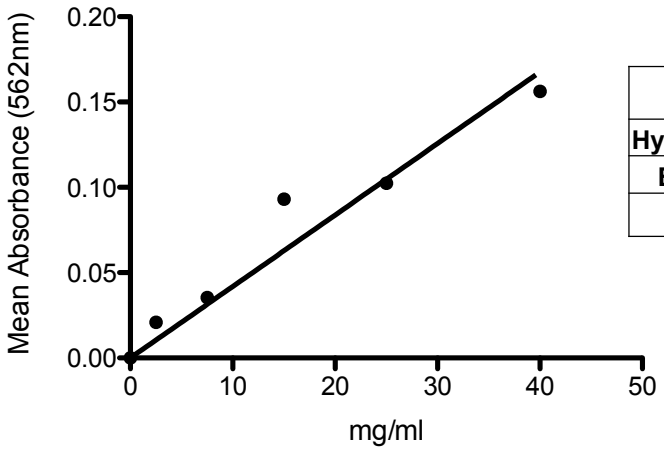
5.2. SDS-PAGE and western blot of brain extracts

Whole brain extracts (hypothalamus, all brain and brainstem) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose by western blot and probed with patient sera/commercial antibodies. SDS-PAGE and Western blot of organ extracts have previously been used to detect autorectivity in autoimmune patient sera (Hanson & Horowitz, 1992; Latif, Baker, & Dunn, 1993). Such methods are commonly used to confirm the presence of autoantibodies responsible for paraneoplastic neurological syndromes including Hu and Yo (Grisold et al. 1995; Hughes et al. 2010). Separation of brain proteins by SDS-PAGE results in a proteomic map that is based on size; autoantibodies commonly produce identifiable patterns of immunoreactivity, although it is well recognised that pathogenic antibodies might not react with denatured epitopes (Vincent et al. 2011). Anticipated band sizes of candidate autoantigens and positive controls have been summarised in **Tables 1.4 and 1.5.**

Method development

Following careful dissection of the relevant brain areas and homogenisation, protein concentrations of each extract were quantified by bicinchoninic acid (BCA) protein assay. By interpolating mean protein absorbance against a best-fit line of standard points, protein concentrations for each homogenate were estimated (**Figure 5.12**); all concentrations were

approximately 20 mg/ml. SDS-PAGE and western blot were then performed for each brain region, loading the same amount of protein each time.



Sample	Absorbance 512 nm
Hypothalamus	0.076
Brainstem	0.082
All brain	0.082

Figure 5.12: **Quantification of brain homogenate concentration by BCA**
Protein concentration of each brain homogenate was quantified by interpolation of absorbance (512nm).

Prior to serum screening, optimisation experiments were performed. These aimed to establish the optimal serum dilutions for screening and to determine the appearance of antibody binding patterns with commercial antibodies and with sera from patients with other autoimmune neurological diseases (**Figure 5.13, a**). A 1/200 serum dilution was chosen instead of 1/600; bands were observed in a healthy control patient and an NMDAR antibody positive control patient at both dilutions, although were clearest at 1/200. Disease control sera (NMDAR, Hu and Yo) were tested at 1/200 dilution. Hu and Yo (paraneoplastic antibodies) had distinguishable patterns.

Hu antibodies are characteristically identifiable by a broad band at around 40 kDa, as observed in all brain regions tested. Yo antibodies are usually against three proteins, sized 34, 52 and 62 kDa respectively, producing a characteristic triplet of bands (Hughes et al. 2008). For Yo sera, a triplet of bands was observed in the hypothalamus only, although the observed size was slightly smaller than anticipated. No strong bands were observed in the NMDAR antibody positive patient serum.

Fig 5.13

Of the commercial antibodies for candidate antigens tested ($GABA_AR\alpha3$, LGI1, CASPR2, Contactin2 and TRIB2), strong bands were only observed for $GABA_AR\alpha3$ and LGI1. Weak bands were observed with CASPR2 and Contactin2, and no bands at all for TRIB2. These bands were frequently observed at different molecular weights to those reported in the literature (**b**); different autoantibodies did not reliably appear to produce identifiably specific binding patterns as anticipated. This point was illustrated by probing for orexin in blots of brain extracts (**a**) versus blots of orexin peptide (**c**). Orexin is reported to be 3kDa in size. However in the brain extracts, only a 40kDa band was observed, which was strongest in the hypothalamus. Probing an orexin peptide blot with the same commercial antibody only revealed a 3kDa band.

Screening

Twenty randomised patient sera were screened simultaneously on the western blots of the three brain extracts at 1/200 dilution. 12/20 patient sera were from orexin-deficient children. The remaining sera were selected on the basis of prior CBA/I.F. positivity. Each serum was randomly assigned and incubated with the numbered strips from each brain region. Following DAB staining, strips were re-assembled into the original blots, dried and photographed for analysis. Contrast and brightness were equally increased to eliminate the weakest bands. Initial inspection of the strips was performed blinded. Following decoding strips were grouped according to age and orexin status; no bands were found to be consistently present in orexin-deficient children that were not also present in other groups (**Figure 5.14**). Bands were observed with almost all sera tested, suggesting that polyclonal IgG in human serum is commonly reactive to a number of neuronal epitopes. However, it did appear that the strips with the most intense bands (sera 44, 43, and 34) were generally from orexin-deficient children. Additionally, differential binding patterns were observed between brain regions. Using patient 34 as an example, a triplet of bands clearly visible in “All brain” and “Hypothalamus” was not present in “Brainstem”.

Despite the apparent lack of identifiable pattern, the molecular weights (kDa) of bands in

Fig 5.14

each strip were approximated to the nearest 5kDa by comparison with the adjacent molecular weight markers and potted against a $\log_2$ axis (**Figure 5.15**). Approximation of molecular weight became increasingly inaccurate with decreased migration into the gel, as gradations in the molecular weight markers exponentially increased. This analysis did not reveal any clear differences between orexin-deficient children and other groups. Any given band could correspond to a wide variety of proteins of a similar migration present in brain extracts.

Finally, total band intensity above background was calculated for each serum tested on the hypothalamus extract strips. This was achieved using ImageJ Analyse->Blots as used for Total IgG quantification and described previously in **Figure 3.3.** However, instead of calculating the area under the peak corresponding to 50kDa IgG heavy chain, the peaks corresponding to bands highlighted in **Figure 5.15** were quantified. This analysis confirmed observations apparent by visual inspection of the blots: the highest density of binding was observed with sera 44, 43, 34 and 8. **Figure 5.16** plots these findings. These four sera had a total binding density of more than one standard deviation above the population mean. Sera 44 and 8 had total band densities more than two standard deviations above the population mean. A table of prior findings in these sera is also included in the figure. All four of these sera were from children, three of whom had deficient levels of CSF orexin. Although Serum 8 was grouped as 'orexin-normal', with a level of 177 pg/ml levels were technically intermediate. High Total IgG levels were not consistently found in these sera, but all had prior positivity on either cell-based assay or immunofluorescence (see table in **Figure 5.16**).

Results for paediatric sera are summarised in **Table 5.2** and all adult serum results are included in **Appendix 6**

Fig 5.15 and 16

Table 5.2

5.3. Summary and discussion

This chapter reports the use of two immunological screening tools with which narcoleptic samples were tested. No consistent evidence of autoimmunity was found. Following studies of orexin and MCH neuron distribution, morphology and postnatal development in rats, all available sera and CSFs were screened for reactivity against antigens expressed in brain sections containing orexin neurons. High levels of background observed in the initial screen were successfully removed by preadsorption, although no consistent patterns of immunoreactivity were observed. Some sera were also tested on western blots of brain extract; binding was observed in most sera and no specific bands were identified with narcoleptic samples. However, three of the four sera with the greatest total binding were from orexin-deficient children. Had a consistent band been observed, N-terminal sequencing after further purification on, for instance 2-D gels, could have been used to identify the peptide responsible (Pankuweit, Portig, Lottspeich, & Maisch, 1997).

