

## Immunological and Genetic Aspects of Narcolepsy

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Narcolepsy has long been known to be associated with specific Human Leukocyte Antigen variants, and therefore expected to be an autoimmune disorder. For many years, it proved difficult to detect evidence of such a process, but recent studies are making progress on genetic susceptibility factors, as well as potential environmental triggers. New results are also emerging on the presence of specific autoantibodies in the disorder, as well as phenotypic and potential pathophysiologic overlap with other autoimmune disorders. These results are the focus of this review.

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### NARCOLEPSY

Narcolepsy is an increasingly recognized sleep disorder characterized by excessive daytime sleepiness, symptoms of dissociated/abnormal rapid eye movement (REM) sleep, disrupted nocturnal sleep, and cataplexy. Although daytime sleepiness is the most disabling symptom, significantly disrupted sleep at night results in a similar total amount of sleep over 24 hours compared to controls. Many of the hallmark symptoms of narcolepsy [sleep paralysis, hypnagogic hallucinations, sleep-onset REM periods (SOREMPs)] are also seen in the general population, especially in the context of sleep deprivation, however cataplexy (brief episodes of bilateral muscle weakness triggered by emotions) is highly specific to narcolepsy. The most typical triggers for cataplexy are joking, laughter, and to a lesser extent, anger. Attacks may vary in severity ranging from jaw sagging or head drooping, to knees buckling, or total body paralysis lasting up to several minutes, with consciousness preserved throughout the episode. The unifying feature of these symptoms is that they all reflect a fundamental dysregulation of normal transitions between sleep and wake. Disease onset is most often in childhood, peaking between 10 and 25 years of age, and once established, the disease is life-long. Recent work has resulted in substantial insights on key genetic factors contributing to susceptibility and how these relate to the immunological processes underlying the pathophysiology of the disease.

Narcolepsy-cataplexy occurs with a fairly consistent prevalence of approximately 0.02-0.03% in various Caucasian groups throughout the world, with a somewhat higher prevalence in Japanese (0.16%) and individuals of African descent, and a lower prevalence in Israeli Jews (0.002%).<sup>1,2</sup> These prevalence rates partially correspond with higher frequency of the susceptibility factor DQB1\*06:02 (discussed below). This factor has a high frequency in African Americans (38%) and lower frequency in Ashkenazi Jews (3-6%), although not in Japanese (12%). The prevalence of narcolepsy without cataplexy has been difficult to characterize, as the diagnosis lacks specificity (relying primarily on the presence of SOREMPs), and patients are likely to be underdiagnosed or misdiagnosed. Population based studies indicate that 1-3% of the population report sleepiness with multiple SOREMPs detected by mean sleep latency test (MSLT) testing.<sup>3,4</sup>

Familial occurrence of narcolepsy-cataplexy was reported in the initial 1887 report by Westphal,<sup>5</sup> and until recently, it has been considered to be a familial disorder. Many previously report-

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ed affected family members were instances of unrecognized obstructive sleep apnea, and it is now understood that narcolepsy is primarily a sporadic disorder, although one with strong genetic susceptibility factors. Approximately 8-10% of patients report another family member with narcolepsy/cataplexy, indicating that familial clustering occurs at low levels. The risk to first-degree relatives is 0.9-2.3%, which is low, but nevertheless 10-40 fold greater than in the general population.<sup>1,6</sup> Increased rates of narcolepsy-like symptoms (excessive sleepiness, hypnagogic hallucinations, sleep paralysis) are also seen in family members, but overall risk is difficult to quantify due to the high prevalence of these in the general population.<sup>1</sup> Twin studies in narcolepsy indicate a very low concordance rate. Among 21 published case-reports of monozygotic twins, only 25% were concordant for narcolepsy-cataplexy (32% concordance when narcolepsy either with or without cataplexy was considered).<sup>1</sup>

These family and twin studies support the action of genetic variants conferring disease susceptibility, as well as the need for environmental triggers to initiate disease. As described in detail in this review, several significant susceptibility loci have now been identified for narcolepsy, and likely underlie part of the familial risks for the disease. While a specific Human Leukocyte Antigen (HLA) allele is clearly involved (DQB1\*06:02, described below), this locus does not account for all of the increased risk observed in first-degree relatives.<sup>7</sup> Non-HLA susceptibility genes are therefore being sought in context of multiplex families, and as co-factors in sporadic narcolepsy. Multiplex families with narcolepsy occurring across multiple generations have been rarely identified, and 30% of these show no association with HLA DQB1\*06:02, suggesting the action of high penetrance non-HLA alleles.<sup>1</sup> The small size of these families together with their rare occurrence has precluded identification of these variants through traditional linkage studies. Recently, several non-HLA susceptibility genes have been identified through Genome Wide Association (GWA) studies of sporadic narcolepsy cases, and like HLA, these also have important roles in immune function.

