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Common variants in *P2RY11* are associated with narcolepsy

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Growing evidence supports the hypothesis that narcolepsy with cataplexy is an autoimmune disease. We here report genome-wide association analyses for narcolepsy with replication and fine mapping across three ethnic groups (3,406 individuals of European ancestry, 2,414 Asians and 302 African Americans). We identify a SNP in the 3′ untranslated region of *P2RY11*, the purinergic receptor subtype $P2Y_{11}$ gene, which is associated with narcolepsy (rs2305795, combined $P = 6.1 \times 10^{-10}$, odds ratio = 1.28, 95% CI 1.19–1.39, $n = 5689$). The disease-associated allele is correlated with reduced expression of P2RY11 in $CD8^+$ T lymphocytes (339% reduced, $P = 0.003$) and natural killer (NK) cells ($P = 0.031$), but not in other peripheral blood mononuclear cell types. The low expression variant is also associated with reduced P2RY11-mediated resistance to ATP-induced cell death in T lymphocytes ($P = 0.0007$) and natural killer cells ($P = 0.001$). These results identify P2RY11 as an important regulator of immune-cell survival, with possible implications in narcolepsy and other autoimmune diseases.

Narcolepsy-cataplexy affects 1 in every 2,000 individuals and is primarily caused by the loss of around 70,000 hypocretin (hcrt, also known as orexin)-producing neurons in the hypothalamus[1,2]. The disease is associated with *HLA-DQB1*06:02* (ref. 3) and a *TRA@* (encoding the T cell receptor alpha) locus[4]. Further, autoantibodies against Tribbles homolog 2 (Trib2), a protein expressed in hcrt cells, have recently been detected in the sera of some individuals with recent-onset narcolepsy[5–7]. These findings strongly suggest narcolepsy is caused by an autoimmune attack on hcrt neurons. This disease may offer a unique model to study immune surveillance of neurons, a topic of growing importance.

Following on our recently published genome-wide association study (GWAS) of 807 individuals with narcolepsy-cataplexy (cases) and 1,074 *HLA-DQB1*06:02*–positive controls, we conducted replication of ten suggestive loci from this study in an additional 1,525 individuals of European ancestry (594 cases and 931 controls). We selected SNPs having $P < 5 \times 10^{-6}$ for replication. A total of 18 SNPs, representing ten genomic regions, met this criterion (**Supplementary Table 1**). Of these SNPs, only one, rs4804122, was significantly associated in the current replication study after Bonferroni correction for 18 markers ($P < 0.01$; see **Table 1** for nominal P value). This SNP is located downstream of *P2RY11* on chromosome 19 in a region of high linkage disequilibrium (LD) spanning several genes (*PPAN*, *P2RY11*, *EIF3G* and *DNMT1*; **Fig. 1**).

The last of these 18 markers is rs9275523, a SNP located between *HLA-DQB1* and *HLA-DQA2* ($P = 5.1 \times 10^{-6}$, odds ratio (OR) = 0.59, minor allele frequency = 0.071 in 799 cases and minor allele frequency = 0.116 in 1,068 controls). The association with this marker is consistent with prior data indicating that the genetic influences of HLA on narcolepsy predisposition are not mediated solely through *DQB1*06:02* heterozygosity[3]. *DQB1*06:02* homozygotes and *DQB1*06:02/03:01* heterozygotes are, for example, at higher risk for narcolepsy as compared to *DQB1*06:02* heterozygotes in general, whereas *DQB1*06:02/06:01*, *DQB1*06:02/05:01* and *DQB1*06:02/06:03* heterozygotes are at a decreased risk for narcolepsy[3,8]. Heterodimerization of DQA1*01:02 and DQB1*06:02 with other DQA1 and DQB1 alleles of the DQ1 group may account for these protective effects by reducing the abundance of the disease susceptibility DQA1*01:02/DQB1*06:02 heterodimer[9]. We therefore expected the finding of a secondary association in the *HLA-DQ* locus, and this finding further indicates a complex influence of *HLA-DQ* or other loci in high LD with *HLA-DQ* on narcolepsy. A recently published study also reported an association of a SNP located in the *HLA-DQA2* region, rs2858884, with narcolepsy, independent

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Table 1 Association of rs4804122 with narcolepsy

Cohorts (*n*)	MAF cases (*n*)	MAF controls (*n*)	OR (95% CI)	Nominal *P*	*P* (BD)
European ancestry					
Original sample (1,881)	0.383 (614)	0.468 (911)	0.71 (0.61–0.82)	3.69×10^{-6}	0.285
Replication sample (1,525)	0.391 (603)	0.453 (907)	0.77 (0.66–0.89)	5.42×10^{-4}	0.252
All (3,406)[a]	0.395 (1,391)	0.459 (1,968)	0.76 (0.69–0.84)	1.16×10^{-7}	0.173
Asians					
Chinese (1,269)	0.264 (582)	0.279 (681)	0.93 (0.78–1.10)	0.389	0.617
Japanese (869)	0.221 (424)	0.245 (432)	0.87 (0.70–1.09)	0.224	N/A
Koreans (276)	0.238 (105)	0.240 (169)	0.99 (0.66–1.49)	0.967	N/A
All (2,414)	0.245 (1,111)	0.262 (1,282)	0.91 (0.80–1.04)	0.172	0.894
African Americans					
All (302)	0.314 (113)	0.307 (189)	1.04 (0.72–1.48)	0.851	N/A
All samples (6,122)	0.328 (2,615)	0.377 (3,439)	0.83 (0.76–0.89)	1.03×10^{-6}	0.1568

MAF, minor allele frequency; BD, Breslow Day test (performed in combination with Mantel-Haenszel test of association).
[a]Including genotypes for 174 cases, 150 controls from original sample that were typed on the Affymetrix 500K Array Set.

of *DQB1*06:02* (ref. 8). In our initial GWAS sample of 1,881 individuals of European ancestry, rs2858884 had a nominal *P* value of 0.013, which was well below the association observed for rs9275523 (**Supplementary Table 1**).