5.3.1. Indirect immunofluorescence (I.F.)

I.F. of rat brain sections is a well-established tool used to identify antibody binding. Although reported to be less sensitive than the antigen-specific CBA for AQP4 antibodies (Waters et al. 2012), I.F. is theoretically capable of detecting reactivity to any antigen expressed in the rat brain. Indeed, other diagnostic laboratories in the field of neurological autoimmunity routinely screen all patient samples on brain sections prior to testing with hypothesis-driven candidate antigen approaches (Lancaster et al. 2011). Specific binding patterns are reported for NMDAR, LGI1 and CASPR2 antibodies (Dalmau et al., 2008; Irani, Alexander, et al., 2010; Lancaster et al., 2011). No well-defined pattern has been reported for Contactin2. Several studies have reported immunoreactivity of narcolepsy patient serum to orexin neurons in rat brain tissue using immunohistochemical techniques (Cvetkovic-lopes et al., 2010; Knudsen et al., 2007; Overeem et al., 2006). However, the reported binding is far from convincing; none of these studies found the binding to be specific for orexin neurons. In the largest study (Overeem et al., 2006), which included 76 loosely-defined narcolepsy patients and 63 controls, ‘consistent’ lateral hypothalamic staining was reported in two patients but

also two controls. The I.F. screening of narcoleptic patients reported in this thesis addressed several of the limitations of the prior studies. Here, several of the samples were taken close to disease onset, after which levels of autoantibodies may subside. Secondly, none the prior studies have screened CSF samples. Finally, all prior studies used HRP-based immunostaining, a method widely agreed to be less sensitive than I.F. (Hughes et al. 2008).

The use of I.F. to screen samples does have several limitations. Firstly, using frozen brain sections, both intracellular and extracellular epitopes are exposed. However, as discussed previously, only extracellular epitopes are easily accessible to the immune system and therefore likely candidates of primary autoantibody attack. Intracellular patterns that one might expect to observe include anti-nuclear antibodies (ANA), reportedly present in 13.3% of adult healthy controls at 1:80 dilution assessed by I.F. (Tan et al., 1997). Additionally, as the cytoplasmic TRIB2 was reported in 21-26% of narcolepsy patients and 1-2% of controls, one might expect cytoplasmic patterns of binding in some patients. Indeed, prior to PALP preadsorption, cytoplasmic binding patterns were most frequently observed in orexin-deficient children, an observation which may be accounted for by TRIB2 antibodies, although this was not confirmed.

The use of rodent tissue to perform such staining is a contentious matter. Not all human autoantigens are present on rat tissue, and monkey tissue may be more sensitive. Additionally, although not reported in the brain, some have reported heterophile antibodies using rodent tissue (Hawkins, McDonald, & Dawkins, 1977). These are human antibodies that give false-positive staining patterns on animal tissues, and commonly titrate-out in excess of 1:1000. Despite these pitfalls, it is important to note that all recent antigen discoveries in the CNS have used rodent tissue as the first step (Graus et al., 2010). The use of monkey tissue is much more ethically and financially restricted, and presents issues of its own; endogenous monkey antibodies share 98% homology with human antibodies, often resulting in high levels of background fluorescence (Hughes et al. 2008). Also, ABO blood reactions may generate confusing patterns. Optimal fixation of sections prior to serum

screening was achieved with 4% formaldehyde solution. However, it is widely understood that the resulting covalent cross-linking masks or damages some antigens (Werner, Chott, Fabiano, & Battifora, 2000). Antigen retrieval methodologies can mitigate these issues, but were not used here.

In order to interpret the widespread binding patterns observed by I.F., a subjective scoring system was used as a semi-quantitative method of assessing binding in different brain regions. However, this scoring of non-specific patterns may not have been appropriate. In a further attempt to quantify the observed binding, end-point titrations were performed, although this was equally uninformative. Had any consistent I.F. patterns been observed, other quantitative methodologies would have been used, for example using ImageJ to calculate mean fluorescence above background.

In an attempt to reduce the non-specific patterns observed after the initial I.F. screen, PALP preadsorption was used. Although this appeared to work well for all positive control sera tested, reducing background whilst preserving specific signal, this may have not been the case for all antigens. This method relies on the assumption that no human neuronal-relevant autoantigens are also expressed in the pigeon liver. In any case, almost all orexin neuron binding observed in the initial I.F screen was abolished following preadsorption; meanwhile, other seemingly irrelevant blood vessel and ependymal patterns became apparent in several sera.

One possibility is that the putative autoantigen in narcolepsy is expressed outside of the hypothalamus, or at too low levels to be detected. Orexin neurons project most densely to the locus coeruleus and brainstem areas controlling REM sleep (Peyron et al., 1998). In retrospect, had sagittal sections been used, orexin cell bodies and their brainstem projections could have been assessed simultaneously.

Finally, no patterns of CSF binding were observed at 1:1 PBS dilution. Neat CSF was not tested in order to conserve sample. As described previously, the IgG ratios found in the orexin-deficient children were commonly around 1:100; in such healthy controls, screening CSF at a 1:1 dilution would correspond to a 1:200 serum dilution in terms of relative IgG concentration. These low IgG levels could partially account for the lack of binding observed.

5.3.2. SDS-PAGE and western blot

The main limitation of SDS-PAGE as a screening tool for autoantibodies is that prior to gel separation, proteins are denatured by boiling and β-mercaptoethanol and sodium dodecyl sulphate treatment, destroying tertiary structure. Antibodies for some conformational epitopes may therefore not be detected. While such methodologies are appropriate for detecting paraneoplastic antibodies such as Hu and Yo (Hughes et al. 2008), antibodies for NMDAR were not observed.

Even some of the commercial antibodies that are raised against linearised epitopes did not produce the anticipated binding patterns. For example, the anti-orexin A commercial antibody did not produce a 3 kDa as expected, but instead a 40 kDa band was present. A possible explanation for this is that whilst the orexin A concentration was too low to be detected in the brain extract, commercial antibodies non-specifically bound to another unrelated 40 kDa peptide. A more unlikely explanation is that the 40 kDa immunoreactive band may represent a precursor form of orexin or perhaps orexin associated with it's receptor. This would account for the band being most intense in the hypothalamus.

Almost all of the sera tested, including healthy controls, had clearly immunoreactive bands. This suggests that the majority of people have autoantibodies against denatured neuronal antigens. Although high levels sensitivity would generally be considered a good thing, clearly this makes interpretation difficult. The presence of bands in all samples tested could partially be accounted for by the lack of cell-surface antigen specificity of this technique; as with I.F., both intracellular and extracellular antigens would have been present on the western blot.

The majority of sera tested produced immunoreactive bands between 50 and 70kDa, further confounding interpretation of the blots. Each of these bands could represent one of hundreds of polypeptides around that molecular weight in rat brain extracts; unlike I.F., where antigens are arranged and concentrated two dimensionally, the one-dimensional separation of peptides by size resulted in substantial overlap in peptide bands. Had a consistent pattern of band immunoreactivity been observed in narcoleptic samples, molecular weights would have been more accurately estimated by measuring the distances of the bands and plotting these against logarithmic measurements of the molecular weight markers. Additionally, two-dimensional gel electrophoresis could have been performed using isoelectric focusing; two peptides are unlikely to be similar in size and isoelectric point.

Given the absence of any consistent band, total band intensities were quantified. Although this method was not specific for any particular antigen, it did yield interesting results. 3/4 of the highest total band densities were found in post-vaccination narcoleptic children. This finding perhaps provides evidence of increased autoreactivity in these patients against neuronal epitopes, even though band density did not correlate with total IgG levels.

