OXFORD

ASSOCIATION STUDIES ARTICLE

A rare P2X7 variant Arg307Gln with absent pore formation function protects against neuroinflammation in multiple sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic relapsing-remitting inflammatory disease of the central nervous system characterized by oligodendrocyte damage, demyelination and neuronal death. Genetic association studies have shown a 2-fold or greater prevalence of the HLA-DRB1*1501 allele in the MS population compared with normal Caucasians. In discovery cohorts of Australasian patients with MS (total 2941 patients and 3008 controls), we examined the associations of 12 functional polymorphisms of P2X7, a microglial/macrophage receptor with proinflammatory effects when activated by extracellular adenosine triphosphate (ATP). In discovery cohorts, rs28360457, coding for Arg307Gln was associated with MS and combined

analysis showed a 2-fold lower minor allele frequency compared with controls (1.11% for MS and 2.15% for controls, $P = 0.0000071$). Replication analysis of four independent European MS case–control cohorts (total 2140 cases and 2634 controls) confirmed this association [odds ratio (OR) = 0.69, $P = 0.026$]. A meta-analysis of all Australasian and European cohorts indicated that Arg307Gln confers a 1.8-fold protective effect on MS risk (OR = 0.57, $P = 0.0000024$). Fresh human monocytes heterozygous for Arg307Gln have >85% loss of 'pore' function of the P2X7 receptor measured by ATP-induced ethidium uptake. Analysis shows Arg307Gln always occurred with 270His suggesting a single 307Gln–270His haplotype that confers dominant negative effects on P2X7 function and protection against MS. Modeling based on the homologous zP2X4 receptor showed Arg307 is located in a region rich in basic residues located only 12 Å from the ligand binding site. Our data show the protective effect against MS of a rare genetic variant of P2RX7 with heterozygotes showing near absent proinflammatory 'pore' function.

Introduction

Chronic inflammation in multiple sclerosis (MS) is thought to result from damage to oligodendrocytes and axons, which leads to demyelination and neuronal death (1). The relapsing-remitting subtype (RRMS) is the most frequent form, but many patients eventually show a slow progression of disability, termed secondary progressive MS (SPMS). Some 15% of patients follow a progressive course without remission termed primary progressive MS (PPMS) suggesting a neurodegenerative component. The pathology of RRMS and SPMS is characterized by localized inflammatory foci (plaques) in both white and gray matter of the central nervous system (CNS) containing activated microglia, infiltrates of T cells and macrophages and a reduction in oligodendrocyte numbers (2,3). Genetic association studies have shown a strong influence of HLA class 2 antigens with a 2-fold or higher prevalence of the HLA-DRB1*1501 allele in the MS population compared with healthy Caucasians (4), whereas other risk loci have more modest effects (5,6). Recent studies have demonstrated proinflammatory effects of the P2X7 receptor in various autoimmune and inflammatory conditions including asthma (7), intestinal and salivary gland inflammation (8,9) and crescentic glomerulonephritis (10). Animal models also show that P2X7 plays a major role in inflammation because mice lacking the P2RX7 gene show less anticollagen-induced arthritis and reduced chronic inflammatory pain (11,12). However, recent genome-wide association studies (GWASs) have failed to show an association of common functional polymorphisms of P2RX7 with MS (5,6,13).

The purinergic P2X7 receptor is highly expressed in cells of monocyte/macrophage lineage and exerts proinflammatory effects when activated by extracellular adenosine triphosphate (ATP) (14). Postmortem studies show that this receptor is highly upregulated both on activated microglia and oligodendrocytes in the brain of patients with MS (15). Activation of P2X7 by ATP induces pore formation in cells of monocytic lineage, which triggers a cascade of proinflammatory effects including formation of the NALP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome, secretion of cytokines IL-1β and IL-18 (14,16–19) as well as activation of pathways leading to nuclear factor kappa beta (20,21). In other cells expressing P2X7 such as oligodendrocytes (which lack NALP3 components), sustained exposure to ATP over several minutes irreversibly activates a program of apoptotic cell death which may be delayed by many hours (22). The relative proinflammatory effects of P2X7 and its genetic variants in monocytic cells can be measured by the ATP-induced uptake of ethidium, a fluorescent dye that can permeate the dilated state of the P2X7 channel (23). Recent evidence shows that P2X7 in the absence of its ligand can also act as a scavenger receptor (24–26), which functions to remove apoptotic cells from the CNS before their necrotic death, thus minimizing inflammation (27). We and others have documented around one dozen non-synonymous polymorphisms in the human P2RX7 gene

which impart wide variation in the function of this receptor in the Caucasian population (28–31). Of these functional polymorphisms, we genotyped five rare variants [mean allele frequency (MAF) <3%] and seven common variants (MAF >5%) in three consecutive Australasian case–control cohorts with MS. These polymorphisms affect various aspects of receptor function including trafficking, cell surface expression, ligand binding and cytoskeletal anchoring (Supplementary Material, Table S1) (32).