Our next step was to attempt replication of the new chromosome 19 association in other ethnic groups. Notably, rs4804122 had no effect in a sample of 2,414 Asians and 302 African Americans (**Table 1**). This led us to explore differential LD patterns for this marker across ethnic groups. Based on data from the International HapMap Project, we selected five SNPs in high LD with rs4804122 in individuals of European ancestry but which were in lower LD with rs4804122 in Asians. We also genotyped one additional marker, rs3745601, a functional SNP located in *P2RY11* which was previously reported to be associated with myocardial infarction and elevated levels of C-reactive protein[10], even though this SNP is in low LD with rs4804122. We genotyped these six SNPs in 3,406 individuals of European ancestry (1,401 cases and 2,005 controls), 2,414 Asians (1,130 cases and 1,284 controls) and 302 African Americans (113 cases and 189 controls). A SNP located in the 3′ untranslated region (3′UTR) of *P2RY11*, rs2305795, showed the most significant association with narcolepsy across all ethnic groups (**Table 2** and **Fig. 1**). The rs2305795 association was significant in individuals of European ancestry ($P = 5.19 \times 10^{-8}$), in Asians ($P = 0.042$), and also in the combined study sample (combined $P = 6.1 \times 10^{-10}$, OR = 1.28). These associations remained significant after Bonferroni correction for the six fine-typing markers in the replication sample. The replication of this association across ethnic groups also illustrates the value of transethnic mapping in narcolepsy, as previously found in *HLA* and *TCR@* studies[3,4,11]. We found no significant interaction between rs2305795 and previously identified loci (data not shown).

To determine whether the presence of the disease-associated SNP was correlated with a significant change in the expression of any of the genes in the linkage region (**Fig. 1**), we quantified mRNA expression in peripheral blood mononuclear cells (PBMCs) of *PPAN*, *P2RY11*, *PPAN-P2RY11*, *EIF3G* and *DNMT1* in 60 individuals with narcolepsy and 56 control subjects of European ancestry (**Supplementary Table 2**). Expression of P2RY11 mRNA correlated strongly with rs2305795 genotype (52.6% lower expression with the disease-associated allele, $P = 0.002$; **Fig. 2a**) and sex (lower expression in females, $P = 0.039$) but not disease status, *HLA-DQB1*06:02* genotype, age or body mass index (BMI). The lack of effect of disease status is not surprising, considering the current narcolepsy-cataplexy model suggesting rapid hypocretin cell destruction with minimal residual immune response once the destruction is complete (a 'hit and run' hypothesis). A weaker correlation was also found with DNMT1 mRNA expression (lower expression with the disease-associated allele, $P = 0.029$). As expression of DNMT1 positively correlates with P2RY11 independently of rs2305795 ($r^2 = 0.48$, $P < 0.0001$), this effect is likely secondary. We found expression of the readthrough PPAN-P2RY11 transcript[12] to be very low

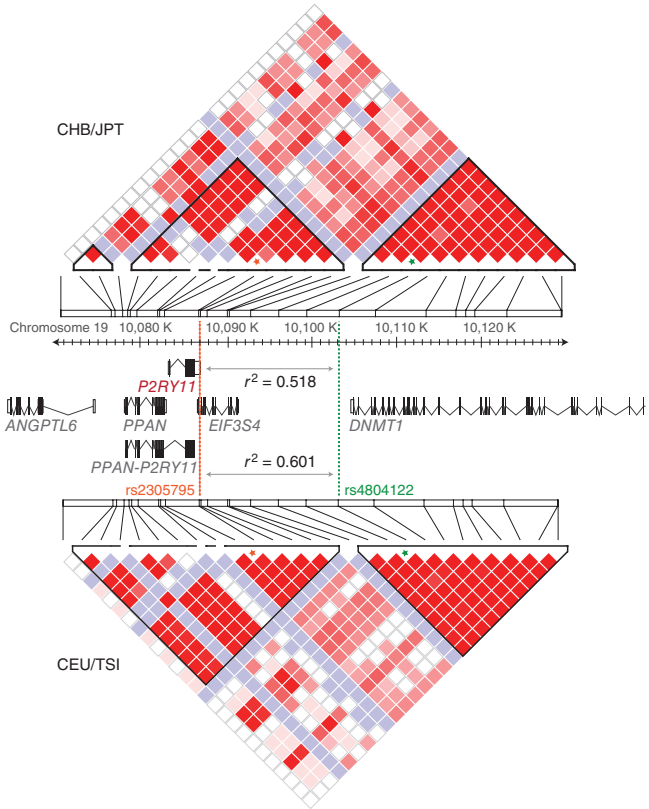

Figure 1 Risk locus on 19q13.2, showing gene organization and linkage disequilibrium in the region of interest (10,071,000–10,130,000 bp). At top, the *D′* and LOD-based LD plot using data from the combined Chinese and Japanese populations (CHB/JPT). Below, the *D′* and LOD-based LD plot for individuals of European ancestry only (CEU/TSI). We calculated *D′* values from the HapMap v3R2 CHB, JPT, CEU and TSI populations. In addition, r^2 values between the original marker, rs4804122 (green), and the best transethnic marker, rs2305795 (orange), derived from our own data are indicated. rs2305795 falls in the 3′UTR of P2RY11.