6. Preliminary data: Neuronal cultures

Neuronal cultures were attempted as a final method to assess antibody binding in narcoleptic sera. It was hoped that orexin neurons themselves could be cultured, and effects of narcoleptic serum on live neurons assessed. Unlike I.F. screening on frozen brain sections, serum was applied to intact, live neurons prior to formaldehyde fixation, a cross-linking process that may disrupt some antibody-antigen interactions and expose non-pathogenic intracellular antigens.

6.1. Primary hippocampal cultures

The laboratory routinely uses primary (dissociated) cultures of hippocampal neurons to test for autoantibody binding (Irani, Bera, et al., 2010). Prior to attempting orexin-containing hypothalamic cultures, hippocampal cultures were first used to test narcoleptic sera. Hippocampal neurons express a wide variety of neuronal antigens commonly targeted in neurological autoimmune diseases, including the VGKC related LGI1, CASPR2 and Contactin2 as well as NMDA receptors. Following fixation and permeabilisation, a MAP2 commercial antibody was used as a marker; healthy live neurons strongly express this antigen. Positivity is confirmed by assessment of the degree of serum/MAP2 colocalisation. As for I.F. screening, a subjective scoring system (0: no binding, 1= weak binding, 2=moderate binding, 3= strong binding) was used.

After checking the neuronal expression of candidate antigens with positive control sera (**Figure 6.1, a),** 50 sera were screened on hippocampal neurons, including 23 orexin-deficient children. Sera with prior CBA/I.F. positivity and controls were also included. Representative examples of patient binding are illustrated in **Figure 6.1, b.** No positivity was observed in adults or healthy controls **(c)**. 4/23 orexin-deficient children's and 3/5 orexin-normal children's sera bound to hippocampal neurons. Of the seven positive results, four sera had also been previously positive on CBA or I.F (**Figure 6.1 d**). This compares to 11/38 of all paediatric sera investigated. Positivity did not seem to differentiate between CSF

Figure 6.1 a, b

Figure 6.1 c, d

orexin levels. These results await confirmation by repeat experiment by a blinded colleague.

As an extension of the above hippocampal cultures, dissociated hypothalamic neuronal cultures were also attempted. Several approaches were tried, using neurons from different rat ages and with different culture conditions. Although live neurons were present on the coverslips after 7-14 days in culture (as they stained with MAP2), no orexin immunoreactive neurons appeared to be present. Due to the harshness of trituration steps required for primary cultures, neurons are necessarily harvested from P0 rats. However, as shown in prior I.F. experiments, orexin neuronal immunoreactivity did not appear until P8 (**Figure 5.3, a**). This could partly account for the lack of orexin neurons observed in these cultures.

6.2. Organotypic hypothalamic slice cultures

Orexin neurons have been successfully cultured in organotypic slice cultures of rat hypothalamus (Katsuki & Akaike, 2004). The main advantage compared to primary neuronal cultures is that damaging trituration steps are not required, preserving brain structure and neuronal synapses. This means cultures can be harvested from older animals in which orexin neuron immunoreactivity has had time to develop.

6.2.1. Method development

Initial attempts with P9 rats using a method adapted from multiple sources proved successful (Gogolla, Galimberti, Depaola, & Caroni, 2006; Katsuki & Akaike, 2004). After one week in culture, both orexin and MCH neurons were clearly visible, most densely populated around the fornix (**Figure 6.2**). By bisecting each coronal slice along the midline and culturing halves separately, test/control staining experiments could be conducted. Due to slice thickness, confocal microscopy was necessary, producing 'optical slices'. For all confocal imaging, the 'Smart Setup -> Best signal' model was adopted and settings calibrated. Pinhole size, exposure time and field size were kept constant, although offset and gain were adjusted prior to each photography session. Although this setup was slower than other image capture

Figure 6.2, 6.3, 6.4

models, this gave the best signal and minimised bleed-through between channels. A standardised methodology of counting neurons in a particular slice was adopted; by photographing two confocal microscope stacks immediately superiomedial and superiolateral to the fornix, the number of neurons in a 2.5 mm^2 area could be ascertained. **Figure 6.3** illustrates how neurons were counted in successive 5 μm depth intervals through each slice.

The viability of orexin and MCH neurons was assessed by simply counting the number of immunoreactive cell bodies present in the cultures. Although this approach assumes that these neurons only remain immunoreactive whilst still alive, it has been successfully used elsewhere to quantify orexin and MCH neuronal killing (Katsuki & Akaike, 2004). Other standard approaches to quantify cell viability include the use of nuclear exclusion dyes such as propidium iodide (PI); PI only enters dead, permeabilised cells, fluorescently staining nuclei red and leaving live cells unstained. PI was investigated as an alternative marker for orexin neuron viability, but was found to give inconsistent results; all neurons at the base of sections were PI stained (**Figure 6.4**), suggesting that they were not alive. However this was clearly not the case, as several immunoreactive orexin and MCH neurons were still present. It seems more likely that excess PI added in culture became trapped at the base of the thick sections, and was not sufficiently removed by the gentle wash steps performed prior to fixation.

NMDA killing of orexin and MCH neurons

Prior to looking at the effects of patient serum on organotypic cultures, the excitatory neurotransmitter *N*-Methyl-D-aspartic acid (NMDA) was used to assess whether this would cause orexin neuronal killing. Katsuki & Akaike, 2004 report that NMDA is selectively excitotoxic to orexin neurons. They claim that following 24-hour incubation of slice cultures with NMDA (10-100uM) and 72-hour post-incubation recovery, the number of orexin neurons was markedly decreased whereas MCH neurons were relatively spared. Repetition of this experiment did not yield the same results (**Figure 6.5**). Although there was a significant reduction in the percentage of orexin neurons present relative to respective control halves

Fig 6.5

following NMDA incubation, similar reductions were also observed for MCH neurons (**Figure 6.5, b)**. Neuronal morphology was also altered by NMDA treatment (**a**). Following NMDA incubation, remaining neurons appeared smaller with fewer processes. The non-specific nature of NMDA killing was further indicated by the significant reduction in slice thickness measured on the confocal microscope; slices treated with 100μM NMDA were 40% as thick as their control halves (**c**).

The inverse correlation of orexin and MCH numbers and increasing NMDA dose suggests that death of orexin neurons can be quantified in this manner. However, these findings do not concur with the findings of other groups; orexin neurons were
not found to be selectively vulnerable to NMDA cytotoxicity.

6.2.2. Serum binding and killing of orexin and MCH neurons

Aquaporin-4 positive patient serum and complement

Prior to using narcoleptic patient serum, antibody-mediated damage to organotypic cultures was first modelled with an established neurological autoimmune condition. Aquaporin-4 antibodies have previously been shown to result in astrocyte lesions when provided with a source of human complement in slice cultures (Zhang, Bennett, & Verkman, 2011). Astrocyte damage is commonly measured by loss of glial fibrillary acidic protein (GFAP) immunoreactivity, an astrocyte marker expressed in healthy cells.

Overnight incubation of slice cultures with aquaporin-4 antibody positive sera (1/100 final dilution) and a source of human complement (healthy patient serum, 1/10 final dilution) was found to result in widespread astrocyte lesions (**Figure 6.6**). Whilst clear GFAP staining was present in slices incubated with healthy control serum and complement, serum binding to blood vessels and loss of GFAP staining were observed with aquaporin-4 positive serum. These findings suggest that the observed loss of astrocytes was due to complement-mediated destruction via the alternative pathway.

Figure 6.6, 6.7

Paediatric narcolepsy patient serum and complement

Sera 51 and 38 were selected to be incubated with the slice cultures. These sera were chosen as they were both from post-vaccination children with narcolepsy-cataplexy. Whilst Serum 50 was from an orexin-deficient child, Serum 38 was from a child with normal orexin levels. Both of these sera had previously been positive on CASPR2 CBA and I.F.