Results

Association of the Arg307Gln variant of P2X7 with MS in Australasia

In consecutive case–control cohorts, we genotyped 12 functional P2RX7 variants in three case–control cohorts totaling 2941 patients and 3008 normal subjects, all of Caucasian descent from Australia and New Zealand (ANZ). In the first discovery cohort, we found a significant association between MS and a low-frequency polymorphism Arg307Gln, described a decade previously by our group (29) [odds ratio (OR) = 0.48, $P = 0.0083$, Table 1]. In the second discovery cohort of subjects, we genotyped the same 12 single nucleotide polymorphisms (SNPs) in 1160 patients and 1252 controls and again found an association of MS with the Arg307Gln polymorphism (OR = 0.56, $P = 0.0106$, Table 1). In the third discovery cohort, we genotyped 697 patients and 1117 controls and again found an association of MS with Arg307Gln (OR = 0.47, $P = 0.011$, Table 1). Combined analysis of all discovery cohorts (Table 2 and Fig. 1) yielded a minor allele frequency for Arg307Gln of 1.11% for MS compared with 2.15% for normal controls (OR = 0.51, $P = 0.0000071$), which was statistically significant at the Bonferroni-adjusted threshold. No homozygotes for Arg307Gln were found in cases or controls. None of the common variants of P2RX7 showed any tendency to an association except for Arg270His (rs7958311) which showed a suggestive association with MS in the combined analysis and which did not meet the Bonferroni-adjusted threshold (Table 2). We observed that heterozygotes for the Arg307Gln SNP were always found on a haplotype that included the minor allele of Arg270His and our previous data show that this 270His SNP in isolation imparts partial loss of 'pore' function on the receptor (31). Pairwise linkage disequilibrium (LD) analysis using the program Haploview showed LD between Arg270His and Arg307Gln [$D' = 1.0$, Log of Odds (LOD) = 7.55] and suggested that the functional effect of a single 307Gln–270His haplotype containing two loss-of-function variants may form the haplotype of P2X7 showing association with MS (Fig. 1).

Another functional SNP (30), Ile568Asn (rs1653624), showed a trend toward association with MS that was not statistically significant (Tables 1 and 2). A rare loss-of-function variant Tyr315Cys (rs28360472) in the adjoining P2RX4 gene (33) also showed a trend toward association with MS with an OR suggesting increased risk (OR = 1.30, $P = 0.079$).

Table 1. P2X7 genotyping in separate Australian cohorts of MS and Australian Caucasian controls

SNP	RS ID	Discovery Cohort 1 (1072 cases versus 721 controls) Minor allele frequency					Discovery Cohort 2 (1160 cases versus 1252 controls) Minor allele frequency					Discovery Cohort 3 (697 cases versus 1117 controls) Minor allele frequency				
		MS	Control	χ^2	P	OR	MS	Control	χ^2	P	OR	MS	Control	χ^2	P	OR
HLA-DR15	rs9271366	0.3205	0.1711	27.47	**1.6E−07**	**2.284**	0.3386	0.153	203	**4.9E−51**	2.834	0.3384	0.1827	45.47	**1.55E−11**	2.289
ENDPT1	rs10748643						0.4443	0.4547	0.6442	0.4222	0.9546	0.4511	0.4747	1.638	0.2005	0.9095
P2RX7																
151 + 1	rs35933842	0.01077	0.008511	0.4428	0.5058	1.268	0.006466	0.01094	1.26	0.2617	0.5885	0.01084	0.004695	2.362	0.1243	2.323
V76A	rs17525809	0.06625	0.05827	0.731	0.3925	1.147	0.07143	0.06289	0.5349	0.4646	1.146	0.06968	0.07025	0.004119	0.9488	0.9913
G150R	rs28360447	0.01471	0.01677	0.2255	0.6349	0.8752	0.02137	0.01878	0.4108	0.5216	1.14	0.01865	0.01596	0.3607	0.5481	1.172
H155Y	rs208294	0.4618	0.4553	0.09972	0.7522	1.026	0.4449	0.4545	0.4516	0.5016	0.9618	0.4461	0.4391	0.1676	0.6822	1.029
R270H	rs7958311	0.2437	0.2595	0.972	0.3242	0.9196	0.2341	0.2422	0.173	0.6775	0.9563	0.254	0.2743	1.749	0.186	0.9008
R276H	rs7958316	0.01744	0.01371	0.6297	0.4275	1.277	0.02092	0.02158	0.02535	0.8735	0.9687	0.02224	0.02512	0.296	0.5864	0.8826
R307Q	rs28360457	0.01029	0.02113	6.958	**0.008344**	0.4817	0.01239	0.02198	6.522	**0.01065**	0.5583	0.01004	0.02126	6.362	**0.01166**	0.4671
A348T	rs1718119	0.4023	0.3954	0.1559	0.693	1.029	0.4075	0.3897	1.584	0.2082	1.077	0.3908	0.3787	0.512	0.4743	1.052
T357S	rs2230911	0.06595	0.07225	0.5257	0.4684	0.9067	0.07974	0.07321	0.2829	0.5948	1.097	0.06759	0.08116	2.175	0.1403	0.8206
Q460R	rs2230912	0.1779	0.1573	2.569	0.109	1.159	0.173	0.1604	0.5303	0.4665	1.094	0.1662	0.1681	0.02228	0.8814	0.9862
E496A	rs3751143	0.1886	0.1799	0.4203	0.5168	1.06	0.1827	0.19	0.1726	0.6778	0.9525	0.1826	0.1712	0.7436	0.3885	1.082
I568N	rs1653624	0.02434	0.01466	4.008	**0.04528**	1.677	0.02775	0.01918	3.899	**0.0423**	1.475	0.0208	0.02367	0.3113	0.5769	0.8763
P2RX4																
Y315C	rs28360472	0.01697	0.00901	4.002	0.04545	1.897	0.01681	0.01597	0.05224	0.8192	1.053	0.01722	0.01258	1.254	0.2628	1.375

Significant P values are shown in bold.