Table 2 Fine typing of SNP markers in the chromosome 19 region

Cohort (*n*)	SNP	Position (bp)	Associated allele	Freq cases (*n*)	Freq controls (*n*)	OR (95% CI)	χ^2 (MH[a])	P (MH[a])	*P* (BD)
	rs11666402	10,080,076	G	0.476 (1,313)	0.437 (1,816)	1.18 (1.07–1.31)	10.34	0.00130	0.933
	rs12460842	10,083,195	G	0.603 (1,283)	0.546 (1,593)	1.25 (1.12–1.39)	16.27	5.50×10^{-5}	0.338
	rs3745601	10,085,548	A	0.134 (1,216)	0.109 (1,587)	1.27 (1.08–1.50)	7.99	0.00470	0.061
European ancestry (3,406)	**rs2305795**	**10,087,052**	**A**	**0.608 (1,311)**	**0.542 (1,802)**	**1.33 (1.20–1.47)**	**29.64**	**5.19×10^{-8}**	**0.482**
	rs4804122[b]	10,102,944	T	0.605 (1,391)	0.541 (1,968)	1.32 (1.19–1.45)	28.09	1.16×10^{-7}	0.173
	rs11880388	10,114,573	A	0.536 (1,312)	0.496 (1,828)	1.16 (1.05–1.30)	9.068	0.00260	0.832
	rs2228611	10,128,077	A	0.538 (1,316)	0.497 (1,839)	1.18 (1.06–1.30)	9.345	0.00224	0.850
	rs11666402	10,080,076	G	0.421 (558)	0.406 (644)	1.03 (0.87–1.21)	0.093	0.7610	0.487
	rs12460842	10,083,195	G	0.758 (501)	0.721 (631)	1.20 (0.99–1.45)	3.549	0.0596	0.884
	rs3745601	10,085,548	A	0.885 (104)	0.712 (288)	1.38 (0.99–1.93)	3.646	0.0562	0.358
Asians (2,414)[c]	**rs2305795**	**10,087,052**	**A**	**0.728 (1,105)**	**0.689 (1,249)**	**1.20 (1.06–1.37)**	**8.209**	**0.00417**	**0.770**
	rs4804122[b]	10,102,944	T	0.755 (1,111)	0.738 (1,282)	1.10 (0.96–1.25)	1.862	0.1724	0.894
	rs11880388	10,114,573	G	0.590 (563)	0.579 (638)	1.09 (0.92–1.28)	0.897	0.3437	0.310
	rs2228611	10,128,077	G	0.594 (561)	0.577 (644)	1.11 (0.94–1.32)	1.637	0.2007	0.199
	rs11666402	10,080,076	G	0.273 (108)	0.256 (117)	1.09 (0.72–1.66)	0.162	0.6875	NA
	rs12460842	10,083,195	G	0.574 (95)	0.474 (116)	1.49 (1.01–2.17)	4.146	0.0417	NA
	rs3745601	10,085,548	A	0.122 (74)	0.121 (112)	1.01 (0.53–1.91)	0.00098	0.9749	NA
African Americans (302)	**rs2305795**	**10,087,052**	**A**	**0.684 (106)**	**0.621 (116)**	**1.32 (0.89–1.96)**	**1.952**	**0.1624**	**NA**
	rs4804122[b]	10,102,944	C	0.314 (113)	0.307 (189)	1.04 (0.72–1.48)	0.035	0.8514	NA
	rs11880388	10,114,573	G	0.491 (106)	0.430 (114)	1.28 (0.88–1.86)	1.632	0.2014	NA
	rs2228611	10,128,077	G	0.472 (107)	0.430 (115)	1.18 (0.81–1.72)	0.772	0.3795	NA
	rs11666402	10,080,076	G	0.449 (1,979)	0.421 (2,577)	1.14 (1.04–1.24)	8.482	0.00359	0.820
	rs12460842	10,083,195	G	0.643 (1,892)	0.600 (2,439)	1.25 (1.14–1.37)	22.31	2.32×10^{-6}	0.662
	rs3745601	10,085,548	A	0.313 (1,394)	0.290 (1,987)	1.28 (1.10–1.48)	10.87	0.00098	0.159
All samples (6,122)	**rs2305795**	**10,087,052**	**A**	**0.664 (2,522)**	**0.603 (3,167)**	**1.28 (1.19–1.39)**	**38.28**	**6.14×10^{-10}**	**0.727**
	rs4804122[b]	10,102,944	T	0.672 (2,615)	0.623 (3,439)	1.22 (1.12–1.32)	23.87	1.03×10^{-6}	0.157
	rs11880388	10,114,573	A	0.499 (1,981)	0.481 (2,580)	1.08 (0.99–1.17)	2.962	0.08524	0.133
	rs2228611	10,128,077	A	0.500 (1,984)	0.482 (2,598)	1.08 (0.99–1.17)	2.817	0.09327	0.096

[a]A Mantel-Haenszel (MH) test was performed on all cohorts except for the African American cohort (NA in the table) in order to account for diverse subgroups. Breslow Day (BD) test of heterogeneity results are presented in the context of MH testing. For ease of comparison of ORs, results for the associated allele (rather than the minor allele) are presented for each cohort. Most significant SNPs in each cohort are indicated in bold. [b]Original GWA SNP. [c]Differences in Asian genotype counts reflect samples genotyped at Stanford versus samples genotyped at Stanford combined with those genotyped in China.

