The Autoimmunity Hypothesis in Narcolepsy

Moriah Thomason, Matt Huggins, Steve Beier, and Trent Watkins

Summary

Since the finding in 1983 that narcolepsy-cataplexy has a strong genetic association with a particular HLA class II haplotype, it has been hypothesized that most sporadic cases result from autoimmunity. Recent evidence indicates that the disease results from the death of the small number of hypocretin (hcrt)-producing cells in the lateral hypothalamus, strongly suggesting that an autoimmune attack against these cells is the most common cause of narcolepsy. We propose to validate this widely-held view with experimental evidence that will suggest potential autoantigens, provide an autoimmune mouse model, and show direct evidence of autoimmunity in narcoleptic patients.

Specific Aims:

1) To determine the potential autoantigens expressed in hypocretin-producing cells.
2) To develop a mouse model for autoimmune narcolepsy by expressing the HLA susceptibility haplotype in the appropriate humanized background and immunizing the mice with antigens from hcrt-producing cells.
3) To identify signs of autoimmunity against hypocretin-producing cells in human narcoleptics.

Background and Significance

Narcolepsy is a disease of unknown etiology characterized by excessive daytime sleepiness (EDS), episodes of muscle weakness (cataplexy) triggered by emotional stimulation, and abnormalities of rapid eye movement (REM) sleep (International Classification of Sleep Disorders, 1997). Narcolepsy has a profound influence on the quality of life and safety of affected individuals. Attacks are frequent and incapacitating (Goswami, 1998). It has an estimated prevalence of 20 to 60 per 100,000 in Western countries (Aldrich, 1992; Mignot, 1998; Thorpy, 2001). Men and women are affected equally. Age at onset lies between 15 and 30 years in the majority of patients. Excessive daytime sleepiness (EDS) alone or in combination with hypnagogic hallucinations and/or sleep paralysis is the presenting symptom in approximately 90% of patients (Guilleminault et al., 1974). Approximately 70% of patients have cataplexy, 30% have hypnagogic hallucinations, and 25% have sleep paralysis (Overeem et al., 2001).

Hypocretins

Recent evidence indicates narcolepsy results from the death of the small number of hypocretin-producing cells in the lateral hypothalamus (Siegel, 1999, Kilduff et al., 2000, Thannickal, 2000). Hypocretins, also called orexins, are hypothalamic neuropeptides involved in sleep and energy homeostasis. The Hcrt gene encodes two hypocretin/orexin peptides. They are type 1 and type 2, (or in the case of orexin) A and B, and are 33 and 28 amino acids long, respectively. Cells that produce hypocretin (Hcrt)/orexin (OX) are anatomically localized to the lateral region of the hypothalamus. (Sakurai et al., 1998).

The projections of the Hcrt cells are widely distributed in the brain and all levels of the spinal cord. Effects of Hcrt peptides are excitatory. Furthermore, they have neuromodulatory effects on both GABA- and glutamate-mediated neurotransmission in hypothalamic neurons (van den Pol et al., 1998). The most dense extrahypothalamic input is to the locus coeruleus (LC) (Peyron et al., 1998). Activation of LC neurons supports the idea that the Hcrt/orexin system might have a neuromodulatory effect on arousal states, and it has also been suggested that LC neurons play a key role in muscle tone (Wu et al., 1999). In addition to food intake regulation, energy metabolism and arousal state mediation, the hypocretin system has been implicated in neuroendocrine, cardiovascular and gastrointestinal control.

HLA Association

A genetic component of this disorder has been established in both humans and dogs. In humans, a strong association to human leukocyte antigen (HLA) class II has been established. Across diverse ethnic backgrounds, more than 85% of narcoleptic individuals share a specific HLA allele, HLA-DQB1*0602. This contrasts to presentation of this allele in only 12 to 38% of the general, non-affected population (Mignot, 1998).

The highly polymorphic HLA gene products are major histocompatibility complex (MHC) molecules responsible for the presentation of peptide fragments of antigens to the immune system. Almost all known HLA-linked diseases have been shown to be autoimmune in nature. This, taken with evidence that environmental triggers may be involved, has led to much speculation that narcolepsy is an autoimmune disorder (Mignot, 1998, Siegel, 1999, Thannickal, 2000). Because of the recent discovery of Hcrt system damage in narcolepsy, the autoimmune focus in this field has new impetus. We are now aware that a very specific cell type in an anatomically specific region of the brain is attacked in the human disease. Typical onset in the second or third decade of life makes it likely that this disease is the result of neurodegeneration. The link to HLA makes a very good case for an immune attack, potentially transient in nature, which targets this specific cell-type. However, there does not yet exist clear evidence for immune attack on the Hcrt receptors or cells in human narcolepsy (Hinze-Selch et al., 1998). The cause of the Hcrt cell loss associated with narcolepsy and the putative role of immune attack in this pathology remain to be determined.