### Animal Models of Narcolepsy:

#### The Role of Hypocretins

Narcolepsy with clear cataplexy has been repeatedly reported in dogs, and attempts to establish a canine model of the disease began in the 1970s. As in humans, canine narcolepsy is primarily sporadic, but following identification of multiple affected Doberman puppies from the same litter, as well as several affected Labrador retrievers, a breeding colony was successfully established at Stanford to study this recessive mutation.<sup>8</sup> Canine narcolepsy was found to be very similar to the human disorder. In both the sporadic and genetic forms, dogs exhibited typical cataplexy triggered by food or play, had decreased bout length for sleep and wake, and had short REM latency. Affected Dobermans, Labradors and Dachshunds, were found to carry mutations in the hypocretin receptor 2 gene following an extensive positional cloning effort.<sup>9</sup> The

three identified mutations each resulted in complete loss of receptor function and thus impairment of postsynaptic hypocretin neurotransmission. In parallel experiments, a narcolepsy phenotype (including sleep fragmentation, cataplexy-like episodes, and sleep onset REM periods) was also produced in prepro-hypocretin (alternatively named orexin) knockout mice.<sup>10</sup> This ligand gene encodes two highly related peptides, released by proteolytic cleavage and termed hypocretin-1 and -2. These peptides then activate two related G protein-coupled receptors called hypocretin/orexin receptors 1 and 2.<sup>11,12</sup> Hypocretin-receptor deficient rodents were created,<sup>13</sup> demonstrating that the hypocretin receptor-2 knockout animals, and animals lacking both receptors had a narcolepsy phenotype, but receptor-1 knockout animals have a very mild phenotype with only slight sleep fragmentation abnormalities. Additional transgenic models were later created to study the effects of loss of orexin/hypocretin-containing cells as opposed to selective loss of the neuropeptide.<sup>14,15</sup>

### Hypocretin Deficiency in Human Narcolepsy

The identification of hypocretin receptor defects in canine narcolepsy led directly to our current understanding of the pathophysiology in humans, by directing focus to the function of the hypocretin system. Studies of cerebrospinal fluid (CSF) revealed low or undetectable levels of hypocretin-1 peptide in the CSF of sporadic narcolepsy cases, and post mortem brains from narcolepsy cases had profound decreases in expression of preprohypocretin, and peptide content.<sup>16-18</sup> In situ hybridization showed a remarkably specific loss of hypocretin hybridization signal, with no apparent loss or damage to co-mingled melanin concentrating hormone expressing cells.<sup>17</sup> Subsequent studies of narcolepsy brain tissue indicated similar reductions in expression for genes co-expressed in hypocretin cells including dynorphin, Narp and IGFBP3, suggesting loss of the cells.<sup>19-21</sup>

Mutation screening was also performed to search for variants causing hypocretin system dysfunction, but as occurrence is typically sporadic, HLA-associated, and involved apparent loss of cells, studies focused on rare DQB1\*06:02 negative, and familial cases, which would be more likely to carry mutations. One mutation was identified, introducing a highly charged Arginine into the signal peptide of the preprohypocretin protein in a highly unusual case of early onset disease which was DQB1\*06:02 negative and manifested cataplexy at 6 months.<sup>17</sup> Functional analysis suggested abnormal trafficking of the mutant protein precursor, likely resulting in toxicity to the cells, which was supported by undetectable hypocretin-1 in the CSF. Multiple studies of typical DQB1\*06:02-positive sporadic cases of narcolepsy, using a variety of methods (sequencing of candidate genes, and GWA studies) have not revealed mutations or single nucleotide variants linked with hypocretin ligand or receptor genes predisposing to narcolepsy.<sup>17,22-25</sup> Thus, while defects in hypocretin neurotransmission underlie narcolepsy, this is not due to loss of expression or function of the hypocretin system loci, but rather to dramatic impairment or loss of the

-70,000 cells producing the peptide within the hypothalamus. As noted above, canine narcolepsy is highly analogous to human narcolepsy: the majority of identified cases are sporadic, and are associated with low CSF hcr-1 levels.<sup>26</sup> As three distinct hypocretin receptor 2 mutations have been identified in dogs, it is therefore somewhat mysterious that no such mutations have yet been identified in humans. HCRTR2-mutant narcoleptic dogs show typical cataplexy, but mice lacking HCRTR2 exhibit less cataplexy than hypocretin/ orexin ligand knockout mice, or mice lacking both receptors.<sup>13</sup> It may thus be that HCRTR2 mutations in humans result in a diminished phenotype. The HLA association (described in detail below), the highly selective pattern of hypocretin cell loss, and the lack of identified mutations point to an underlying autoimmune disease mechanism for narcolepsy.