In all cohorts, we genotyped a single SNP in the major histocompatibility complex locus (rs9271366), which was used as a proxy for HLA-DRB1*1501 in our previous study (5). In the three Australasian MS cohorts, the HLA-DRB1*1501 SNP was associated with MS with OR of 2.3, 2.8 and 2.3, respectively, and overall P-value of 1.71E−71 (Table 1). In two of the three discovery cohorts, we genotyped a promoter SNP, rs10748643, in the ENDPT1 gene which codes for a microglial and endothelial NTPDase and which has been associated with inflammatory bowel disease (34). Although NTPDase may influence the ambient concentrations of ATP, the ligand for P2X7, our data show that this SNP lacked association with MS (OR = 0.94, P = 0.15). Although the SNP, rs10748643, was not present on the platform used in International Multiple Sclerosis Genetics Consortium (IMSGC) studies, it is in LD with several other variants in the region which lacked an association with MS (13).

Frequency of Arg307Gln shows a latitudinal gradient in Europe and is associated with protection against MS

We genotyped the same P2RX7 variants in four independent replication cohorts from Sweden, Germany, Northern Ireland and Spanish Basque totaling 2140 patients and 2634 normal subjects, all of Caucasian descent. The allele frequency of Arg307Gln showed a latitudinal gradient among population-based controls being lowest in Sweden (0.011) and progressively greater in Germany (0.014), Northern Ireland (0.021) and greatest in the Spanish Basque region (0.025). In each European case–control cohort, the frequency of Arg307Gln in MS cohorts was always lower than the country-based controls, but due to small numbers, no single country case–control comparisons achieved significance (Supplementary Material, Table S2). However, on combining all four European case–control cohorts, the Arg307Gln allele was significantly associated with protection against MS (OR = 0.69, P = 0.0258) and this result replicates the Australasian discovery cohorts (Table 2). No homozygotes Gln307Gln were found except for a single control subject in the Spanish Basque cohort and in whom rs7958311 was homozygous His270His. Analysis of the combined European cohorts in Table 2 revealed a second significant association in which the SNP, Ala348Thr (rs1718119), was associated with increased risk of MS (OR = 1.15, P = 0.0011). Our group has previously identified this SNP on a gain-of-function haplotype for which Gln460Arg (rs2230912) is a marker or tag SNP (31). In the European cohorts, this tag SNP rs2230912 was also associated with MS (OR = 1.21, P = 0.0180). These data show a gain-of-function haplotype of P2X7 increases the risk of MS, a finding that mirrors the ANZgene discovery analysis showing the protective effect of a non-functional P2X7 against MS. Finally, combined analysis of the three discovery (Australasian) and four replication (European) (Table 2) cohorts yielded an overall association of Arg307Gln with MS (OR = 0.58, P = 0.0000014). Meta-analysis results of the summary statistics from all Australasian and European cohorts concur with the combined analysis of individual-level results (OR = 0.57, P = 0.0000024). Overall, these data strongly implicate the Arg307Gln variant of P2X7 in a protective effect on MS risk.

Association of Arg307Gln with clinical subtype and HLA-DRB1*1501 status

Despite the loss in statistical power due to diminished sample sizes, we assessed the Arg307Gln associations in clinical and HLA genotype subgroups using the combined Australian cohorts (n = 2165 patients). For clinical subtypes of MS, the Arg307Gln

Table 2. P2X7/P2X4 genotyping in combined Australasia and European cohorts of MS cases and controls

SNP	ANZ cohorts (2941 cases versus 3008 controls) Minor allele frequency					European cohorts (2140 cases versus 2634 controls) Minor allele frequency					Combined cohorts (5081 cases versus 5642 controls) Minor allele frequency				
	MS	Control	χ^2	P	OR	MS	Control	χ^2	P	OR	MS	Control	χ^2	P	OR
HLA-DR15	0.3322	0.1595	319.7	**1.7E−71**	2.62	0.3119	0.1595	309.4	**3.0E−69**	2.39	0.3236	0.1595	665.7	**8E−147**	2.52
ENDPT1	0.4461	0.4623	2.052	0.152	0.93	0.4373	0.4408	0.115	0.735	0.98	0.4414	0.4502	1.322	0.2501	0.96
P2RX7															
151 + 1	0.0093	0.0079	0.407	0.5232	1.17	0.0065	0.0060	0.03534	0.8509	1.07	0.0085	0.0070	0.907	0.3407	1.21
V76A	0.0690	0.0656	0.402	0.5257	1.05	0.07214	0.0843	2.298	0.1295	0.84	0.0699	0.0733	0.586	0.4439	0.95
G150R	0.0183	0.01734	0.159	0.6901	1.05	0.01689	0.0158	0.1641	0.6854	1.06	0.0177	0.0166	0.369	0.5432	1.06
H155Y	0.4513	0.4487	0.078	0.7796	1.01	0.4557	0.4439	1.323	0.25	1.05	0.4532	0.4464	0.978	0.3227	1.03
R270H	0.243	0.2646	5.49	0.01913	0.89	0.2719	0.2901	1.831	0.176	0.91	0.2507	0.275	10.43	**0.0012**	0.88
R276H	0.0199	0.02136	0.272	0.6018	0.93	0.02224	0.0207	0.2669	0.6054	1.07	0.0209	0.0210	0.003	0.9551	0.99
R307Q	0.0110	0.02153	20.17	**7.08E−06**	0.51	0.01334	0.0192	4.966	**0.0258**	0.69	0.0120	0.0204	23.34	**1.36E−06**	0.58
A348T	0.4016	0.387	2.622	0.1054	1.06	0.4095	0.3766	10.67	**0.0011**	1.15	0.4049	0.382	11.6	**0.00065**	1.10
T357S	0.0711	0.07689	1.128	0.2881	0.92	0.0845	0.0688	3.987	0.04584	1.25	0.0747	0.0737	0.052	0.8193	1.01
Q460R	0.1732	0.1632	1.655	0.1983	1.07	0.169	0.1436	5.591	**0.0180**	1.21	0.172	0.1556	6.973	**0.008273**	1.12
E496A	0.1849	0.1771	0.940	0.3323	1.05	0.1876	0.2304	12.2	**0.00047**	0.77	0.1856	0.1977	3.289	0.06976	0.92
I568N	0.02486	0.0196	3.708	0.0541	1.27	0.0203	0.0199	0.01819	0.8927	1.02	0.0229	0.0198	2.567	0.1091	1.16
P2RX4															
Y315C	0.01706	0.01314	3.078	0.0793	1.30	0.01403	0.01044	2.557	0.1098	1.349	0.0157	0.0118	6.015	**0.0141**	1.334