Specific Aim 1: To identify potential autoantigens expressed by hypocretin-containing neurons of the hypothalamus.

Hypothesis:

Hypocretin-containing neurons express a unique antigen that specifically interacts with the HLA class II molecule DQB1*0602, allowing for presentation to the immune system that may stimulate an autoimmune attack.

Experiments:

Experiments designed to identify candidate antigens will utilize two transgenic mice in concert with two established peptide-identification schemes to identify potential autoantigens that are both uniquely expressed by hcrt neurons and contain epitopes capable of binding to DQB1*0602.

1) Hcrt-neuron ablation mice

Transgenic mice expressing the truncated Machado-Joseph disease gene product (ataxin-3) under the preprohypocretin promoter have been previously described (Hara et al., 2001). These mice lack hcrt-expressing neurons with no apparent effect on other cell types. Gene expression of these mice will be compared to w/t animals as described below.

2) GFP-expressing mice

Transgenic mice expressing GFP under the preprohypocretin promoter have recently been developed (E. Mignot, personal communication). Hypothalamic regions of these mice will be dissociated and subject to fluorescence activated cell sorting (FACS) to obtain a homogeneous population of hcrt-containing neurons. These neurons will be used in this and later specific aims.

3) Suppression subtractive hybridization (SSH)

The Clontech PCR-select™ kit will be used to created a subtracted cDNA library and identify novel, differentially expressed genes. Briefly, cDNA will be generated from total RNA isolated from whole hypothalamic regions of w/t and hcrt-neuron ablated mice (control). These libraries will be subject to *Rsa I* digestion and each library divided into two pools. Each w/t pool will have a different adaptor/primer sequence added and be allowed to hybridize with control cDNAs. The pools will be mixed and subject to a second hybridization, the addition of primers to bare ends and two rounds of PCR. cDNAs expressed differentially in the hcrt-containing neurons will, exclusively, undergo exponential amplification. Amplified cDNAs will be separated on agarose gels and sequenced.

4) TEPITOPE analysis

We will use a commercially available software package (TEPITOPE) to predict peptide-binding properties of the HLA-II allele DQB1*0602. This program uses extensive crystallographic and binding studies of HLA ligands for 25 alleles to generate a virtual matrix for the binding groove of the particular MHC-II molecule being studied. The matrix describes the contribution of nine independent pockets to the binding of peptides. Weighted sums of each pocket are then used to calculate binding probabilities for each of ~8000 nonamers. This data can then be used to search sequence databases for likely antigens.

Possible outcomes and interpretations

From the SSH, we expect to obtain a library of many cDNAs expressed differentially, or exclusively, in hcrt-containing neurons. This library will almost certainly contain a mix of novel and known genes products. The sequence binding data from the TEPITOPE analysis will be analyzed to obtain a 'best-fit' peptide motif.

Genes known to be expressed in other tissue will be removed from the SSH results and the remaining sequences will be searched for epitopes with characteristics matching those of peptides identified by the TEPITOPE program. This will provide a list of candidate antigens whose expression by

hcrt-neurons will be confirmed by RT-PCR of cDNA produced from FACS-sorted hcrt/GFP neurons (see GFP mice above). These experiments will result in a 'short list' of candidate autoantigens that can be used to supplement subsequent specific aims.

Problems and pitfalls:

It is possible that the list of candidates identified through the above procedures will be too large for efficient implementation into specific aims 2 and 3. There are other approaches that may serve to pare the list to a more manageable size. For example, a phage display library of random nonamers could be applied to a column with bound DQB1*0602. The sequences of binding peptides could then be used to refine the 'best-fit' epitope described above and reduce the number of candidate proteins.

The utility of SSH to this application lies in the ability to identify novel gene products as well as the lack of bias inherent to other schemes. However, if the protocol fails to provide reasonable data, we may be able to employ one of many other approaches. For example, cDNA microarrays or other differential display methods might provide increased sensitivity and/or a quantified measure of differential expression levels.

Specific Aim 2: Development of an autoimmune mouse model

A proven strategy for developing robust animal models of autoimmune disorders with known MHC-II linkages has been the introduction of these disease-associated human MHC-II alleles (HLA) into "humanized" transgenic mice, engineered for compatibility the human MHC-II molecule (see Fugger, 2000, for review). This approach has been used to design transgenic autoimmune models of rheumatoid arthritis, multiple sclerosis, and insulin-dependent diabetes mellitus. In each of these instances, mice expressing disease-associated MHC-II developed a more robust autoimmune response upon immunization with known autoantigens or co-stimulatory factors, than non-transgenic mice (Madsen et al., 1999a).