### HLA GENE ASSOCIATIONS IN NARCOLEPSY

The HLA genes are located within an extended span of human chromosome 6p containing 239 annotated loci,<sup>27</sup> also referred to as the major histocompatibility locus (MHC). In addition to the classical Class I and II genes, the MHC region contains over a hundred non-HLA loci, many of which also have roles in the immune response. The critical role of specific class II HLA antigens in the development of narcolepsy was first noted in the Japanese population, when it was noted that 100% of cases had DR2 and DQ1, whereas this haplotype was found in only 30% of controls.<sup>28</sup> Class II HLA antigens are heterodimeric glycoproteins that are primarily located on antigen presenting cells (dendritic and phagocytic cells), and function to bind processed foreign peptides and present them to T cells by engaging the T cell receptor. The initial association noted in Japanese was rapidly confirmed in individuals of European descent<sup>29,30</sup> but was found to be more variable in African Americans, where there is greater haplotype diversity.<sup>31</sup> The core susceptibility locus was identified by analyzing high resolution HLA haplotypes in various ethnic groups, which revealed that DQB1\*06:02 is the most specific marker for narcolepsy in all ethnic groups. In Japanese and Europeans, the predisposing DQB1\*06:02 allele occurs together with DQA1\*01:02 on a haplotype with DRB1\*15:01. In African Americans, however, these DQB1 and DQA1 alleles are found on haplotypes with a variety of DRB1 alleles, particularly DRB1\*15:03, DRB1\*15:01, DRB1\*11:01 and DRB1\*08:06.<sup>32,33</sup> The specificity of DQB1\*06:02 versus DR2 in narcolepsy was again highlighted in a small study of Korean patients and controls, where two common DR2 haplotypes were identified (DRB1\*15:01, DQB1\*06:02 and DRB1\*15:02, DQB1\*06:01).<sup>34</sup> All cases of narcolepsy with typical cataplexy were DQB1\*06:02 positive, versus 24% controls, however from the perspective of DR2, the additional common haplotype reduced this ratio to 100% vs. 33%.

DQB1\*06:02 is clearly the most important factor predisposing

to narcolepsy, and is found in 90% of narcolepsy cases. Nevertheless, the allele is quite common, ranging in frequency across ethnic groups from 12% in Japanese to 38% in African Americans, and is therefore not sufficient for the development of the disease. Furthermore, the association between narcolepsy and DQB1\*06:02 is particularly strong only in cases with typical cataplexy, and much decreased, or absent in cases without cataplexy.<sup>35-37</sup> Less than 1% of cases with low hypocretin levels are DQB1\*06:02 negative, suggesting disease heterogeneity among DQB1\*06:02-negative subjects, and those without cataplexy.

Indeed, it is not clear whether the DQB1\*06:02 allele acts alone, or in combination with other HLA variants to confer susceptibility, although additional HLA gene variants certainly modulate the DQB1\*06:02 effects. The genes encoding DQB1 and DQA1 (encoding DQ $\beta$  and DQ $\alpha$ , respectively) are located adjacent to each other within a 20 kb segment of the Major Histocompatibility Complex (MHC) class II region on human chromosome 6. The protein products form a  $\alpha\beta$  heterodimer known as the HLA DQ antigen localized on the cell surface, which binds foreign or self antigens and presents these to the T cell receptor to effect either an immune response or tolerance. Not surprisingly, given the close proximity, the DQB1\*06:02 and DQA1\*01:02 alleles are in nearly complete linkage disequilibrium in all populations, making it very difficult to study independent contributions of the two genes, or whether they are actually required to act together to confer narcolepsy susceptibility. The DQA1\*01:02 allele occurs in conjunction with a variety of non-DQB1\*06:02 haplotypes, and these do not increase risk for narcolepsy, therefore DQA1\*01:02 by itself does not predispose to the disease.