(93.5% lower than P2RY11 expression) and to vary with sample storage conditions, and thus, we did not further analyze it. Gene expression levels of PPAN and EIF3G did not correlate significantly with rs2305795 or disease status (**Supplementary Table 3**). These results indicate that the A allele of rs2305795, the disease-associated allele, decreases P2RY11 mRNA expression in PBMCs.

P2RY11 is a member of a large family of more than 20 purinergic receptors. Purinergic signaling plays a fundamental role in immune regulation, modulating proliferation, apoptosis and chemotaxis in lymphocytes, monocytes and polymorphonuclear granulocytes[13,14]. Unlike most other purinergic receptors, P2YR11 is a low-affinity receptor and detects high concentrations of extracellular ATP. P2RY11 is unique among purinergic receptors, as it is coupled to both Gq and Gs, with its activation leading to increases in both cAMP and IP_3 (ref. 15). In healthy tissue, ATP is mostly localized intracellularly (millimolar range) and not extracellularly (nanomolar range). During inflammation, however, ATP levels rise in the extracellular space[16] and produce a cascade of concentration-dependent effects on the immune system. At lower concentrations, ATP induces immune-cell chemotaxis through the stimulation of P2Y2 and P2Y6 receptors[17–19]. High levels of ATP are typically cytotoxic, an effect mediated by the P2X7 receptor[20–23]. Because P2RY11 is a pseudogene in rodents, it has been difficult to study its function. P2RY11 expression is widespread and is pronounced in both human brain and white blood cells[24]. In neutrophils, stimulation of P2RY11 delays apoptosis[25], and P2RY11 has also been shown to inhibit the migratory capacity of monocyte-derived dendritic cells and $CD1a^+$ dermal dendritic cells[26]. In addition to the lack of *P2RY11* in rodents, functional studies have also been hampered by a lack of specific ligands. Most studies have used NF157, a partially selective antagonist[27]. Only recently have a more specific antagonist (NF340) and an agonist (NF546) been developed[28].

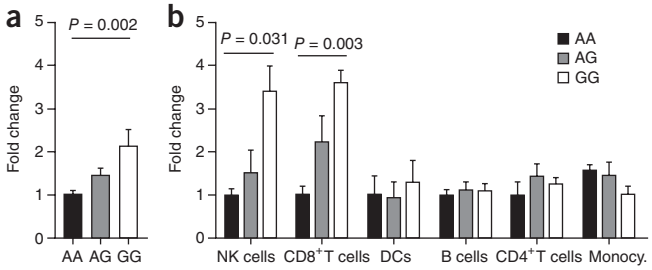

Figure 2 P2RY11 mRNA expression in PBMCs. (**a**) Expression in PBMCs from 116 subjects with various rs2305795 genotypes (mean + s.e.m., 60 cases and 56 controls; AA, $n = 49$; AG, $n = 51$; GG, $n = 16$). As we observed no direct effect of disease status on P2RY11 expression, subjects were grouped by genotype. The P2RY11 rs2305795 AA genotype resulted in a 53% reduction in P2RY11 expression compared to the rs2305795 GG genotype and was associated with increased risk of narcolepsy. (**b**) P2RY11 expression by rs2305795 genotype in various immune cell subsets (mean + s.e.m., $n = 7$–8 normal controls per genotype category). NK cells, $CD56^+$ natural killer cells; B cells, $CD19^+$ B cells; Monocy., $CD14^+$ monocytes; DCs, myeloid/plasmacytoid dendritic cells. Shown are Bonferroni-corrected one-way analysis of variance (ANOVA) *P* values.

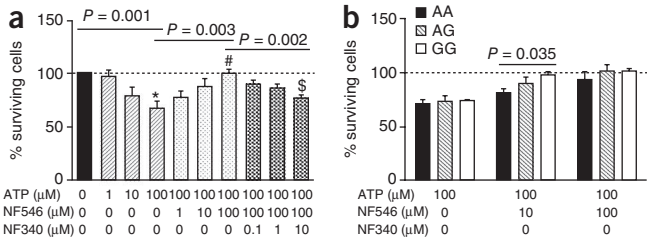

Figure 3 PBMC cell death induced by ATP was inhibited by the stimulation of P2RY11 and varied by rs2305795 genotype. (**a**) The effect of ATP on cell viability and the dose response of co-incubation with the P2RY11-specific agonist NF546 and the antagonist NF340 (mean + s.e.m., $n = 7$–8 rs2305795 AG control subjects). Overall one-way ANOVA P values are shown, with Tukey's post test. *, significantly different from control with no ATP, $P < 0.01$; #, significantly different from treatment with 100 μM ATP but no NF546, $P < 0.01$; $, significantly different from treatment with 100 μM ATP and 100 μM NF546, $P < 0.01$. (**b**) Effect of the rs2305795 genotype on the percent of cells rescued from ATP-induced cell death by P2RY11 stimulation. Ten micromolar NF546 has a less potent effect on cell survival after ATP-induced cell death with the rs2305795 AA genotype compared to the rs2305795 GG genotype. Heterozygote subjects fall in between. Mean + s.e.m., $n = 9$ subjects in each group.