Significant P values are shown in bold.
In European cohorts, the case and control number for SNP 151 + 1, V76A, R270H, T357S, Q460R and E496A are 999 and 1320, respectively.

variant was associated with RRMS (OR = 0.59, P = 0.000034) and with less significance with PPMS (OR = 0.60, P = 0.025) or SPMS (OR = 0.52, P = 0.0205) although patient numbers for PPMS and SPMS were small (Table 3 and Supplementary Material, Table S3). An association of Arg307Gln with MS was seen for both male and female patients (Supplementary Material, Table S4). We also examined for possible dependency of the Arg307Gln association on the HLA-DRB1*1501 allele by stratifying all genotyped cohorts into HLA-DRB1*1501 positive and negative subgroups. The P2X7 Arg307Gln variant showed an association with MS both in HLA-DRB1*1501 negative patients (OR = 0.57, P = 0.00038) and in HLA-DRB1*1501 positive patients (OR = 0.52, P = 0.00028) (Supplementary Material, Table S5).

Dominant negative effect of Arg307Gln on P2X7 pore formation function in monocytes

Our previous work has shown that mutant P2X7 307Gln constructs expressed in HEK 293 cells show total absence of function when exposed to the physiological ligand ATP (29). We examined the effect of this variant on P2X7 'pore' formation in CD14$^+$ monocytes from normal subjects who were heterozygous for Arg307Gln using ATP-induced ethidium$^+$ uptake as an assay for P2X7 'pore' function. Uptake of ethidium$^+$ was nearly abolished (>85% loss) into cells from subjects heterozygous for Arg307Gln, whereas monocytes from subjects wild-type at all functional polymorphic positions showed brisk uptake of ethidium$^+$ dye via the P2X7 receptor (Fig. 2 and Supplementary Material, Fig. S1). These data show that the Arg307Gln variant in heterozygous dosage exerts a dominant negative effect on the proinflammatory actions of P2X7 and this may be a major factor in the protective effect of the variant in MS. In contrast, monocytes from peripheral blood which were heterozygous for the loss-of-function Ile568Asn variant showed a reduced P2X7-mediated ethidium$^+$ uptake ('pore' function) approximating half wild-type value (Fig. 2A). Our laboratory has described two gain-of-function P2X7 haplotypes in the Caucasian population (termed P2X7-2 and P2X7-4) which contain Ala348Thr (31). We studied monocytes harboring haplotype P2X7-4 in homozygous dosage containing the gain-of-function variant Ala348Thr (rs1718119) as well as the marker SNP Gln460Arg (rs2230912) and another weaker gain-of-function variant His155-Tyr (rs208294). These monocytes showed a P2X7-mediated ethidium$^+$ uptake which was more than double the wild-type value (Fig. 2A).

Surface expression and phagocytosis mediated by P2X7 is not impaired in the Arg307Gln variant

We and others have shown an unsuspected function for the P2X7 receptor which in the absence of ATP directly recognizes and regulates the engulfment of particles and apoptotic cells (24–26). We asked if this scavenger function of P2X7 may differ between the polymorphic variants and in some way contribute to the association of Arg307Gln with MS. This innate phagocytic property of P2X7 was assessed by a quantitative assay in which HEK 293 cells transfected with P2X7 constructs (either wild-type or variant) were incubated with 1.0 µm fluorescent yellow-orange (YO) beads and the uptake of beads quantitated using real-time flow cytometry (35). Transfected cells expressing the 307Gln variant of P2X7 showed a phagocytic function equal to wild-type cells despite a near complete absence of ATP-induced ethidium$^+$ uptake (Fig. 2A–C). All other P2X7 variant constructs showed bead phagocytosis equal to wild-type P2X7 consistent with

Figure 1. Genetic association of functional P2X7 variants with MS. (**A**) Significance of the association for each SNP is shown as −log P at each of the variant amino acid positions shown in the X-axis. All polymorphisms are exonic except a splice site variant at +1 position of intron 1 termed 151 + 1. (**B**) Haploview analysis shows the LD between each pair of the 12 SNPs in terms of D′ values. Separate analyses are shown for all MS and all normal control subjects. Plots of relative D′ and LOD scores for patients (upper) and controls (lower) are generated by Haploview program. Standard color scheme is displayed: bright red (D′ = 1; LOD ≥ 2), blue (D′ = 1; LOD < 2), shades of pink/red (D′ < 1; LOD ≥ 2) and white (D′ < 1; LOD < 2). The numbers are D′ score (×100). The LD pattern for R307Q in the haplotype analysis is similar for MS and normals and shows that the association of R307Q with MS is independent of adjoining functional variants.

strong expression on the cell surface of all variants except the P2X7 568Asn (homozygous) construct (Fig. 2C). This 568Asn construct showed both poor bead uptake and reduced P2X7-induced ethidium⁺ uptake consistent with failure of trafficking of P2X7 receptors to the cell surface (Fig. 2B and C). These data show that the Arg307Gln variant of P2X7 is expressed on the cell surface and has phagocytic capacity equal to wild-type P2X7.