There are clear dosage effects for HLA DQB1\*06:02, and additional HLA alleles have been identified that modulate narcolepsy risk in DQB1\*06:02 heterozygotes. Carriers of two DQB1\*06:02 alleles (homozygotes) have an additional 2-4 fold risk for narcolepsy compared to heterozygotes, and may also exhibit more severe symptoms,<sup>38</sup> thus the effect is not merely dominant/additive.<sup>7,39,40</sup> There are numerous alleles that increase predisposition, including DQB1\*03:01, DQA1\*06, DRB1\*04, DRB1\*08, DRB1\*11, and DRB1\*12.<sup>7,39</sup> The most prominent of these susceptibility alleles is DQB1\*03:01, which yields consistently high relative risks in all ethnic groups. Surprisingly, the predisposing effect was present on a wide variety of DQA1 haplotypes, suggesting that antigen binding activity may be specified by unique variations encoded by DQB1\*03:01 (independent of those present on the DQ $\alpha$  chain). Alternatively, susceptibility may result through a mechanism independent of DQ $\alpha$  DQ $\beta$  pairing.<sup>39</sup>

A series of HLA alleles have been identified that provide significant protective effects in DQB1\*06:02 heterozygotes, including DQB1\*06:01, DQB1\*05:01, and DQA1\*01 (non-DQA1\*01:02). Analysis of the structure of DQB1\*06:01 indicates that very minor changes in the peptide-binding pocket may determine disease risk, since this variant is structurally very similar to DQB1\*06:02, differing by only 9 amino acids. Three of these are located

at the T cell receptor binding surface, and 5 contribute to binding pockets. In a crystallographic study comparing DQB1\*06:02 vs \*06:011, the latter allele was predicted to have a significantly different hydrogen binding pattern in pocket P9, and a significantly reduced volume in the P4 pocket in DQ\*06:011. These would have substantial impact on peptide specificity.<sup>41</sup> Studies by several groups using different methods have reported protective effects of DQA1\*01:03-DQB1\*06:03,<sup>7</sup> most recently, through a genome-wide association study in narcolepsy.<sup>42</sup> It is notable that two of the most highly protective haplotypes contain DQA1\*01 alleles that are non-DQA1\*01:02 i.e. DQA1\*01:03/DQB1\*06:01, and DQA1\*01:01/DQB1\*05:01.<sup>39</sup> This has led to speculation that the protective effect may be mediated through DQA1 and DQB1 alleles encoding proteins that can pair with DQA1\*01:02 and DQB1\*06:02. DQ $\alpha$  and DQ $\beta$  chains from the same family are structurally very similar, and have been shown to form stable trans-heterodimers *in vitro*.<sup>43</sup> This indicates that both DQB1\*05 and DQB1\*06 can form heterodimers with DQA1\*01 subtypes since they all belong to the general DQ1 family, but not with other DQA1 alleles. According to this model, the DQA1\*01, non-DQA1\*01:02 allele encoded on the alternative chromosome, could pair with DQB1\*06:02, reducing the incidence of the pathogenic DQA1\*01:02-DQB1\*06:02 heterodimer through competition. Similar trans-pairing effects have been documented in the pathophysiology of celiac disease and type 1 diabetes, two other DQB1-associated autoimmune diseases.<sup>44,45</sup>

### Non-HLA, Non-HCRT Genes in Narcolepsy

Linkage analysis has been a very powerful method for identifying mutations in Mendelian disorders segregating in families, where successes usually depend on large numbers of families with many affected individuals across several generations. To date, linkage studies to identify susceptibility genes in familial narcolepsy have largely been disappointing, and suggested loci have not been replicated.<sup>46,47</sup> While these small and rare families have not yielded much information to date, future studies using genome-wide sequencing technologies such as whole exome sequencing, may yet provide a wealth of information. By contrast, GWA studies using high-density SNP arrays have been fruitful. The first narcolepsy susceptibility SNP identified through a genome wide study was identified in a Japanese case control design study.<sup>24</sup> The associated SNP, rs5770917, is located between CPT1B and CHKB on chromosome 22 (discovery set: 222 cases, 389 controls; replication in 159 cases, 190 controls. P values ranged from 1 to  $6 \times 10^{-4}$ ; OR = 1.79). This same SNP was also significantly associated with narcolepsy in a small sample of Korean narcolepsy cases ( $p = 0.03$ , 115 cases, 309 controls), and showed similar trends in cases of European and African descent. In two subsequent large GWA studies of narcolepsy cases and controls of European descent, the chromosome 22 SNP showed no association with narcolepsy susceptibility, although the variant has a very low minor allele frequency in this group (4%).<sup>25,42</sup> Interestingly, this