To further explore how P2RY11 might regulate the immune system, we next quantified receptor expression in CD4$^+$ T cells, CD8$^+$ T cells, CD56$^+$ natural killer cells, CD19$^+$ B cells, CD14$^+$ monocytes and dendritic cells (myeloid and plasmacytoid subsets, that is, a combination of CD1c$^+$, CD141$^+$ and CD304$^+$ cells). P2RY11 expression has previously been shown to be higher in dendritic cells as compared to monocytes and CD4$^+$ T cells[29], but in that previous study, P2RY11 expression in CD8$^+$ and CD19$^+$ cells was not measured. We found that P2RY11 expression is widespread in immune cells but is notably higher in CD8$^+$ cells compared to dendritic cells (in which expression is 63.0% lower), CD19$^+$ B cells (65.5% lower), CD4$^+$ T cells (75.0% lower) and CD14$^+$ monocytes (84.8% lower). Further, the effect of the disease-associated allele, the A allele of rs2305795, on gene expression was apparent in both CD8$^+$ T cells and natural killer cells (72% and 70% reduction across genotypes; **Fig. 2b**) but not in other PBMC subtypes. The smaller genotype effect on expression in PBMCs (**Fig. 2a**) is consistent with a primary effect in CD8$^+$ T cells and natural killer cells, which represent roughly 25% of total PBMCs.

As changes in gene expression do not necessarily translate into functional effects, we next studied whether ATP and P2RY11 have genotype-dependent effects on immune cells. As previously reported[12], we found that increasing concentrations of ATP induce PBMC cell death (**Fig. 3a**), an effect likely mediated by P2X7 receptor stimulation. Using the recently developed P2RY11 agonist NF546 and the antagonist NF340, we further discovered that P2RY11 stimulation mitigates this effect, suggesting that immune-cell death in the presence of high ATP is controlled by a balance of activation of multiple purinergic receptors, including P2RY11. A similar survival effect of P2RY11 stimulation by ATP has been reported in neutrophils[24] and endothelial cells following natural killer cell–mediated killing[30]. Comparing cells with various rs2305795 genotypes, we found that the protecting effect of P2RY11 stimulation was less pronounced in subjects carrying the narcolepsy-associated, low expression rs2305795 A allele genotype, as demonstrated by the lower P2RY11-induced survival in PBMCs with this genotype (**Fig. 3b**). To determine whether these effects varied by immune cell subsets, we used fluorescence activated cell sorting (FACS) and found significant genotype effects in natural killer cells ($P = 0.001$), CD8$^+$ T cells ($P = 0.0007$) and CD4$^+$ T cells ($P = 0.009$), but not in monocytes or B cells (**Fig. 4**). This result is in line with the expression data reported in **Fig. 2b**, although we also found genotype-dependent effects in CD4$^+$ T cells, a population without P2RY11 expression differences, a finding possibly reflecting differential P2RY11 responses in various CD4$^+$ T cell subsets.

How could reduced P2RY11 function, associated with the rs2305795 A allele, be involved in narcolepsy susceptibility? Our results demonstrate clear effects of the polymorphism on immune-cell viability. A possible pathway may thus be modulation by P2RY11 of immune response to a potential infectious narcolepsy trigger, such as *Streptococcus pyogenes*[31], or a modulatory effect of the autoimmune process leading to hypocretin cell destruction. Although our results suggest a novel function for P2RY11 in T cells and natural killer cells, relevant effects on other cells not measured here are possible, if not likely. For example, P2RY11 induces thrombospondin-1 secretion and inhibits lipopolysaccharide-stimulated interleukin-12 (IL-12) release in monocyte-derived dendritic cells[32], an effect that could have a cascade of indirect effects on the immune system. Further, activation of P2RY11 on dendritic cells induces maturation and stimulates IL-8 release[33]. As IL-8 is an important mediator of neutrophil chemotaxis, modulation of the innate immune system could also be involved. Indeed, it has recently been shown that P2RY11 stimulation also modulates natural killer cell chemotaxis in response to CX(3)CL1 and CXCL12 (ref. 30). Finally, direct effects of P2RY11 on hypocretin cell apoptosis or microglial activation are also possible, as virtually nothing is known regarding localized expression and function of P2RY11 in the human brain.

In summary, we report on an association of rs2305795 in the 3′UTR of P2RY11 and narcolepsy. This receptor is highly expressed in CD8$^+$ T cells and natural killer cells and modulates immune cell viability. Additional studies of this receptor in narcolepsy and other autoimmune diseases are warranted.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

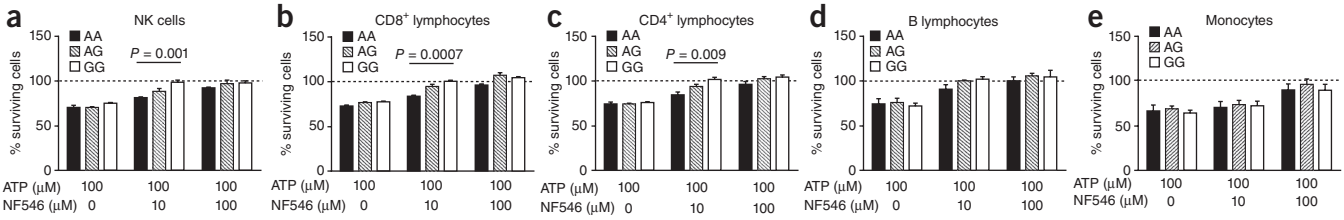

Figure 4 Effect of ATP and P2RY11 co-stimulation on different immune cell subtypes. PBMCs were co-incubated with 100 μM ATP and the P2RY11-specific agonist NF546 in different doses and the effect on different cell fractions was determined by FACS. We saw an effect from the rs2305795 genotype in T lymphocytes and natural killer (NK) cells but not in B lymphocytes and monocytes. Shown are mean + s.e.m., $n = 8$ per column and P values are from one-way ANOVAs. Dashed lines represent 100% survival.