Modeling of P2X7R shows Arg307 in close proximity to ATP-binding site

Using the recently determined crystal structure of zP2X4R, we constructed a model for the P2X7R and analyzed the relative position of the extracellular SNPs investigated in this study. Except for Ala348, which is nested in the transmembrane domain facing

Table 3. P2X7/P2X4 genotyping in Caucasian relapsing remitting multiple sclerosis cases and controls from Australasia and European cohorts

Protein variant	Australasia (n = 2079 versus 3008)		European (n = 1474 versus 2634)		Combined (n = 3553 versus 5642)	
	P	OR	P	OR	P	OR
P2XR7						
151 + 1	0.6914	1.114	0.8083	1.1	0.4997	1.161
V76A	0.8895	1.013	0.1817	0.8544	0.3116	0.929
G150R	0.7336	0.9477	0.5187	1.122	0.8577	1.021
H155Y	0.7073	1.016	0.2049	1.06	0.2565	1.036
R270H	0.07023	0.9088	0.2836	0.9273	**0.01414**	0.9021
R276H	0.59	0.9251	0.5987	1.086	0.9464	0.9929
R307Q	**1.1E−05**	0.464	0.2002	0.7937	**3.4E−05**	0.5948
A348T	0.3782	1.037	**0.0131**	1.124	**0.01977**	1.075
T357S	0.4688	0.9396	0.1516	1.186	0.7896	1.019
Q460R	0.1274	1.096	**0.006588**	1.261	**0.003039**	1.156
E496A	0.5857	1.033	**6.75E−05**	0.7264	**0.0194**	0.8961
I568N	0.2043	1.192	0.5864	1.091	0.1841	1.149
P2RX4						
Y315C	0.1468	1.272	0.07281	1.438	**0.01995**	1.348

Significant *P* values are shown in bold.

the pore of the channel, five other SNPs are within the extracellular domain (Fig. 3). From the three SNPs closest to the ATP-binding site, two are located in the head domain (Gly150 and His155), which is known to be involved in activation and desensitization of P2X receptors (37). The third residue, Arg307, appears just above the highly conserved Lys311 on an antiparallel beta sheet secondary structure. Because position 307 is measured at a distance of 12 Å away from the ligand binding site (Lys311), replacement of this Arg with a Gln is unlikely to directly affect ATP binding. However, together with Lys145, Arg307 forms a secondary pocket and creates a highly positive environment suitable for binding of a coagonist or a second ATP molecule (Fig. 3).

Discussion

A rare variant of the P2X7 receptor, Arg307Gln, was associated with MS in all three Australasian cohorts (combined cohorts P = 0.0000071) with an OR of 0.51 predictive of a protective effect against developing MS. Our first discovery cohort was drawn from subjects in our ANZgene GWAS in whom principal component analysis showed little evidence of population stratification (5). Patients for the second and third discovery cohorts were of Australasian Caucasian parentage and came from centers that participated in IMSGC studies with an estimated genomic inflation factor (λ) of 0.96 showing insignificant population stratification (Supplementary Material, Tables S1–S7, Ref. 13). In the three Australasian control cohorts, the Arg307Gln mean allele frequencies were constant between cohorts (e.g. 0.02113, 0.02198 and 0.0213). The discovery cohorts show a significant protective effect of Arg307Gln on developing MS for a large cohort of 2941 Australasian patients. We studied four European MS case–control cohorts and in all four, the mean frequency of Arg307Gln in MS was always lower than the country-based controls. In each cohort from Sweden, Germany, Northern Ireland and Spanish Basque, the Arg307Gln variant consistently exerted the largest protective effect with ORs between 0.61 and 0.88 (Supplementary Material, Table S2). This protective effect of Arg307Gln was significant for all European countries when analyzed together (OR = 0.69, P = 0.0258). Previous GWAS platforms have not included rs28360457 because of its low frequency and imputation can be

unsuccessful if the SNP is not in strong LD with common SNPs on the genotyping platform. We examined the LD relationship of rs28360457 over a 100 kB region spanning *P2RX7* and found LD with rs7958311 (D′ = 0.93, LOD = 7.55) and also stronger LD with a number of intronic SNPs, none of which were present on the IMSGC platform (Supplementary Material, Table S6). This lack of a good proxy for rs28360457 together with its low frequency in Northern Europe and Scandinavia may explain why this SNP has not previously been associated with MS despite the large size of its effect.

The Arg307Gln allele was associated with all three subtypes of MS, consistent with an effect of this loss-of-function allele to decrease secretion of proinflammatory cytokines from activated microglia/macrophages and lessen inflammation associated with acute relapses and progressive disease. Arg307Gln was associated with both HLA-DR15 positive and negative patients consistent with its role as an innate immune receptor rather than some function in presentation of immunogenic myelin peptides to T lymphocytes.