same SNP was associated with Essential Hypersomnia in another small Japanese sample (137 cases, 569 controls).<sup>24</sup> This is a heterogeneous disorder, which may include incompletely penetrant cases of narcolepsy cataplexy, as the rate of DQB1\*06:02 is increased. Both genes flanking the SNP are potentially interesting candidates as potential modulators of narcolepsy and REM sleep effects. CPT1B is a rate-limiting enzyme in long chain fatty acid  $\beta$  oxidation in muscle mitochondria, a pathway implicated in regulation of theta frequency in REM sleep in mouse models.<sup>48</sup> Likewise, CHKB is a kinase involved in phosphatidylcholine synthesis- a precursor to acetylcholine which is a known regulator of REM sleep and wakefulness.<sup>49</sup> Although the minor allele frequency is very different between Japanese and Europeans, it is notable that this SNP was not associated with narcolepsy in a large sample from China (510 DQB1\*06:02 positive cases, 452 controls), where the allele frequency is similar to Japanese. The polymorphism also had no effects on clinical presentation of narcolepsy including severity of objective sleepiness.<sup>50</sup> These conflicting results could nevertheless represent genetic variation among populations, which could be clarified through replication testing of an additional set of Japanese cases and controls.

The T cell receptor alpha (TCRA) is now established as a significant and general susceptibility factor for narcolepsy, based on multiple replications and remarkably consistent odds ratios across studies. Three SNPs within the TCRA locus were found to be significantly associated with narcolepsy in a genome wide study of a large cohort of European descent ( $p = 1.9 \times 10^{-13}$ , OR = 1.87).<sup>25</sup> The discovery sample consisted of 1074 controls and 807 cases of European ancestry, all DQB1\*06:02 positive. The association was replicated in samples from multiple ethnic groups, with highest significance across individuals of European, African, and Asian (Japanese and Korean) descent at rs1154155. Of interest, the population attributable risk for rs1154155C in Caucasians is estimated to be 20%, but this rises to 43% in Asians, where the allele frequency is much higher, which may explain the increased prevalence of narcolepsy in Japan, despite the lower frequency of DQB1\*06:02 in this group.<sup>7</sup> This association has now been found repeatedly, and appears to be an important susceptibility locus in all groups. A second genome wide study in a large sample of European narcolepsy cases (DRB1\*1501 positive, thus presumed DQB1\*06:02+) and controls also observed a significant association for this rs1154155C (discovery + replication samples total 932 cases, 1449 controls;  $p = 5.3 \times 10^{-7}$  OR = 1.54).<sup>42</sup> Furthermore, the TCR alpha finding was also replicated in the Chinese cohort noted above, with rs1154155 showing a dose-dependent effect on susceptibility ( $p < 10^{-4}$ , OR = 1.47).<sup>50</sup> The polymorphism did not show an effect on the severity of daytime sleepiness in this cohort, although an increased age of onset for carriers was suggested (requiring follow-up in additional studies). This finding is similar to the effect of DQB1\*06:02 dose, where homozygosity dramatically increases risk for narcolepsy, with more variable results regarding effects on disease severity and age of onset suggested.<sup>38,40</sup>

The TCRA locus is an obvious biological candidate, as it encodes the alpha chain of the mature T cell receptor  $\alpha\beta$  heterodimer. This protein is localized on the surface of T cells and recognizes antigens bound and presented by class I or class II MHC molecules, and following engagement with the antigen-MHC complex, initiates an immune response. The T Cell receptor loci are highly complex, and undergo somatic cell recombination similar to the immunoglobulin loci to produce the myriad unique receptors in the T cell repertoire which undergo positive and negative selection to be capable of recognizing self MHC, yet not be autoreactive. The TCRA locus contains 46 functional variable (V) and 49 functional joining (J) segments, with the susceptibility SNP located in the J segment region.