Accession codes. mRNA sequences for genes in this study are deposited in the NCBI Nucleotide database under the following accession codes: P2RY11: NM_002566.4; PPAN: NM_020230.4; EIF3G: NM_003755.3; and DNMT1: NM_001130823.1.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

E.M., B.R.K., J.H. and N.R. designed the study with valuable input from R.C.A., H.K., L.S., K.T. and P.Y.K. B.R.K., M. Kawashima, L.L., S.H., R.C.A., H.K., K.W., J.L.E. and T. Miyagawa generated molecular data. A.H. and M.U.K. provided the P2RY11 agonist and antagonist. B.R.K., J.F., J.H., E.M. and N.R. participated in the data analysis. B.R.K. and E.M. wrote the manuscript. J.F., S.W., M. Kvale, D.F.L., N.R. and J.H. read and substantially commented on the manuscript. E.M., F.H., S.K., J.L., X.D., G.P., S.N., S.C.H., Y.H., M.H., B.H., J.M., P.B., D.K., Y.S.H., M.E., A.D., E.R., P.E.H., F. Poli, F. Pizza, B.F., J.H.J., S.-P.L., K.P.S., W.T.L., M.M.O. and P.J. contributed narcolepsy samples. T.J.R., J.W., T.G.N.T., M.D., G.T.N., H.-E.W., G.A.R., C.G., T. Meitinger, P.P. and T.Y. provided samples and/or genotypes. E.M. provided financial support.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Thannickal, T.C. *et al.* Reduced number of hypocretin neurons in human narcolepsy. *Neuron* **27**, 469–474 (2000).
2. Peyron, C. *et al.* A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat. Med.* **6**, 991–997 (2000).
3. Mignot, E. *et al.* Complex HLA-DR and -DQ interactions confer risk of narcolepsy-cataplexy in three ethnic groups. *Am. J. Hum. Genet.* **68**, 686–699 (2001).
4. Hallmayer, J. *et al.* Narcolepsy is strongly associated with the T-cell receptor alpha locus. *Nat. Genet.* **41**, 708–711 (2009).
5. Cvetkovic-Lopes, V. *et al.* Elevated Tribbles homolog 2–specific antibody levels in narcolepsy patients. *J. Clin. Invest.* **120**, 713–719 (2010).
6. Kawashima, M. *et al.* Anti-Tribbles homolog 2 (TRIB2) autoantibodies in narcolepsy are associated with recent onset of cataplexy. *Sleep* **33**, 869–874 (2010).
7. Toyoda, H. *et al.* Anti-Tribbles homolog 2 autoantibodies in Japanese patients with narcolepsy. *Sleep* **33**, 875–878 (2010).
8. Hor, H. *et al.* Genome-wide association study identifies new HLA class II haplotypes strongly protective against narcolepsy. *Nat. Genet.* **42**, 786–789 (2010).
9. Hong, S.C. *et al.* DQB1*0301 and DQB1*0601 modulate narcolepsy susceptibility in Koreans. *Hum. Immunol.* **68**, 59–68 (2007).
10. Amisten, S. *et al.* Increased risk of acute myocardial infarction and elevated levels of C-reactive protein in carriers of the Thr-87 variant of the ATP receptor P2Y11. *Eur. Heart J.* **28**, 13–18 (2007).
11. Matsuki, K. *et al.* DQ (rather than DR) gene marks susceptibility to narcolepsy. *Lancet* **339**, 1052 (1992).
12. Communi, D., Suarez-Huerta, N., Dussossoy, D., Savi, P. & Boeynaems, J.M. Cotranscription and intergenic splicing of human *P2Y11* and *SSF1* genes. *J. Biol. Chem.* **276**, 16561–16566 (2001).
13. Bours, M.J., Swennen, E.L., Di Virgilio, F., Cronstein, B.N. & Dagnelie, P.C. Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol. Ther.* **112**, 358–404 (2006).
14. Di Virgilio, F., Boeynaems, J. & Robson, S.C. Extracellular nucleotides as negative modulators of immunity. *Curr. Opin. Pharmacol.* **9**, 507–513 (2009).
15. Communi, D., Govaerts, C., Parmentier, M. & Boeynaems, J.M. Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.* **272**, 31969–31973 (1997).
16. Bodin, P. & Burnstock, G. Increased release of ATP from endothelial cells during acute inflammation. *Inflamm. Res.* **47**, 351–354 (1998).
17. Chen, Y. *et al.* ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* **314**, 1792–1795 (2006).
18. Elliott, M.R. *et al.* Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* **461**, 282–286 (2009).
19. Ben Yebdri, F., Kukulski, F., Tremblay, A. & Sévigny, J. Concomitant activation of P2Y(2) and P2Y(6) receptors on monocytes is required for TLR1/2-induced neutrophil migration by regulating IL-8 secretion. *Eur. J. Immunol.* **39**, 2885–2894 (2009).
20. Peng, L., Bradley, C.J. & Wiley, J.S. P2Z purinoceptor, a special receptor for apoptosis induced by ATP in human leukemic lymphocytes. *Chin. Med. J.* **112**, 356–362 (1999).
21. Aswad, F., Kawamura, H. & Dennert, G. High sensitivity of CD4+CD25+ regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2X7 receptors. *J. Immunol.* **175**, 3075–3083 (2005).
22. Aswad, F. & Dennert, G. P2X7 receptor expression levels determine lethal effects of a purine based danger signal in T lymphocytes. *Cell. Immunol.* **243**, 58–65 (2006).
23. Taylor, S.R.J. *et al.* Sequential shrinkage and swelling underlie P2X7-stimulated lymphocyte phosphatidylserine exposure and death. *J. Immunol.* **180**, 300–308 (2008).
24. Moore, D.J. *et al.* Expression pattern of human P2Y receptor subtypes: a quantitative reverse transcription-polymerase chain reaction study. *Biochim. Biophys. Acta* **1521**, 107–119 (2001).
25. Vaughan, K.R. *et al.* Inhibition of neutrophil apoptosis by ATP is mediated by the P2Y11 receptor. *J. Immunol.* **179**, 8544–8553 (2007).
26. Schnurr, M. *et al.* ATP gradients inhibit the migratory capacity of specific human dendritic cell types: implications for P2Y11 receptor signaling. *Blood* **102**, 613–620 (2003).
27. Ullmann, H. *et al.* Synthesis and structure-activity relationships of suramin-derived P2Y11 receptor antagonists with nanomolar potency. *J. Med. Chem.* **48**, 7040–7048 (2005).
28. Meis, S. *et al.* NF546 [4,4′-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1 (4-methyl-phenylene)-carbonylimino))-bis(1,3-xylene-alpha,alpha′-diphosphonic acid) tetrasodium salt] is a non-nucleotide P2Y11 agonist and stimulates release of IL-8 from human monocyte-derived dendritic cells. *J. Pharmacol. Exp. Ther.* **332**, 238–247 (2010).
29. Duhant, X. *et al.* Extracellular adenine nucleotides inhibit the activation of human CD4+ T lymphocytes. *J. Immunol.* **169**, 15–21 (2002).
30. Gorini, S. *et al.* ATP secreted by endothelial cells blocks CX3CL1-elicited natural killer cell chemotaxis and cytotoxicity via P2Y11 receptor activation. *Blood* **116**, 4492–4500 (2010).
31. Aran, A. *et al.* Elevated anti-streptococcal antibodies in patients with recent narcolepsy onset. *Sleep* **32**, 979–983 (2009).
32. Marteau, F. *et al.* Involvement of multiple P2Y receptors and signaling pathways in the action of adenine nucleotides diphosphates on human monocyte-derived dendritic cells. *J. Leukoc. Biol.* **76**, 796–803 (2004).
33. Wilkin, F. *et al.* The P2Y11 receptor mediates the ATP-induced maturation of human monocyte-derived dendritic cells. *J. Immunol.* **166**, 7172–7177 (2001).