In slice cultures, both sera appeared to bind to Orexin and MCH cell bodies in the hypothalamus, although serum from a healthy control did not (**Figure 6.7, a**). However, as previously observed in I.F. experiments, these sera also bound to other cells present. Above-background binding to orexin and MCH neurons was quantified using ImageJ for the two test sera and the healthy control (**b**). This confirmed the observation of increased binding in these sera. However, no significant differences were found between the level of binding in orexin and MCH neurons. Finally, incubation of slice cultures with serum and complement did not kill orexin or MCH neurons; in either case a similar number of immunoreactive neurons were present in both test and control halves (**c**).

6.3. Summary and discussion

Neuronal cultures were performed to assess the effects of narcoleptic serum binding in a live *in vitro* system. Preliminary screening of narcoleptic sera with dissociated hippocampal neurons were performed; paediatric but not adult serum were found to bind to live hippocampal neurons, results for which confirmation is eagerly awaited. Subsequent experiments sought to culture orexin neurons themselves for antibody testing. Attempts with dissociated hypothalamic cultures were unsuccessful, perhaps because the cultures were taken at P0 and rat orexin neurons only appear after P8. Therefore, a different approach using orexin-containing organotypic slice cultures from P9 rats was tried. Although orexin neurons survived for over a week in culture, it was only possible to test two sera in duplicate with this methodology, in which neuronal binding did not appear to be specific for orexin neurons. Furthermore, no loss of orexin neurons was observed following incubation of slice

cultures with patient sera and complement. To provide conclusive data, more sera would need to be tested, and more repeats performed.

Time constraints meant that hippocampal neuronal cultures could not be repeated. Nonetheless, these preliminary results seem to highlight hippocampal neuronal binding in 4/23 orexin-deficient children and 3/5 orexin-normal children. There are no prior reports of narcoleptic serum binding to hippocampal neurons, and if confirmed by repeat experiment, these findings would be important. However, they would raise several questions: Why were the orexin-normal children positive? Why did this not correlate with prior I.F. results? Why do these antibodies bind to hippocampal neurons, when the underlying pathology in narcolepsy is focally hypothalamic?

The primary limitation of these hypothalamic slice cultures was the complexity of the method. For each experiment, two weeks' work was required from brain harvesting to visualisation of binding, and cultures were easily and frequently damaged. High-risk steps included: 1. Vibratome sectioning, where coldness of the specimen was vital and attached dura frequently disrupted sections. 2. Transfer and wash steps, where the thin sections were easily damaged. 3. Culture, as infections were not uncommon. 4. Permeabilisation, if insufficient, meant that antibodies could not adequately penetrate the section and were not easily removed in wash steps, resulting in high background and low signal especially at the base of sections. For these reasons, only two sera were tested in this system.

To validate the method prior to testing with narcoleptic patient sera, prior organotypic slice culture experiments reported in the literature were repeated. Katsuki & Akaike, 2004 report that orexin neurons are selectively vulnerable to high doses of NMDA. Using the same conditions, NMDA was found to be cytotoxic to all neurons. It is possible that the range of NMDA dilutions tested was not large enough to demonstrate the reported differential effect. However, these experiments were successful in their primary aim; to demonstrate that orexin neuronal death could be quantified. Meanwhile, Zhang et al., 2011 found clear antibody-

mediated lesions in organotypic cultures following incubation of AQP4 positive patient serum and complement. Although astrocyte loss was successfully demonstrated, unlike narcolepsy, the cellular destruction in NMO is against neuroglia rather than neurons themselves. An additional limitation was that a AQP4+, complement – control was not included, which would be necessary to confirm that the damage observed was complement-mediated.

Subsequently, two narcoleptic sera were tested on the organotypic cultures, both from clinically validated post H1N1-vaccination narcolepsy-cataplexy children with prior positivity on other tests. These studies made the assumption that the putative autoimmune attack in narcolepsy is complement-mediated, and that orexin neuron death would occur within the short time course of the experiment. However, there are several other mechanisms by which antibodies can result in autoimmune disease, including direct functional block or stimulation and antigenic modulation. For example, NMDA receptor antibodies cause cross-linking and receptor internalisation (Hughes et al., 2010); the neurons themselves are not killed, but symptoms can be accounted for by a reduction of cell surface NMDA receptor clusters. In relation to narcolepsy, such an explanation does not seem likely, as the degeneration of orexin neurons themselves has been conclusively demonstrated (Thannickal et al., 2000). Alternatively, opsonised surface antibodies could potentially activate cytotoxic lymphocytes or macrophages. Subsequent perforin and granzyme-B degranulation or endocytosis could account for specific orexin neuron loss (Trambas & Griffiths, 2003). This hypothesis could potentially have been tested using these slice cultures with NK cells.

8. General discussion

This thesis aimed to address the question “Is narcolepsy an antibody-mediated autoimmune disease”. Investigations were performed on a relatively large cohort of sera and CSFs, which included samples obtained from recent-onset and post H1N1-vaccination paediatric cases. Although no consistent evidence of antigen-specific autoantibodies was found in the sera or CSF, there were some interesting findings suggestive of a disordered immune system in some samples, which will be the basis for future work.

In the first instance, this study focused on biomarkers of disease that might be found in samples investigated for narcolepsy and related cohorts. Raised CSF total IgG and CSF oligoclonal bands represent the production of antibodies in the CNS and are found in autoimmune, autoinfectious and demyelinating conditions (Dale & Brilot 2010; Tibbling et al. 1977). Although neither oligoclonal bands were found, nor serum total IgG levels raised in orexin-deficient children investigated in this thesis, CSF IgG levels were raised, as was the serum/CSF IgG ratio. Although the absence of paediatric CSF total IgG reference values hindered the interpretation of absolute levels, these findings may be evidence of neurological inflammation. The highest total CSF IgG levels were found in clinically validated narcolepsy cases who had received Pandemrix® vaccine. This might indicate that the Pandemrix® vaccination itself was responsible. Although no CSF samples were available in a cohort of healthy Pandemrix®-vaccinated children, serum total IgG levels were not raised; these findings could indicate that immune dysfunction in these patients was limited to the CNS rather than systemic disturbances triggered by Pandemrix®.

Recognised as a sensitive and specific narcolepsy biomarker, the loss of the CSF neuropeptide orexin A is thought to be closely linked to the pathogenic processes underpinning narcolepsy (Mignot, 2011). However, levels can also be disturbed in other autoimmune neurological conditions (Baba et al., 2009; Mignot et al., 2002; Tsutsui et al., 2012); in accordance with evidence from prior case reports, patients with CNS autoimmune

diseases were found to have significantly depressed but not deficient levels, although none met the diagnostic threshold for narcolepsy. This suggests that the orexin system and/or CSF flow are disrupted secondary to these conditions, although the selective orexin targeting seen in idiopathic narcolepsy-cataplexy seems unlikely. MCH is another neuropeptide that has been implicated in narcolepsy, and may even be responsible for some narcolepsy symptoms (Peyron et al., 2009). In this study, the highest CSF MCH levels were found in paediatric narcolepsy samples, which may reflect MCH disinhibition close to disease onset. However, unlike orexin A, which clearly delineates narcolepsy-cataplexy patients, MCH did not seem to be discriminatory biomarker. As such, it seems unlikely that CSF levels of MCH are of diagnostic significance, or indicative of a particular disease mechanism.