In addition, to the associations in narcolepsy, the same SNP was significantly associated in the Japanese Essential Hypersomnia sample. The rs1154155C allele was significantly increased among patients that carried HLA-DRB1\*15:01-DQB1\*06:02 vs HLA matched controls ( $p = 0.008$ , 47 cases, 151 controls), but showed no signal among those that were HLA-negative.<sup>51</sup> As noted above, DQB1\*06:02 is increased in frequency in EHS. Together, the HLA and TCR findings suggest that this subset of EHS cases shares a similar genetic basis and autoimmune pathophysiology with narcolepsy cataplexy, likely representing an incompletely penetrant phenotype. DQB1\*06:02 is also at increased frequency in narcolepsy without cataplexy, where approximately 30-40% of cases carry the allele versus 25% in controls. This suggests that approximately 5-15% of these cases may have a hypocretin related pathology, a figure also consistent with CSF hypocretin-1 studies in this subgroup of patients. The HLA region also shows strong signal in GWA studies, although these were not remarkable in the study by Hallmayer et al due to the experimental design which used DQB1\*06:02 positive cases and controls. However, a significantly protective HLA region SNP located upstream of the DQA2 locus was identified in the genome wide study of European narcolepsy cases and controls.<sup>42</sup> The rs2858884 marker was associated with narcolepsy in the discovery sample ( $p = 4.65 \times 10^{-5}$ , OR = 2.1, 562 cases, 702 controls) and replication sample ( $p = 0.038$ , OR = 1.4, 370 cases, 495 controls). The highly protective effect of this SNP is partly explained by strong linkage disequilibrium with DRB1\*13:01-DQB1\*06:03. This DQ variant differs by only two amino acids from DQB1\*06:02, and in the context of DRB1\*13:01, is carried together with DQA1\*01:03, a haplotype with known protective effects. As discussed above, this may be mediated through DQA1\*01:03 trans-pairing with DQB1\*06:02.<sup>7,39</sup>

The precise mechanism underlying the associations of HLA and TCR genes has not been characterized, but rs1154155 or another polymorphism in tight linkage could influence the occurrence of potentially pathogenic VJ $\alpha$  recombinants. When present in context of the DQB1\*06:02/DQA1\*01:02-encoded DQ heterodimer, this very specific TCR idiomorph could then lead to further immune reaction ending in the destruction of hypocretin-producing cells. Together the HLA and T cell receptor associations

clearly demonstrate an autoimmune basis for narcolepsy, as unlike HLA, functional TCRs are only expressed in immune T-cells.

The P2RY11 purinergic receptor gene also plays a role in narcolepsy development, as noted in several studies. This susceptibility locus was initially identified through screening and replication of second-tier significance hits in a study of European and European/American narcolepsy cases and controls.<sup>25</sup> SNP rs4804122 was associated with narcolepsy susceptibility in the discovery sample (although not at genome-wide levels of significance;  $p = 5.92 \times 10^{-5}$ , OR 0.76, 788 cases, 1061 controls), and gave similar results in a Caucasian replication sample ( $p = 5.4 \times 10^{-4}$ , OR = 0.77; 603 cases, 907 controls). However, this marker was not significantly associated in a large sample of Asian cases and controls when tested for replication. The marker is located in a region of high linkage disequilibrium spanning the PPAN through DNMT1 loci on chromosome 19, and comparison of regional linkage disequilibrium suggested alternative markers that could be associated across ethnic groups. One of these, SNP rs2305795A located in the 3' untranslated region of the P2RY11 purinergic receptor gene, showed strong association with narcolepsy across the three ethnic groups tested [Caucasian, African American, Asian (Japanese, Korean);  $p = 6.14 \times 10^{-10}$ , OR 1.28; 2522 cases, 3167 controls].<sup>52</sup> Sub-analysis in the Chinese narcolepsy cohort also showed the same SNP, rs2305795A, was significantly associated in this population ( $p = 0.01$ , OR 1.29; 741 cases, 610 controls). This second study confirms the involvement of the locus in narcolepsy, despite the fact that the first SNP did not meet genome wide significance, and did not replicate outside the Caucasian ethnic group. There is still rather scant information on how this purinergic receptor contributes to narcolepsy susceptibility, because the Y11 form is present as a pseudogene in rodents, and thus little is known about the function of this protein. In humans, the gene is widely expressed, with pronounced levels in the brain and in white blood cells, both appealing targets in the pathophysiology of narcolepsy.<sup>53</sup> Purinergic signaling plays a fundamental role in immune regulation, modulating proliferation, chemotaxis and apoptosis in a variety of lymphoid cell types. P2RY11 is expressed in CD8+ T cells and NK cells, and the A allele of rs2305795 is associated with reduced P2RY11 expression in selected cell populations, and is also associated with reduced ATP-induced apoptosis of immune cells. This locus could therefore mediate narcolepsy susceptibility through a variety of mechanisms including modulation of immune response to an infectious trigger, modulation of an autoimmune process, mediation of neutrophil chemotaxis, or potentially through direct apoptotic effects on hypocretin cells.