Hypothesis:

Mice expressing the HLA-DQB1*0602 allele in a humanized background will develop narcolepsy-cataplexy spontaneously at low incidence. They will be very susceptible to induction of autoimmune narcolepsy by immunization with antigens from hcrt-producing cells.

Experiments:

*1) Generation of mice transgenic for human MHC-II HLA-DQB1*0602*

For the human MHC-II transgene DQB1*0602 to function in the context of the murine immune system, two requirements must be met. First, the mouse T cell co-receptor cd4 must be replaced by human CD4 to fully stimulate T cell activation in response to human MHC-II antigen presentation. Second, because mouse and human MHC-II molecules are capable of forming heterodimers, the endogenous mouse MHC-II loci must be deleted. Mouse lines that carry each of these modifications have been established (Masden et al., 1999b; Killeen et al., 1993). These lines will be intercrossed, ultimately resulting in a mouse line transgenic for human CD4 and lacking endogenous mouse MHC-II($hCD4^{+/+}MHC\text{-}II^{null}$).

As a by-product of Cre-mediated deletion of endogenous mouse MHC-II genes, a loxP site remains present at the mouse MHC-II locus in MHC-II ^{null} mice (Masden et al., 1999b). We will use this preexisting loxP site to insert a single copy of a construct carrying the DQB1*0602 gene into the mouse MHC-II locus (Fugger, 2000). To provide a dimerization partner for the human DQB1*0602 protein, human DQA1*0102 will be expressed from the same construct. The benefits of this site-specific approach are two-fold: the expression of the MHC-II transgene will be driven by endogenous mouse MHC-II regulatory sequences and transgene expression will be free from positional effects.

Briefly, an integration construct consisting of the DQB1*0602/DQA1*0102 genes flanked by loxP sites matching the orientation found in the $MHC\text{-}II^{null}$ mice will be constructed. This construct will be microinjected into $hCD4^{+/+}MHC\text{-}II^{null}$ mouse embryos along with Cre recombinase protein required for

site-specific integration. Transgenic mice lines will be established using standard methods and confirming site-specific integration using PCR analysis.

Once mice lines expressing DQB1*602 have been established, they will be assessed for susceptibility to spontaneous and antigen-induced narcolepsy. We will use a continuous video tracking system established previously to monitor behavioral differences between potentially narcoleptic mice and control populations (Chemmeli et al., 1999). Mice that display behavioral signs of narcolepsy, such as abrupt cessation of motor activity, will be monitored more closely using EEG/EMG to confirm episodes of cataplexy, disruption of REM sleep, and to rule out confounding neuropathology, such as seizure. In addition, brain sections will be assayed by immunohistochemistry for the loss of hcrt-producing cells and the appearance of inflammatory markers, such as MHC-II and CD4, in the hypothalamus.

2) Autoantigen-induced narcolepsy

Transgenic mice will be challenged with potential autoantigens (in complete Freund's adjuvant) in an attempt to generate an autoimmune response and narcoleptic pathology in DQB1*0602 transgenic mice. Three sources of autoantigen will be tested for the ability to induce narcolepsy in the DQB1*0602 mouse line:

1) Protein extracts prepared from hypocretin-secreting cells, isolated by FACS-sorting of hcrt/GFP cells as described above.
2) Peptides identified in Aim 1 as potential DQB1*0602 binding partners.
3) Hypocretin peptide alone.

Potential outcomes and limitations

If the DQB1*0602 allele is sufficient to confer susceptibility to spontaneous or autoantigen-induced narcolepsy in these mice, it would support the hypothesis that autoimmunity plays a role in the etiology of narcolepsy. Once an autoimmune model of narcolepsy is established, autoantigen specific T cell receptors could be cloned from reactive T cells, facilitating the identification of hypocretin-associated antigens capable of triggering an autoimmune attack.

In the previously established mouse models of insulin-dependent diabetes mellitus, generation of an immune response required the co-expression of the costimulatory molecule B7. It is possible that expression of DQB1*0602 alone will not be sufficient to generate susceptibility to narcolepsy and that unknown costimulatory factors are required to generate an autoimmune response.

Specific Aim 3: Are there signs of autoimmunity against hypocretin-producing cells in narcoleptic patients?

Previous efforts have not shown signs of significant immune dysregulation in narcoleptic patients. Two major impediments have hindered those previous studies. Prior to the recent finding of hcrt-neuron death in narcolepsy, there were no putative targets, leaving researchers to look for general signs of inflammation. Secondly, patients were analyzed only when symptomatic, perhaps long after the putative autoimmune destruction of hcrt-neurons, when the immune response has returned to normal. Our approach addresses both of these issues.