There are several plausible candidate antigens for a putative autoantibody attack in narcolepsy-cataplexy and some of these were tested. Previous studies have shown that the orexin system itself is not the target of autoimmune attack, as antibodies directed against orexin A and B peptides and receptors 1 and 2 were not found (Tanaka et al. 2006; Deloumeau et al. 2010). This study specifically sought to investigate extracellular receptors and associated proteins that are expressed on orexin neurons and/or implicated in other neurological autoimmune conditions with sleep disturbance. In the narcolepsy cohort, although a few low-titre positives were found, these were irregularly scattered between disease groups, and as such are unlikely to indicate a consistently targeted autoantigen. Of particular note, no antibody-positive narcolepsy samples were identified following testing with NMDA receptor or $GABA_A$ receptor α3 subunit, which seemed the most likely candidates. In a condition for which clinical, serological and pathological findings seem to be so closely allied, aetiology and pathogenesis of individual cases are likely to be similar across the disease. Therefore, one would expect a causative autoantibody for narcolepsy to be identifiable in the majority of orexin-deficient cases when samples are taken close enough to disease onset. These studies did not identify such an antibody, despite the availability of recent onset samples. Previously identified candidates not investigated in this thesis include TRIB2 (Cvetkovic-lopes et al., 2010; Kawashima et al., 2010; Toyoda et al.,

2010). Although anti-TRIB2 antibodies are reported in around one quarter of cases, they are unlikely to be of pathogenic significance, as these antibodies are directed against a ubiquitous cytosolic enzyme. A recent study identified the secreted protein insulin-like growth factor binding protein 3 (IGFB3) as another potential candidate, as it is involved in sleep regulation, expressed in orexin neurons, and is down-regulated in narcolepsy (Honda et al. 2009). Although no positive sera or CSF were identified when screened by western blot in Honda et al.'s study, a cell-based assay of IGFB3 would have been a more suitable method to test this antigen. Despite the possibility of new candidate antigens, the sporadic identification of antigen positivity reported in this thesis combined with the previous identification of TRIB2 are most likely autoimmune epiphenomena in narcolepsy-cataplexy patients rather than primary antibody-mediated events.

Indirect immunofluorescence immunohistochemistry (I.F.) and western blot of brain extracts are standard autoantibody screening tests that were preformed on the narcolepsy samples. Despite significant efforts to optimise methods, specific patterns of autoantibody reactivity remained elusive. Two other groups have previously reported the immunohistochemical binding of narcoleptic serum to orexin neurons (Knudsen et al., 2007; Overeem et al., 2006). However, similar to the findings of this study, binding was not specific to orexin neurons and was also reported in healthy controls. In this study, similarly confounding autoreactive bands were identified when screening brain extracts by western blot. Therefore, antibody-searching techniques that use rat brain may be limited where high background signal caused by irrelevant antibodies may obscure genuine pathological antibody binding patterns.

It is now widely understood that a repertoire of 'physiological' neurological autoantibodies exists, usually directed against cytoskeletal and nuclear components. Such autoantibodies may even perform beneficial regulatory roles; some human anti-myelin antibodies have been found to promote re-myelination by anti-apoptotic signalling (Howe et al., 2004). On the other hand, the presence of anti-brain antibodies in healthy controls does not preclude pathogenicity; other factors, such as blood-brain barrier integrity must be important. For

instance, high titres of pathogenic myelin oligodendrocyte glycoprotein (MOG) antibody from MS patients only caused demyelination in mice after sensitisation with myelin proteins to permeabilise the BBB (Litzenburger et al., 1998). Despite the lack of specificity of anti-CNS binding observed in this thesis, it is important to note that all recent discoveries of neurological autoantibodies have used rat brain tissue as an initial step; relevant autoantibodies may have simply not been present in these samples, or at too low a concentration to enable detection.

Neuronal cultures have previously been used to investigate antibody binding and their functional effects in other neurological autoimmune diseases (Dalmau et al., 2008; Irani, Bera, et al., 2010). However, there are no prior reports of their use in narcolepsy. In this study, preliminary data on hippocampal cultures seems most promising, as neuronal reactivity was exclusively identified in orexin-deficient paediatric samples. Curiously, this antibody binding was also seen in children with normal orexin levels. Although it seems unlikely in all cases, antibodies in some of these children could represent a narcolepsy-cataplexy disease time point that precedes the destruction of all orexin neurons and subsequent CSF orexin deficiency. However, testing one of these sera for orexin neuron-specific binding with organotypic slice cultures showed no evidence of specific orexin neuron binding or complement-mediated antibody destruction. These findings have not been repeated, and further work on more samples is required.

A time-honoured approach for establishing pathogenicity of autoantibodies has been the application of Koch's postulates, a set of four criteria borrowed from the field of microbiology (Koch, 1932). In relation to narcolepsy, Scammell 2006 proposes that analogous criteria would be: 1. The demonstration of autoantibodies in the majority of patients but not controls. 2. The identification of the antibody target to be orexin neurons or related structures. 3. Demonstrable benefit of immunomodulatory therapies. 4. Induction of narcolepsy-cataplexy following passive transfer of antibodies and active immunisation with antigen in animal models. In relation to criteria 1 and 2, no consistent autoantibodies have been identified by *in*

vitro studies, such as this one. Meanwhile, in response to criteria 3, clinical evidence from trials of immunomodulatory treatments is conflicting; whilst one case study reported the normalisation of CSF orexin levels following intravenous immunoglobulin treatment (Dauvilliers et al. 2009), others report no benefit even when administered close to disease onset (Knudsen et al., 2012). Finally, no passive transfer experiments have been reported with animal models. In other neurological autoimmune diseases, including Guillian-Barré syndrome, myasthenia gravis and Lambert-Eaton myasthenic syndrome, and the recently discovered NMDA receptor encephalitis, analogous criteria for causality have been completely or mostly fulfilled. Despite almost 30 years of searching, not one of the above criteria has been convincingly fulfilled for narcolepsy. The most compelling evidence of autoimmunity in narcolepsy remains the tight HLA association that first stimulated autoimmune speculation (Honda et al. 1983).

8.1. Conclusion

In conclusion, it remains unclear whether narcolepsy is an antibody-mediated autoimmune disease. In accordance with decades of largely fruitless searching in the field, this study did not find compelling evidence of consistently pathological autoantibody, even in recent onset and post-H1N1 vaccination samples. Although this thesis found some evidence of autoantibodies, these are perhaps most likely explained as secondary inflammatory epiphenomena. In the context of the growing body of largely negative findings when using conventional antibody-searching techniques, insight from novel methodologies is required, and alternative mechanisms of orexin neuronal death must be considered. The delineation narcolepsy aetiology remains a Holy Grail in the field of neuroimmunology.

8.2. Future directions

Increasingly, the focus of narcolepsy research has shifted away from thus-far unsuccessful antibody-searching techniques and has instead focused on finding an explanation for the HLA and TCR genetic associations. It is hoped that understanding these associations will

provide insight into the processes underlying disease susceptibility and provide aetiological clues.

Current aetiological hypotheses for narcolepsy have attempted to integrate the genetic associations with epidemiologically identified disease associations. Some of the first investigators to identify the association of H1N1 vaccination and narcolepsy were surprised to find that many post-H1N1 cases were also positive for ASO (Dauvilliers et al. 2010). Other groups have also identified an association with streptococcal throat infections (Aran et al., 2009). In a recent response to a letter published in *Sleep*, Dr. Mignot, the most eminent and respected investigator in the field, speculates that two factors are necessary for narcolepsy development (Mignot, Dauvilliers, & Montplaisir, 2011). Firstly, a streptococcal-induced molecular mimicry component could be mediated through antigen presentation by DQB1*0602/DQA1*0102 to a specific TCR idiotype. In this model, streptococcal autoantigens could cross-react with orexin neurons in an analogous situation to that in rheumatic heart disease (Church et al. 2002). Secondly, non-specific triggers of disease, such as strong vaccine adjuvants or infection could increase blood brain barrier permeability allowing antibody entry, re-activate dormant T cell clones, or induce brain-specific HLA interactions.