## HLA ASSOCIATIONS AND AUTOIMMUNITY

The binding and presentation of processed antigens by HLA

proteins is critical to the development of tolerance in the thymus (by deletion of self-reactive T cells) and to determination of an immune response in the periphery, and not surprisingly, the vast majority of autoimmune diseases have shown associations with HLA variants. In most cases, a series of predisposing and protective alleles at different loci have been identified, indeed the key alleles involved can be difficult to discern due to the complex and extensive linkage disequilibrium patterns of the MHC region. The identification of the tight association with DR2/ DQB1\*06:02 immediately suggested an autoimmune mechanism for narcolepsy, which is also consistent with the peripubertal pattern of onset, and low rates of concordance in monozygotic twins. Notably, the DQB1\*06:02 allele shows the tightest of any HLA-autoimmune disease associations, being nearly ubiquitously present in narcolepsy, and conferring nearly absolute dominant protection against the autoimmune destruction of insulin-secreting pancreatic  $\beta$  cells in type I diabetes.<sup>6,54</sup> Some clinical features of narcolepsy are less typical of autoimmune disorders: females are not at increased risk, and it has been exceedingly difficult to find direct evidence of humoral or cellular autoimmune attack.<sup>6,55,56</sup> The first direct and replicated evidence of an autoimmune process in narcolepsy has been the recent identification of reactive autoantibodies against the Tribbles 2 homolog (TRIB2) protein in sera of narcolepsy cases.<sup>57-59</sup> In three independent studies, autoantibodies were found among 14-26% of DQB1\*06:02 positive cases with cataplexy, but were rare in cases without cataplexy and were found in 2-4% of controls. Difficulty in detecting such processes in the past may have reflected the typically long delay between disease onset and diagnosis, emerging only now that the diagnostic delay is becoming reduced. Nevertheless, it is not clear how these antibodies relate to the disease process, as there is no direct evidence that the antibodies injure hypocretin neurons, and TRIB2 expression is widespread in the brain and other tissues.<sup>60</sup> In addition, subsequent investigations have not continued to identify these autoantibodies.<sup>61</sup> Alternative models for the role of these antibodies could include hypocretin cell death (through another cause) causing the release of intracellular TRIB2 protein and leading to the production of autoantibodies, a process known as epitope spreading; both hypocretin cell death and production of TRIB2 antibodies could also be independent results of an inflammatory response; or finally, TRIB2 antibodies could be the result of an unrelated inflammatory response widespread in the population, but restricted to a timeframe in which more recent onset cases were identified and collected versus controls.

## POTENTIAL ENVIRONMENTAL TRIGGERS

The search for environmental triggers is currently a fast moving and exciting field, and recent studies indicate that upper airway infections may be key players. Studies repeatedly suggest a role for Streptococcal infections, and increased levels of streptococcal

antibodies (anti-streptolysin O) have been noted in patients compared to age-matched Caucasian controls when analyzing serum samples taken within 3 years of narcolepsy onset. This association then dissipates when studying samples from cases with longstanding disease.<sup>58,62,63</sup> In a questionnaire-based study, narcolepsy was also more common among people reporting a diagnosis of strep throat before the age of 21.<sup>64</sup> These recent findings support early reports of increased Streptococcal antibodies in narcolepsy which had remained controversial.<sup>62,65,66</sup> These findings are interesting in context of a recent study on the presentation of cataplexy very close to disease onset in pediatric cases, which appears to be somewhat different from cataplexy after the disease phenotype has become stable. In these incipient cases, cataplexy is more complex and manifests not only with "negative" motor features (partial/facial or generalized hypotonia, head drops and falls) but also with various additional "active" motor abnormalities ranging from facial grimaces, eyebrow raising, perioral/ tongue movements, to stereotypies, swaying of head and/or trunk, and dyskinetic/dystonic movements reminiscent of chorea.<sup>67</sup> Both negative and active behaviors were significantly associated with narcolepsy versus neurologically normal controls, and were enhanced in patients by emotional triggers, consistent with the classic definition of cataplexy. Active motor features were more prevalent close to disease onset, resolving within several years of onset, suggesting an overlapping movement disorder occurring close to narcolepsy onset in children, and then resolving with time. In this study, the active motor phenomena were also increased in patients with higher titers of ASO (> 400), which is of particular interest when considering commonalities of these active cataplexy features with Sydenham chorea, various tics, and PANDAS, which are considered to be post streptococcal autoimmune disorders of the central nervous system.<sup>68</sup> These all share an episodic course, present with motor and behavioral symptoms, and may disappear within several years. Indeed, cases of nascent narcolepsy in children have been initially misdiagnosed as Sydenham chorea, Huntington disease, and PANDAS. It will be of interest to determine whether choreic features of incipient cataplexy represent a post-streptococcal disorder.