Hypothesis:

Human narcoleptic patients will have circulating antibodies and memory T cells directed against components of hcrt-producing cells, while controls will show minimal reactivity.

Experiments:

1) Search for autoantibodies:

The first set of experiments is designed to find autoantibodies in the serum of narcoleptic patients and controls that are directed against hcrt-producing cells. Three simple, complementary approaches will

be used. In each case, the human serum will be used as a primary antibody probe against components of hcrt-producing cells.

For the ELISA, 96-well plates will be coated with a crude protein extract from FACS-sorted GFP/hcrt cells and then probed with human serum. In addition, the same experiments will be performed using candidate autoantigens identified in Aim 1 in order to provide greater sensitivity and specificity. For Western blot experiments, the crude lysates of FACS-sorted GFP/hcrt cells will be separated by electrophoresis and transferred to membranes for probing with the serum.

The third approach will use the human serum to immunostain cryosections through the lateral hypothalamus of human and rat brains. While less sensitive and quantitative, this approach may allow for detection of binding that only occurs in a more native form. Co-staining with a mouse anti-hypocretin antibody will confirm that the patients' antibodies are targeting hcrt-producing cells.

2) Search for autoreactive T cells:

The autoantibody experiments proposed above are simple and necessary, but they may fail to show evidence of an inactive, past autoimmune attack. Here we overcome that limitation by searching for memory T cells, cells that show evidence of a past attack on hcrt-cell antigens. We will take advantage of the fact the memory T cells, having been previously activated *in vivo* by Signal 1 (HLA-antigen-TCR) and Signal 2 (B7-CD28) no longer require Signal 2 for stimulation (Ashton-Rickardta, 1999).

Peripheral blood monocytes (PBMCs), which include all types of T cells and autologous antigen-presenting cells (APCs), will be isolated from the blood of narcoleptics and controls using Ficoll-Paque Plus. $2.5x10^5$ cells/well (96-well plates) will be stimulated with hcrt-cell antigens and proliferation will be determined by [^{3}H]thymidine incorporation. In half of the wells, anti-CD28 antibody will be added to prevent the co-stimulation (signal 2) required for naive T cell responses, so that only CD28-independent memory T cell responses will be observed (Lovett-Racke *et al.*, 1998). Three kinds of hcrt-cell antigens will be used: extracts from FACS-sorted hcrt/GFP-neurons, candidate autoantigens (Aim 1), and human lateral hypothalamus extract (Langevin and Iverson, 1980). Tetanoid toxin will be used as a positive control memory T cell proliferation, as nearly everyone has been immunized for tetanus.

Possible results and interpretations:

The most compelling result would be that narcoleptics have significant levels of antibodies and memory T cell responses directed against hcrt-producing cells, while control patients have little or no autoantibodies or T cell responses. This result would provide the first strong, credible evidence of autoimmunity in human narcolepsy.

Another possibility would be to find autoantibodies and/or reactive T cells in both patients and controls (or in anyone expressing HLA-DQB1*0602). This possibility might suggest that, as found for PLP in MS, everyone (or anyone with this haplotype) is capable of mounting an immune response against his/her hcrt-producing cells, but only some other genetic and environmental interactions can result in pathology (Burns *et al.*, 2001).

Problems and pitfalls:

It has been hypothesized that previous efforts to find autoimmunity in narcoleptic patients have failed, at least in part, because the autoimmune attack that destroyed the hcrt-producing cells was short-lived, so that antibodies are no longer being produced at the time of assay. The search for autoantibodies proposed here may face similar difficulties. One way to avoid this problem will be to concentrate the search for autoantibodies on young, recently-diagnosed patients.

The search for memory T cells may prove to be more effective, as it will provide current evidence of a past autoimmune attack. Since not all memory T cells persist in the absence of antigen, it again may be useful to concentrate on recently-diagnosed patients. While the method that we propose is the simplest, it may lack sensitivity, since the cells of interest constitute only a tiny portion of the PBMCs and there may be noise from unrelated proliferating T cells (*e.g.*, from mouse antigens and/or other antigens in our crude mixtures). Alternative, more-sensitive methods are possible and have been shown to produce

similar results in MS (Sholz *et al*, 1998). A combination of positive and negative selection can be used to dramatically enrich for $CD4^+CD45RO^+$ memory helper T cells. These cells can then be stimulated in vitro by APCs isolated from our humanized mice expressing HLA-DQB1*0602 (Aim 2).

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