The integration of genetic and epidemiological findings with novel structural analyses is already proving an insightful approach to better understand the pathological roles of disease-associated MHC II polymorphisms (for a review, see Jones et al. 2006). For example, the association of coeliac disease with HLA DQ2 can now be explained by the superior ability of DQ2 to bind digested gluten peptides (Kim et al., 2004). In narcolepsy, comparison of the crystal structures of disease-associated DQB*0602 and protective DQB*0601 revealed intriguing structural differences (Siebold et al., 2004). Altered peptide binding between these two otherwise closely related MHC types seems to be central to the positive and negative associations. These findings could help in the identification of putative pathogenic peptides.

Ultimately, such an understanding may assist in the pharmacological blocking of aberrant MHC II-TCR signalling, which may underpin narcolepsy pathogenesis.

Around the time of the last H1N1 pandemic, the Spanish Flu of 1918, Von-Economo described encephalitis lethargica, a neurological disorder that presented with pharyngitis followed by severe sleep disturbances, movement disorder and neuropsychiatric signs (Von-Economo 1930). Since the 1920's, cases of the disease have been sporadic, and many now speculate an aetiological association between the disease and flu (Dale et al. 2004). Infrequent contemporary cases of the condition further suggest an autoimmune pathogenesis, with elevated ASO titres, CSF oligoclonal bands, and brain autoantibodies reported including those directed against NMDAR (Kiley & Esiri 2001; Dale et al. 2004; Dale et al. 2009). It is tempting to speculate that the recent rise in paediatric narcolepsy-cataplexy cases following H1N1 vaccination could be aetiologically related to the more symptomatically polymorphic encephalitis lethargica; narcolepsy-cataplexy may represent the tip of the iceberg of post-infectious neurological autoimmune pathologies.

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Appendices

Appendix 1: Solutions

All routine laboratory reagents was obtained form Sigma-Aldrich® and media for cell culture from GIBCO® unless stated otherwise.

Solutions

Lauria Bertoni (LB) media (1 litre)

- 10g Bacto™Triptone (BD Biosciences®)
- 5g Bacto™Yeast Extract (BD Biosciences®)
- 5g NaCl (Normapur, BDH)
- 1 litre final volume of deionized water
  Autoclaved

LB Agar (250ml)

- 2.5g Bacto™Triptone (BD Biosciences®)
- 1.25g Bacto™Yeast Extract
- 1.25g NaCl
- 3.75g Bacto™ Agar
- 1 litre final volume deionized water
  Autoclaved

1x Tris Borate EDTA (TBE)

- 27g TrisBase (0.9M)
- 13.75g Boric Acid (0.9M)
- 10ml 0.5M EDTA (0.01mM)
- 2500ml final volume deionized water

1% Agarose Gel

- 1g agarose
- 100ml final volume1x TBE (above)
  Microwave 2 minutes medium power, allow to cool in 50^0 water bath
- 5ul Ethidium Bromide
  Poured immediately into Gel chamber and allowed to set

Solubilisation Buffer (SB)

- 3.03g Tris-HCl (25mM)
- 1.19g HEPES (5mM)
- 109.54g sucrose (320mM)

  Made up to 1000mls reagent grade water, pH to 7.4 and kept at –20°C

Cell culture medium

HEK cell culture media:

- 500ml Dulbecco's Modified Eagle Medium (DMEM)
- 50ml Foetal Calf Serum (FCS)
- 5ml 100x antibacterial/antimycotic solution (PSA)

Cell-based assay wash medium:

- 500ml DMEM
- 2.3g HEPES (Sigma-Aldrich®)
  Made up sterile and 100ml aliquots for non-sterile use

Complete MEM

- 500ml 1x MEM medium
- 50ml Foetal Calf Serum (FCS)
- 5ml 100x PSA

Complete Neurobasal Medium (NB) for hippocampal primary cultures

- 500ml Neurobasal medium
- 5ml 100x PSA
- 0.5mM L-Glutamate
- 10ml 50x B27 supplement (Invitrogen®)

Organotypic slice culture medium:

- 50ml 1x MEM medium
- 25ml 1x HBSS
- 25ml Horse serum, heat inactivated for 30 minutes at 52^0
- Supplemented with 1ml 100x PSA
- 6.5mg/ml Glucose
- 2.3 mg/ml HEPES
- 0.5mM L-Glut

  Filter sterilized

Appendix 2: Commercial antibodies

Primary antibodies

Antibody	**Manufacturer and code**	**Dilution used** (IS= Immunostaining; IF= Immunoflurescence, WB= Western Blot)
Polyclonal rabbit anti-CASPR2	Abcam®	1/1000 (WB)
Monoclonal Mouse anti-GFAP (GA5) (Alexa Fluor® 488 Conjugate): 3655	Cell Signalling Technology®	1:100(IF)
Monoclonal mouse anti-MAP-2: M 9942	Dako®	1:1000(IF)
Polyclonal Rabbit anti TRIB2: T3572	Epitomics®	1/100 (IF), 1/200 (WB)
Polyclonal Rabbit anti-MCH : H-070-47	Phoenix Pharmaceuticals®	1:200 (IS) 1:400 (IF)
Monoclonal mouse anti-Cotactin 2 (F1): sc-376780	Santa Cruz Biotechnology®	1/200 (WB)
Polyclonal goat anti-Orexin A (C19): sc-31403	Santa Cruz Biotechnology®	1:100(IS), 1:300 (IF), 1:400 (WB)
Polyclonal rabbit anti-$GABA_A$ Receptor (α-3 subunit): G4291	Sigma-Aldrich®	1:200 (IF), 1:400 (WB)
Polyclonal goat anti-$GABA_A$ Receptor (α1 subunit): sc-31403	Santa Cruz Biotechnology®	1:100(IF), 1:300 (IF)
Polyclonal goat anti-$GABA_A$ Receptor (β1,2,3 subunits): sc28794	Santa Cruz Biotechnology®	1:100(IF), 1:300 (IF)
Polyclonal rabbit anti LGI1: PRS4531	Sigma®	1/200 (WB)

Secondary antibodies

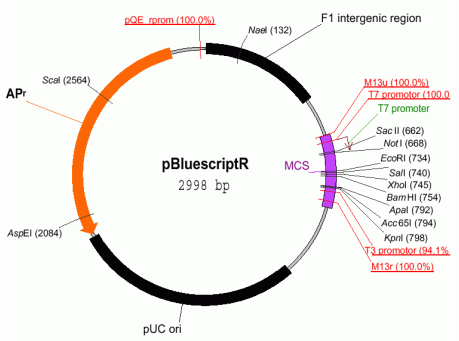
Antibody	**Manufacturer**	**Dilution used**
Polyclonal Rabbit Anti-Goat Immunoglobulins -HRP: P0449	DAKO®,	1/200(IS), 1/2000(WB)
Polyclonal Goat Anti-Rabbit Immunoglobulins –HRP: P0448	DAKO®	1:100 (IS), 1:2000(WB)
Polyclonal Rabbit anti-human-HRP: A0423	DAKO®	1/2000 (WB)
AlexaFluor 568 Goat anti-Human IgG (H+L): A21090	Invitrogen®	1/1000 (IF)
AlexaFluor 488 Goat anti-Human IgG (H+L): A11013	Invitrogen®	1/1000 (IF)
AlexaFluor 568 Donkey anti-Goat IgG (H+L): A11057	Invitrogen®	1/1000 (IF)
AlexaFlour 488 Donkey anti-Rabbit IgG (H+L): A21206	Invitrogen®	1/1000 (IF)
AlexaFluor® 633 Donkey anti-Goat IgG (H+L): A21086	Invitrogen®	1/1000 (IF)
DyLight™488-conjugated Affinipure Donkey Anti-Human IgG (H+L):709-485-149	Jackson ImmunoResearch Laboratories®	1/200 (IF)