Other emerging evidence indicates that exposure to antigens from the H1N1 subtype of influenza A virus may also trigger the disease. An apparent increase in recent onset narcolepsy cases was noted in major narcolepsy centers in France, Canada and the US following the 2009 H1N1 swine flu pandemic.<sup>61</sup> Thirty-one cases with DQB1\*06:02-positive narcolepsy/ hypocretin deficiency were identified and studied for past H1N1 exposure or vaccination. Two of the cases described developed narcolepsy following documented or likely H1N1 viral infection. Fourteen cases developed disease between 2-8 weeks of H1N1 vaccination, and 11 had received a vaccine formulated with an unusual adjuvant system containing squaline and alpha tocopherol (Pandemrix). There were several notable clinical features including rapid development of severe sleepiness and severe cataplexy, as well as atypical age at onset (5 cases over age 38, and 2 cases under age 5). Of

interest, eleven post vaccination cases showed high ASO titers suggesting recent or recurrent streptococcal infections, but none had detectable anti-TRIB2 antibodies. Two additional studies have followed the initial findings, reporting 9-fold and 6.6 fold increased risk of narcolepsy in children and adolescents aged 4-19 after immunization with the Pandemrix vaccine in Finland and Sweden, respectively.<sup>69,70</sup> These breaking results indicate that streptococcal antigens, and/or H1N1 antigens could potentially trigger narcolepsy in predisposed individuals, spurring active investigation to substantiate the link. The action of a specific peptide antigen in the initiation of narcolepsy is suggested by both the highly constrained DQB1 (and potentially DQA1) sequences, and the selected T Cell Receptor sequences that have been implicated. As such, pathologic H1N1 epitopes may be created from infection with the virus, or through an interaction of the adjuvant with epitopes present in the vaccine (that does not occur in the non-adjuvanted formulations). Alternatively, H1N1 could be a non-specific trigger, acting through a mechanism more related to the strength of the immune response than to specificity per se, as the adjuvanted formulation of the vaccine is associated with a stronger immune response. Likewise, streptococcus infection could act as a non-specific factor by leading to superantigen stimulation of dormant autoreactive T-cell clones. In addition, either of these factors could thus potentially act indirectly by increasing blood brain penetration.

## SUMMARY REMARKS

While long suspected to be an autoimmune disease, narcolepsy can now be formally considered as an autoimmune disease based the strong predisposing genetic variants within the HLA and TCR loci and the identification of increased levels of specific autoantibodies near disease onset. The typically sporadic occurrence of narcolepsy, together with studies in twins and rare families had previously demonstrated a complex pathophysiology for the disease, which would require environmental triggers acting on a susceptible (DQB1\*06:02 positive) background, or more rarely, the action of more highly penetrant gene variants acting through a DQB1\*06:02-independent mechanism. Recently, specific candidates for these antigenic triggers have been identified and are under active investigation to determine whether the effects are antigen-specific, or indirect. To date, highly penetrant genes have only been identified in exceptional cases, as in the single detected HCRT mutation, however additional non-HLA/ non-HCRT narcolepsy loci are likely to be identified in the future through whole genome sequencing, or exome sequencing studies of rare families segregating narcolepsy per se, or in conjunction with a spectrum of features (for example narcolepsy/cerebellar ataxia/deafness<sup>71,72</sup>). These may prove to have modulatory effects on the presentation of the more prevalent autoimmune narcolepsy phenotype in conjunction with HLA and TCR factors. Additional susceptibility loci

such as P2RY11 are already emerging, which appear to play a role in modulating the immune response but act outside the immunological synapse of HLA/antigen/T cell receptor. These are exciting functional candidates, and are under active study with respect to influence on immune function. In addition, recent results in the genetics of other autoimmune diseases have revealed combined effects of clusters of immune-related susceptibility genes implicating specific immune pathways leading to disease: these should be actively studied in the context of narcolepsy.<sup>73-75</sup> Finally, variants at HLA DQ, TCRA and other susceptibility loci may also prove to be useful in the identification phenotypic subsets in more heterogeneous disorders such as narcolepsy without cataplexy and hypersomnias, as has been done in Essential Hypersomnia, which are likely to share overlapping pathophysiology.

## Conflicts of Interest

The authors have no financial conflicts of interest.

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