A second SNP, rs7958311 (Arg270His), showed a weak association with MS (P = 0.019, OR = 0.89), and analysis showed that the 307Gln variant always occurred on a haplotype which included the minor allele of rs7958311. Our haploview analysis in Figure 1 shows that Arg307Gln (rs28360457) is most highly associated with MS and is the major SNP driving the association of P2RX7 with MS. However, Arg307Gln is only one of the five functional SNPs which confer major loss of function on the P2X7 receptor and it is unclear why heterozygotes for Arg307Gln exert a dominant negative effect on receptor function, whereas other SNPs with major functional effects in transfection experiments (Supplementary Material, Fig. S1B) show a linear gene dosage effect on P2X7 'pore' function. The Arg270His variant is a functional SNP giving partial loss of P2X7 pore function (31) and the dominant negative effect of heterozygous Arg307Gln on P2X7 pore function may represent a 'double loss-of-function' effect resulting from inheritance of a single (heterozygous) 307Gln–270His haplotype. In a recent study of chronic pain due to osteoarthritis, an association was reported between reduced pain intensity and the variant 270His allele of P2X7 (38). This Canadian study did not examine rare SNPs particularly rs28360457 (Arg307Gln) which
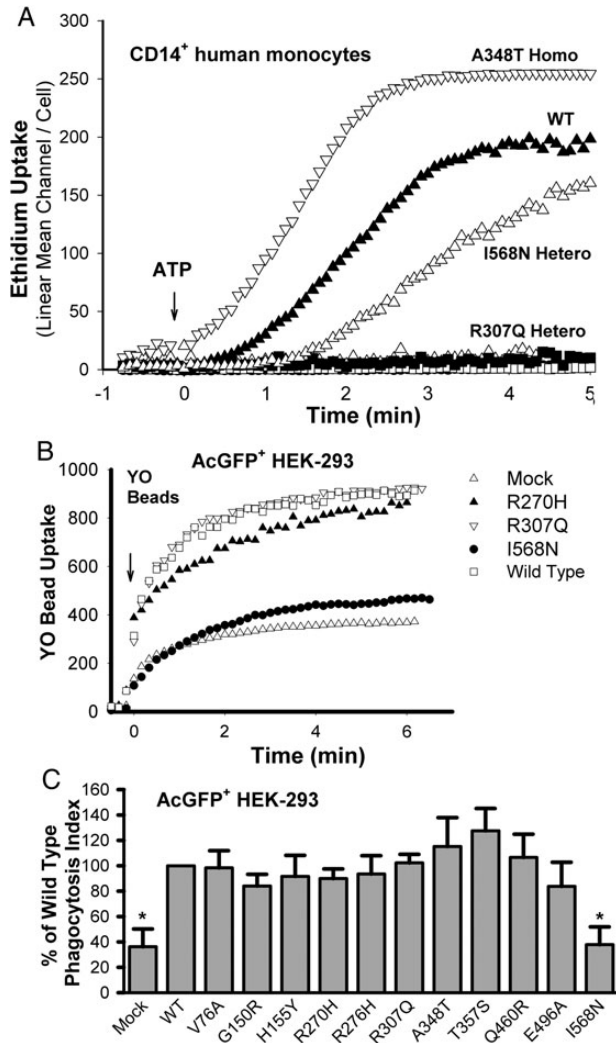
Figure 2. Heterozygous Arg307Gln abolishes P2X7 pore function in human monocytes but does not affect P2X7 scavenger function. (**A**) P2X7 pore formation in fresh human monocytes: human peripheral blood mononuclear cells were collected from subjects with typical wild-type P2X7, a subject homozygous for the gain-of-function haplotype P2X7-4, a subject with typical heterozygous Ile568Asn and subjects with heterozygous Arg307Gln (n = 3). Cells were separated by Ficoll density centrifugation and stained with allophycocyanin-conjugated anti-CD14 monoclonal antibody, washed and resuspended at 2×10^6 in 1.0 ml of Na medium. Ethidium bromide (25 μM) was added followed at 40 s by addition of 1.0 mM ATP. The mean fluorescence intensity (MFI) of P2X7-mediated ethidium uptake into CD14$^+$ monocytes was measured at 5 s intervals by two-color flow cytometry. (**B**) P2X7-mediated bead phagocytosis in HEK-293 cells transfected with wild-type or mutant (307Gln, 270His or 568Asn) constructs each fused with AcGFP. pAcGFP-N1 vector was used as the mock control. Uptake of 1.0 μm YO beads (as MFI) by gated AcGFP$^+$ cells was measured in the absence of ATP (**C**). P2X7 pore function and P2X7 phagocytic function in the 11 rare and common exonic variants in comparison to P2X7 wild-type (the 100% value). HEK-293 cells were transfected with wild-type or mutant P2X7 constructs fused with AcGFP and pore formation and uptake of YO beads measured on the same day. *$P < 0.01$, n = 3–5.

in our Australasian study has a far higher impact in conferring protection from neuroinflammation.

Our data show that a gain-of-function haplotype in P2X7 is associated with MS in the European and in the total combined cohorts (Table 2). This haplotype contains two SNPs, Ala348Thr (rs1718119) and Gln460Arg (rs2230912), which are in LD with

each other and lie in a block termed haplotype P2X7-4 (31). Each SNP was associated with an increased risk of MS with an OR of 1.10 and 1.12 and P-values of 0.00065 and 0.00827, respectively, in the analysis of all combined cohorts (Table 2). In the Australasian cohorts, the same two SNPs showed a trend toward increased risk of MS (OR = 1.06, 1.07) which was not significant (Table 2). In both Australasia and Europe, the rare Arg307Gln SNP with near absent function exerts a protective effect on MS, whereas in Europe, a common gain-of-function haplotype of P2X7 confers increased risk of MS but with modest effect size. In both continents, our data show a role for the inflammatory actions of P2X7 in the pathogenesis of MS.