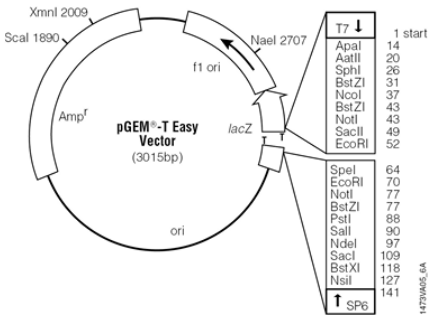
Appendix 3: Molecular genetics

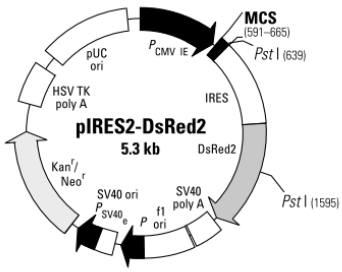
Composition of *in-house* plasmid constructs

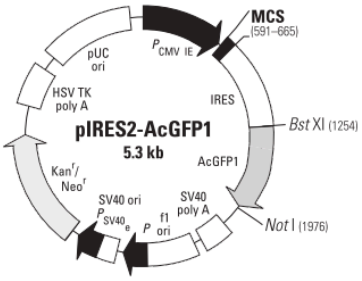
Construct	Vector	Antibiotic Resistance	Florescent tag
NMDAR (NR1/NR2b)	pcDNA3.1 (+)	Ampicillin	None
LGI1 (CASPR2 tail)	pcDNA3.1(+)	Ampicillin	eGFP (cytosolic)
CASPR2	pEGFP-N1	Kanomycin	eGFP (cytosolic)
Contactin2	pcDNA3.1(+)	Ampicillin	None

Vector maps for vectors used in molecular genetics (provided by manufacturers)

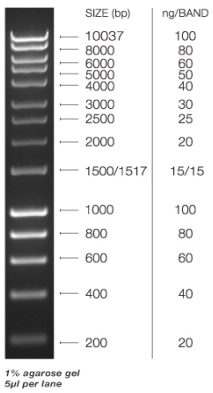






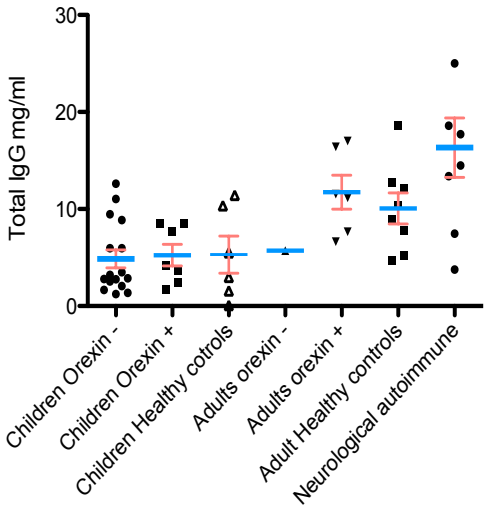


Hyperladder 1

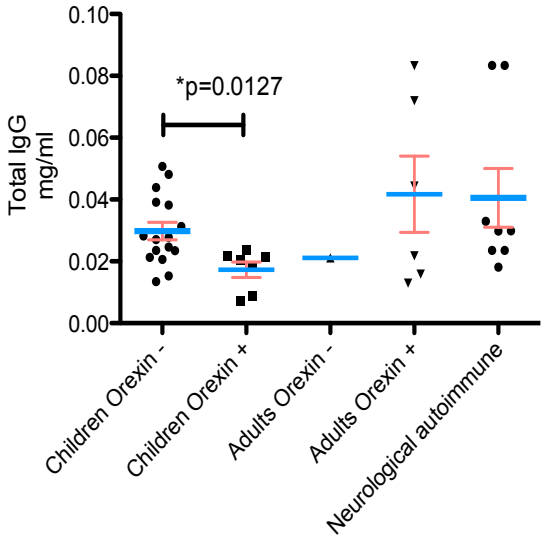


Appendix 4: Total IgG data in all cohorts

a: Serum



b: CSF



All total IgG Data

a: No significant differences were observed between serum IgG in age-matched groups. Increased total IgG observed in adults compared to children. The highest serum IgG was found in patients with autoimmune neurological diseases.

b: CSF IgG was significantly higher in orexin deficient compared to orexin normal children; in several cases, more than a two-fold elevation was observed. Meanwhile, there was wide variation observed in adult CSF IgG levels, a pattern mirrored in autoimmune disease controls.

c: Comparison of serum/CSF IgG ratio's for paired samples of orexin deficient children and orexin normal children also revealed significant differences; orexin deficient children appear to have more IgG in their CSF compared to serum.

Appendix 5: Summary of paediatric narcolepsy cohort data

		Clinical		Biomarkers					Candidate antigen approaches							Screening approaches				
				CSF		IgG (mg/ml)			Serum CBA score					Serum RIA		Serum I.F.				
	Cohort No.	Sex	Age	Orexin A	MCH	Serum	CSF	Serum/CSF IgG ratio	NMDAR 1:20	Caspr2 1:100	LGI1 1:20	CONTACTIN2 1:20	GABAaR α3 1:20	VGKC	GAD	Initial I.F., 1:100 hippo	Orexin co-localisation 1:300	PALP Preabsorbed orexin co-localisation 1:300	Hippocampal neuronal Cultures	Western blot total band density (hypo)
NDCN paired sera/CSF Orexin - Children	30	F	7	10	-	-	-	-	0	0	0	0	0	0	0	0			-	-
	18	F	8	10	-	-	-	-	0.5	0	0.5	0.5	0	0	0	0			0	-
	16	M	10	10	-	-	-	-	0	0	0	0	0	0	0	0			-	-
	33	M	10	10	-	-	-	-	0.5	0	0	0	0	0	0	0			-	-
	3	M	12	10	-	2.9	0.027	105.7	0	0	0	0.5	0	0	0	2n	1c	0	1	1027.6
	53	F	13	10	-	8.9	0.021	416.3	0	0	0	0	0	0	0	0			0	-
	29	F	14	10	-	-	-	-	0	0.75	0	0	0	0	0	1c			0.5	-
	57	M	16	10	-	9.5	0.023	402.7	-	-	-	-	0		0	0			0	-
NDCN paired sera/CSF Orexin + Children	13	F	5	363	-	1.7	0.018	94.3	0	0	0	0	0	0	0	1c			0	-
	55	M	5	288	-	8.5	0.009	979.7	-	-	-	-	0	-	-	0			-	-
	56	M	8	217	-	3.7	0.007	514.3	-	-	-	-	0	-	-	0			0.5	-
	8	F	12	177	213	4.2	0.020	207.3	0	0	0	0	0	0	0	2n	2n	0.5n	1	5746.1
	54	M	12	265	-	8.5	0.021	399.0	-	-	-	-	0	-	-	0			-	-
	12	F	13	301	-	7.7	0.022	351.8	0	0.5	0	0	0	0	0	0			-	-
	5	F	15	262	-	-	-	-	0	0	0	1.5	0	0	0	1n	1n	0	2	-
Swedish post-vaccination narcolepsy-cataplexy	36	M	4	10	-	6.0	0.028	211.0	0	0	0	0	0	0	0	0			0	-
	39	F	8	10	-	1.4	0.013	100.9	0.5	0	0	0.5	0	0	0	0			1	1879.6
	40	F	8	-	-	-	-	-	0	0	0	0	0	0	0	0			0	603.4
	37	M	9	52	-	11.0	0.031	352.9	0	0	0.5	0	0	0	0	0			0	723.5
	*38	F	9	290	-	2.4	0.024	101.0	0.5	1.5	0	1.5	0	0	0	1c	0	1c	2	1742.3
	41	M	11	10	141	1.7	0.051	32.6	0	0	0.5	0	0	0	0	0			0.5	-
	42	M	12	10	263	2.8	0.048	57.8	0	0	0	0	0	0	0	0			0	-
	44	M	14	10	-	12.6	0.038	330.4	0	0.5	0	0.5	0	0	0	0	2c	0.5c	0.5	5112.6
	35	F	15	10	133	3.5	0.021	169.6	0.5	0	0	0.5	0	0	0	0			1	-
	34	F	16	10	-	3.2	0.024	137.0	0	0	0	1	0	0	0	0			0.5	4257.5
	43	M	16	10	217	2.8	0.039	70.4	0	0	0	0.5	0	0	0	0	1.5c	0.5c	0.5	4364.4
Finnish post-vaccination narcolepsy-cataplexy	48	F	9	-	-	-	-	-	0.5	0	0	0	0	0	0	0			1	1119.3
	47	F	12	-	-	-	-	-	0.5	0	0	0	0	0	0	0			0	-
	49	F	13	-	-	-	-	-	0	0	0	1.5	0	0	0	0			0	-
	46	M	14	10	198	2.1	0.015	135.3	0	0	0	0	0	0	0	0			1	534.2
	51	M	17	10	298	1.2	0.028	44.4	0	0.5	0.5	0	0	0	0	1c	1c	1c	0	1974.6
	45	M	18	10	246	6.0	0.044	135.7	0.5	0.5	0	0	0	0	0	0			0	1545.7
	50	F	18	10	-	2.5	0.025	103.4	0.5	1	0	0	0	156	0	1c	1.5c	0.5c	0.5	1197.4
Children Heathy Control sera	HCC1			-		11.4			0	0.5	0	0	0	0	0	0	0	0	0	-
	HCC2			-		3.0			0.5	0	0	0	0	0	0	0.5n	0.5n	0	0	-
	HCC3			-		5.5			0	0	0	0	0	0	0	0	0	0	0	-
	HCC4			-		1.6			0	0	0.5	0	0	0	0	1n	1n	0	0	-
	HCC5			-		0.1			0	0	0	1	0	0	0	0	0	0	0	-