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ONLINE METHODS

Subjects. Narcolepsy-cataplexy cases were selected as previously described[4]. The initial GWAS sample was comprised of 807 cases and 1,074 *DQB1*06:02* positive controls of European descent: 415 cases and 753 controls were recruited from the United States and Canada, and 392 cases and 321 controls were recruited from European centers. Analysis of the GWAS data (549,596 SNPs) was performed as previously described[4]. The replication sample of European descent contained 1,526 individuals, of whom 1,195 were recruited from the United States and Canada (404 cases and 791 controls) and 331 were from Europe (211 cases and 120 controls). The Asian sample included 1,269 Chinese individuals (588 cases and 681 controls), 869 Japanese individuals (437 cases and 432 controls) and 276 Koreans (105 cases and 171 controls). Finally, we studied 302 African Americans (113 cases and 189 controls). Interaction studies were conducted in the initial set and in the replication sets (cases and controls) using a test for epistatic effects implemented in the PLINK software package (v1.06 26, April 2009), which performs a logistic regression including main genotype effects plus an interaction term.

Fine mapping of the chromosome 19 locus and replication of published SNPs. For fine mapping of the associated locus, we used the same case and control samples as described above. Based on LD and r^2 data from the International HapMap Project, we chose five markers in high LD with our initial hit in individuals of European ancestry but which had lower linkage in other ethnic groups. The chosen SNPs were rs11666402, rs12460842, rs2305795, rs11880388 and rs2228611. We also typed the potential functional SNP rs3745601 in *P2RY11*. Previously reported SNP rs2858884 was also genotyped for replication in our sample. For the genotyping, we used predesigned Taqman SNP genotyping assays (Applied Biosystems). Standard D' and $\log_{10}$ odds (LOD) plots were generated using Haploview 4.2. Genotyping was performed at Stanford University except for in the case of the Chinese samples, which were genotyped at Beijing University (L.L.).

Selection of cells for mRNA expression analysis. To compare mRNA expression in controls and narcoleptic subjects, 116 subjects (60 cases and 56 controls) were randomly selected. Twenty-four controls were added on the basis of their rs2305795 genotype (the controls were age, sex and gender matched within genotypic groups). PBMCs were purified using Ficoll-Hypaque gradient centrifugation, total RNA isolated from ~4×10^6 cells (RNeasy Mini Kit #74104, QIAGEN), and RNA concentration and quality were determined by absorbance at 260 nm and 280 nm (SpectraMax M2^e, Molecular Devices). In the 24 subjects selected by genotype, ~1×10^7 PBMCs were sequentially sorted based on CD4, CD8, CD14 and CD19 positivity using Dynabeads (Invitrogen Dynal AS). Two independent replications were performed. In the first, CD19 was separated first, followed by CD14, CD4 and CD8. In the second, CD14 was separated first, followed by CD8, CD4 and CD19. $CD8^+$ T cells and natural killer cells were separated independently from new sets of ~1×10^7 PBMCs. In this experiment, natural killer cells were purified using MACS $CD56^+$ MicroBeads (natural killer cell Isolation Kit #130-092-657, Miltenyi Biotec) and $CD8^+$ T-cells were purified from the $CD56^-$ fraction ($CD8^+$ T Cell Isolation Kit #130-091-154, Miltenyi Biotec). Finally, for purification of dendritic cells, we performed negative selection using Dynabeads Human DC Enrichment Kit (Invitrogen Dynal AS), followed by positive selection using Blood Dendritic Cell Isolation Kit II (Miltenyi Biotec) on ~1×10^8 PBMCs from 21 healthy control blood samples of the three genotypes. The purity of the cell fractions was addressed by quantitative RT-PCR of the different cell surface marker mRNAs (see below) and also, a smaller sample was sorted using flow-compatible Dynabeads (#113.61D, #113.62D, #113.67D, and #125-06D, Invitrogen Dynal AS) and checked by flow cytometry as described below. The MACS Microbead sorted fractions were checked directly with flow cytometry. The purities of the fractions were (mean ± s.d.) CD4: 97.6% ± 0.4%; CD8: 98% ± 1.2%; CD14: 92.3% ± 5.9%; CD19: 76.1% ± 11.2%; and CD56: 89.6% ± 4.9%.