The positively charged arginine at residue 307 is essential for P2X7 'pore' function (29) and this residue is highly conserved across all seven P2X receptors in a range of animal species (39). We performed transfection experiments to examine if the Arg307Gln variant altered P2X7-mediated phagocytic activity but found that the scavenger function of this variant was similar to wild-type. Previously, we proposed that arginine 307 may be involved in the binding of the ATP ligand (29), but homology modeling of hP2X7 based on the recently published structure of the zP2X4 receptor (40) shows that Arg307 is not one of the residues directly interacting with ATP but appears some 12 Å away from the bound ligand (Fig. 3). It is possible that Arg307, which is solvent exposed together with lysine-145, may form a secondary binding pocket, either for an ionic cofactor or for a second ligand molecule (Fig. 3). Several groups have presented evidence for a second ligand or cofactor binding site of low affinity at which either a nucleotide polyphosphate or extracellular anion may act as a necessary cofactor together with ATP (41,42).

There is increasing evidence that disability in MS results from neuroinflammation either on a chronic or relapsing basis (3) and postmortem studies show that the P2X7 receptor is highly upregulated on activated microglia in the brain of patients with MS (15) consistent with the increased secretion of proinflammatory cytokines from the microglia. The genetic protection against MS imparted by Arg307Gln further implicates the function of wild-type P2X7 expressed on microglia/macrophages as a major component in the cascade leading to neuroinflammation. The effect size provided by the near absent function of the Arg307Gln variant approaches that conferred by the HLA-DRB1*1501 allele (but in opposite direction) with an OR of 0.46 for Australasian RRMS and OR of 0.59 for the combined cohorts of MS (Table 3). Other evidence supports an association between P2X7 and MS. In an animal model of MS, pharmacological blockade of P2X7 receptors prevented ATP-induced death of oligodendrocytes and attenuated murine experimental autoimmune encephalomyelitis (43). Recent human studies of P2X7 antagonists in Phase I/II trials have demonstrated acceptable safety and tolerability of these agents (44–46) and trials of antagonists which cross the blood–brain barrier are awaited in neuroinflammatory states (47). In the only case–control association study to date of P2X7 in MS, a Spanish group found a marginal protective effect of two common and hypofunctional P2RX7 alleles, when inherited together (48). In a study of chronic inflammatory pain due to osteoarthritis, an association was reported between reduced pain intensity and the low function 270His variant of P2X7 (38). Although this previous study did not examine rare variants, our haploview analysis shows strong LD between Arg307Gln and Arg270His, further supporting the association of P2X7 variants with inflammatory conditions. Our Australasian data show that inheritance of the loss-of-function polymorphism Arg307Gln in the gene confers protection against the risk of MS (OR for carriers = 0.51, P = 0.0000071) and an effect size that is approaching 2-fold.
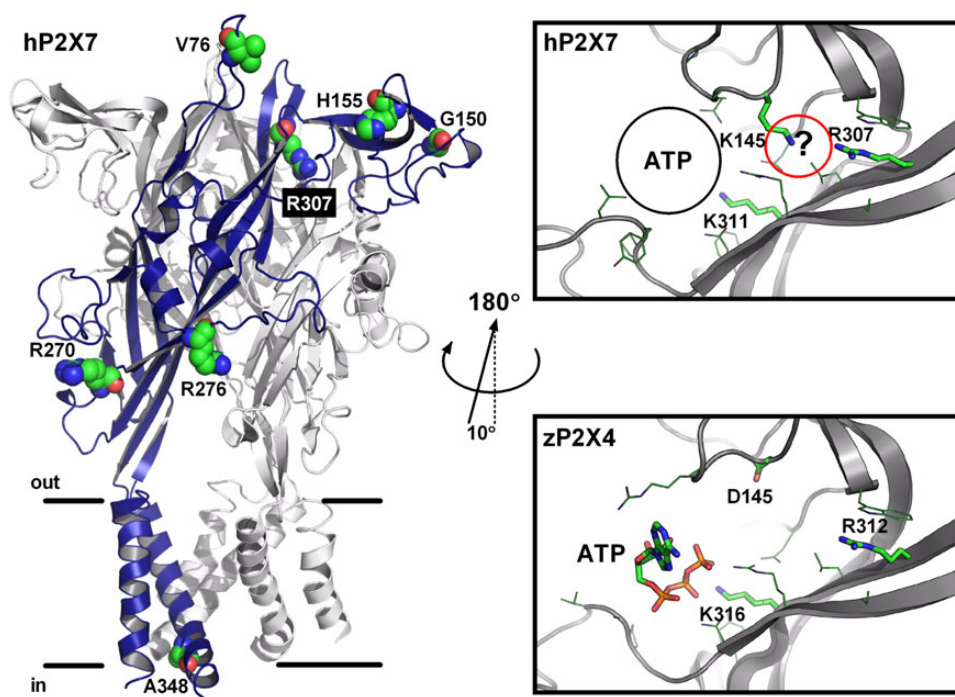
Figure 3. Position of arginine 307 in a homology model of the trimeric human P2X7 receptor. In one monomer depicted in blue, the position of functional polymorphic sites in the extracellular and TM2 domains of hP2X7 is shown by colored residue side chains. The inserts show a magnified view of the ATP-binding site for zebrafish P2X4 (below) and the homologous site for human P2X7 (above). Both K193 and K311 are essential for human P2X7 function (36) and both these homologous residues have been identified in the zP2X4 ATP-binding site (K193 and K 316, respectively). R307 is solvent exposed and together with K145 forms a secondary binding pocket which is remote from the primary ligand binding site. This site is shown in the red circle and may represent a binding pocket either for an ionic cofactor or second ligand molecule in each subunit.