* Patient 38 was noted to be of particular interest: this was a Swedish post-vaccination case of clinically validated narcolepsy-cataplexy who interestingly had normal levels of orexin. Serum form this patent was positive for CASPR2 and Contactin2 CBA, appeared to have a weak cytoplasmic pattern of binding on I.F. and had moderate binding to hippocampal neuronal cultures.

N.B. Undetectable levels of CSF orexin (>40pg/ml) were reported as 10pg/ml for indexing purposes.
Abbreviations: CBA= cell-based assay; RIA= radioimmunoassay; VGKC= voltage-gated potassium channel; IF=immunofluorescence; hippo=hippocampus; hypo=hypothalamus. c=cytoplasmic binding pattern, n=nuclear binding pattern.

Appendix 6: Summary of adult narcolepsy cohort data

		Clinical Data		Biomarkers					Candidate antigen approaches							Screening approaches				
				CSF		IgG (mg/ml)			Serum CBA score					Serum RIA		Serum IF				
	Sample	Sex	Age	Orexin A	MCH	Serum	CSF	Serum/CSF IgG ratio	NMDAR 1:20	Caspr2 1:100	LGI1 1:20	CONTACTIN2 1:20	GABAaR α3 1:20	VGKC	GAD	Initial IF, 1:100 hippo	Orexin co-localisation 1:300	PALP Preabsorbed orexin co-localisation 1:300	Hippocampal neuronal Cultures	Western blot total band density (hypo)
NDCN paired sera/CSF Orexin - Adults	31	F	14	10	-	-	-	-		0	1	0	0	0	0	0			-	-
	11	M	24	10	-	-	-	-	0	0	0	0	0	0	0	0.5n			-	-
	23	M	27	10	-	-	-	-	0	0	0	0	0		0	0			0	-
	7	F	30	10	167	5.7	0.021	270.7	0	0	0	0	0	0	0	2.5n	1n	0.5n	0.5	2898.8
	26	M	30	10	-	-	-	-	0.75	0	0	0	0	0	0	0			0.5	1701.3
	1	M	40	10	-	-	-	-	0	0	0	0	0	0	0	0			-	-
	32	M	53	10	-	-	-	-	0	0.75	0	0	0	0	0	0			0	-
NDCN paired sera/CSF Orexin + Adults	20	M	19	199		7.7	0.022	351.8	0	0	0	0	0	0	0	0			-	-
	28	F	19	385	131	-	-	-	1	0	0	0.5	0	0	0	0			0	-
	14	M	20	387	162	-	-	-	0.75	0	0	0	0	0	0	0			-	-
	24	F	20	516	153	11.2	0.013	858.7	0	0	0	0	0	0	0	0			0	-
	27	F	21	343	-	-	-	-	0	0	0	0	0	0	0	2c	2c	1.5c	0	-
	25	F	23	250	144	-	-	-	0	0	0	0.5	0	0	0	0			-	-
	9	F	24	276	-	-	-	-	0	0.5	0	1	0	0	0	1n			0	
	22	M	28	287	-	-	-	-	0	0	0	0	0	0	0	0			-	-
	6	M	29	162	184	-	-	-	0	0	0	0	0	0	0	0			-	-
	2	F	31	223	183	16.4	0.072	228.1	0	0	0	0.5	0	0	0	2c	2c	2c	0	1627.0
	10	F	33	300		11.5	0.016	727.8	0	1	0	1	0	0	0	2n	2c	1.5c	0.5	568.6
	52	M	33	300	-	-	-	-	-	0	0	0	0	0	0	0			-	-
	4	M	38	194	158	6.6	0.044	149.5	0	0	0	1.5	0	0	0	0	1.5n	1n	-	-
	15	M	43	333	194	17.0	0.083	204.1	1.5	0	0	0	0	0	0	1n	3n	0.5n	0	2039.3
	19	M	44	264	-	-	-	-	0	0	0	0	0	0	0	2n	2n	2n	0	-
	17	F	44	268	-	-	-	-	0	0	0	0	0	0	0	1n			0	-
	21	F	57	242	183	-	-	-	0	0.5	0	0	0	0	0	0			-	-
Known CBA Positive controls included in nrcolepsy	NMDAR	F	-	-		-			2	0	0	0		0		0	2n	2.5n	-	-
	VGKC	M	-	-		-			0	0	0	0		0		3c	3c	3c	-	-
	LGI1,VGKC	F	-	-		-			0.5	1	4	0		964		0.5c			-	-
	CNTN2, VGKC	M	-	-		-			0	0	0	2.5		414		0	1c	1c	-	-
	LGI1,VGKC	M	-	-		-			0	0	2	0		146		1c			0	-
Adult Heathy Control sera	HC1	-	-	-		12.1			0	0	0	0		0		0	2n	2n nucleoli	-	185.6
	HC2	-	-	-		5.3			0	0	0	0		0		0.5c	0.5c	0	0	-
	HC3	-	-	-		8.9			0	0	0	0		0		1n	1n	0	-	-
	HC4	-	-	-		4.7			0	0	0	0.5		0		1n	1n	0	-	2281.0
	HC5	-	-	-					0	0	0	0		0		0		0	-	-
	HC6	-	-	-		12.7			0	0	0	0		0		0	0	0	0	170.7
	HC7	-	-	-		7.8			0.5	0.5	0.5	1		0		0	0	0	0	-
	HC8	-	-	-		18.7			0	0	0	0		0		1n	1n	0	-	-
	HC9	-	-	-					0	0	0	0		0		1n	1n	0	0	790.4
	HC10	-	-	-		10.3			0	0	0	0		0		0	0	0	0	-

N.B. Undetectable levels of CSF orexin (>40pg/ml) were reported as 10pg/ml for indexing purposes.
Abbreviations: CBA= cell-based assay; RIA= radioimmunoassay; VGKC= voltage-gated potassium channel; IF=immunofluorescence; hippo=hippocampus; hypo=hypothalamus. c=cytoplasmic binding pattern, n=nuclear binding pattern.