Gene expression using RT-PCR. Complementary DNA (cDNA) was synthesized from 200 ng of total RNA (cell subsets) or 400 ng of total RNA (PBMCs) using the High Capacity cDNA Reverse Transcription Kit (#4374966, Applied Biosystems). Gene expression was determined by RT-PCR (ABI 7000, Applied Biosystems) using TaqMan gene expression assays (Applied Biosystems). The probe numbers were P2RY11 (Hs01038858_m1), PPAN (Hs00220301_m1), PPAN-P2RY11 (Hs01568729_m1), EIF3G (Hs00959170_m1), DNMT1 (Hs00945899_m1), CD4 (Hs00181217_m1), CD8 (Hs00233520_m1), CD14 (Hs00169122_g1), CD19 (Hs00174333_m1), CD56 (Hs00941830_m1), CD1c (Hs00233509_m1), BDCA-2/CD303 (Hs00369958_m1), NRP1/CD304 (Hs00826128_m1), B2M (#4333766F), UBC (Hs00824723_m1), GAPDH (#4333764F) and ACTB (#4333762F), with the latter four serving as endogenous control genes. RT-PCR of CD4, CD8, CD14, CD19, CD56, CD1c, CD303 and CD304 mRNAs were used to verify the purity of each sorted cell fraction and of the samples. Cell fractions had a 30–69,000-fold difference in expression between wanted and unwanted markers, except in CD19 cells, where the differences were 12–24-fold, and in dendritic cells, where the differences where 5.5–27-fold. Relative quantities of target mRNAs were calculated using the comparative threshold method (Ct-method), with the geometric mean of UBC, GAPDH and ACTB expression as endogenous controls. The s.d. of the fold changes were calculated as s.d. = $2^{\Delta\Delta Ct} \times \ln 2 \times SD(\Delta Ct)$, with $SD(\Delta Ct)$ being the s.d. of ΔCt of all samples in the group.

ATP-induced cell death. PBMCs (1×10^6 cells/ml) from 12 controls selected on the basis of their rs2305795 genotype (age, sex and gender matched between genotypic groups) were incubated for 1 h or 2 h in the presence of ATP in different concentrations (0.1, 1, 10 and 100 μM) and combined with NF546 (0.1, 1, 10 or 100 μM) and/or NF340 (0.1, 1 or 10 μM) (both compounds were synthesized as described previously[27,28]). Both compounds were also tested alone (NF546: 0.1, 1, 10, 100 and 500 μM; NF340: 0.1, 1, 10 and 100 μM), and no effect was seen on cell viability except a tendency towards a decrease with 1 μM NF546. All cell work was performed using Ultra-Low Attachment plates (24W: #3473, 96W: #7007, Costar, Corning Inc.), and care was taken to flush loosely attached cells off the plates for analysis. The cells were counted in a hemocytometer using Trypan blue exclusion of dead and dying cells. All measurements were performed in duplicates. In a second setup, all surviving cells were subsequently analyzed by FACS to determine their immune phenotypes.

FACS analysis of cell phenotypes. The purity of the different cell fractions were checked on a FACscan using the following antibody combinations (antibodies are from BD Biosciences): (i) αCD14-FITC (#555397) and αCD4-PerCP-Cy5.5 (#560650); (ii) αCD8-FITC (#555366), αCD56-PE (#555516) and αCD3-PerCP; and (iii) αCD19-PE (#555413) and αCD3-PerCP. For analyzing the phenotypes of the PBMCs surviving the ATP treatment, we used a 7-marker panel consisting of: αCD14-FITC (#555397), αCD4-PerCP-Cy5.5 (#560650), αCD3-Pacific Blue (#558117), αCD19-APC (#555415), αCD56-PE (#555516) and αCD8-PE-Cy7 (#557746), and also including a Aqua Amine Live/Dead Cell Stain (L34957, Invitrogen). This analysis was performed on a BD LSRII (BD Biosciences) in duplicates. The data was combined with the cell counts as described above.

Statistical analysis. Genotype data was maintained in our database (Progeny Lab 7). Allelic tests of association were performed using the PLINK software package (v1.06 26, April 2009). Genome-wide association analysis of the original Affymetrix sample has been described previously[4]. When studying multiple ethnic groups or subgroups (for example, Taiwanese, Chinese, Japanese and Korean populations), the Mantel-Haenszel test was used together with the Breslow Day test of homogeneity of the OR. For the statistical analysis of expression data, we used a Student's t test, one-way and two-way analysis of variance tests in GraphPad Prism Version 5.00 where appropriate, and general linear regression models in Systat 12 Version 12.00.08, with control for relevant covariates (age, sex and BMI), if significant.

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