Genetic and functional data suggest P2X7 as a promising drug target in this inflammatory disease of the CNS.

Materials and Methods

Study subjects and DNA samples

A total of 1072 MS cases from ANZ of Caucasian ancestry were studied for the first discovery phase with DNA prepared as described previously (5). This cohort was drawn from the AN-Zgene GWAS in which principal component analysis showed little evidence for population stratification with a reported genomic inflation factor (λ) of 1.10 (5), whereas our subsequent cohorts came from centers for which IMSGC estimated a genomic inflation factor after imputation of 0.96 (Supplementary Material, Tables S1–S7, Ref. 13). DNA samples for the second discovery cohorts were provided from Victoria ($n = 769$), Newcastle, NSW ($n = 163$) and New Zealand ($n = 228$). DNA samples for the third discovery cohorts were MS from Newcastle ($n = 203$), Melbourne ($n = 68$) and Tasmania ($n = 426$). Control subjects for the first discovery phase were typed across 13 SNPs and came from Centre for Eye Research Australia ($n = 420$) and healthy subjects from Nepean Hospital, New South Wales, Australia ($n = 301$). Controls for the second discovery phase ($n = 1252$) were de-identified donors from the Australian Bone Marrow Donor Registry (ABMDR) and for the third discovery, cohort were healthy volunteers ($n = 460$) and normal control subjects from the AIBL project ($n = 657$). All cases and controls involved in the discovery and replication phases were included on the basis of reported European Caucasian ancestry. We also genotyped DNA samples of MS patients from Sweden ($n = 301$), Germany ($n = 561$) and Northern Ireland ($n = 629$) and an equivalent number of country-based controls. In addition, we genotyped cohorts from the Basque region of Spain with 989 controls and 649 MS cases provided with ethical permission by the Biobank of The Basque Foundation for Health Innovation and Research (http://www.biobancovasco.org) (see Supplementary Material, Table S7). The Basques live at the western end of the Pyrenees along the Atlantic coast and represent an ancient, homogeneous and genetically isolated population. All Australasian cases and controls were recruited with written and informed consent which was given by the patients for their information to be stored in the study database and used for research. Approval for this research was granted by the Melbourne Health Human Research Ethics Committee, Eastern Health Human Research and Ethics and Australian relevant institutional ethics committees.

Patient phenotyping

Phenotypic assessments of MS cases were conducted according to established clinical (49,50) brain imaging and laboratory (51) criteria. All cases had either definite MS, clinically definite MS or laboratory-supported definite MS according to the McDonald and Poser criteria, respectively. Supplementary demographic information for Australian and New Zealand MS cases has been published (5).

Genotyping and quality control

A total of 12 SNPs were genotyped in the P2RX7 gene (GenBank accession number Y09561) and 1 SNP in P2RX4 (GenBank accession number BC033826) in the discovery and the same SNPs, plus rs10748643, in two of the three discovery and all replication cohorts using the Sequenom MassARRAY system and iPlex Gold

chemistry. All genotyped SNPs in P2RX7 and P2RX4 exert functional effects at the protein level as shown by our laboratory and others (Supplementary Material, Table S1). Individuals with successful genotype calls at <10 SNPs were discarded. In subjects included for analysis genotyping, the failure rate at rs28360457 was 0.4%. For the other SNPs, the failure rates were between 0.2 and 2.0%. All SNPs were in Hardy–Weinberg equilibrium both in cases and controls. Four of the 12 P2RX7 SNPs had been previously typed on the Illumina Human 370CNV platform in the ANZgene study (5) and our Sequenom results replicated the GWAS typing with concordance of 99.9%.

Association testing of SNPs with MS

Pairwise LD was analyzed using the program Haploview (http://www.broad.mit.edu/mpg/haploview). Distribution of genotype frequencies was tested for Hardy–Weinberg equilibrium by a chi-square test. P-values and ORs were calculated using the program PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) (52). The statistical significance threshold was adjusted for multiple testing using Bonferroni correction (0.05/12 SNPs = 0.0041). Meta-analysis was performed using the inverse variance-based methods implemented in the program METAL (www.sph.umich.edu/csg/abecasis/metal/).

P2X7 function in human monocytes

For ethidium$^+$ uptake, mononuclear preparations from normal subjects with known functional polymorphic variants were preincubated with fluorescein isothiocyanate-labeled anti-CD14 monoclonal antibody and ATP-induced uptake of ethidium$^+$ into the gated monocyte subpopulation was measured as described previously (29). For bead uptake, HEK-293 cells were transfected with 3 μg plasmid DNA of wild-type or mutant P2X7 in a pAcGFP-N1 vector, using linear polyethylenimine as described previously (53). Five microliters of 1.0 μm carboxylated YO beads (Polyscience, USA) was added at zero time. The linear mean channel of fluorescence intensity for gated AcGFP$^+$ HEK-293 cells over successive 10 s intervals was analyzed by WinMDI software and plotted against time, as described previously (35).

Homology modeling of P2X7 receptor structure

A model for the human P2X7 receptor was built as already described (54). Briefly, the structure of zP2X4 bound to ATP (4DW1) was used as a template to generate the hP2X7 model using the homology modeling server SWISSMODEL (55). The hP2X7 model was minimized using the GROMOS force-field implemented in the program DEEP VIEW (56). The root mean square deviation between single subunits of the hP2X7 model and zfP2X4.1 structure is 1.85 Å (alignment of 322 atoms).

Supplementary Material

Supplementary Material is available at *HMG* online.

Acknowledgements

Conflict of Interest statement. None declared.

Funding

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Appendix: Membership of ANZgene Consortium

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