Febrile seizures represent a serious adverse event following measles, mumps and rubella (MMR) vaccination. We conducted a series of genome-wide association scans comparing children with MMR-related febrile seizures, children with febrile seizures unrelated to vaccination and controls with no history of febrile seizures. Two loci were distinctly associated with MMR-related febrile seizures, harboring the interferon-stimulated gene IFI44L (rs273259: \( P = 5.9 \times 10^{-12} \) versus controls, \( P = 1.2 \times 10^{-9} \) versus MMR-unrelated febrile seizures) and the measles virus receptor CD46 (rs1318653: \( P = 9.6 \times 10^{-11} \) versus controls, \( P = 1.6 \times 10^{-9} \) versus MMR-unrelated febrile seizures). Furthermore, four loci were associated with febrile seizures in general, implicating the sodium channel genes SCN1A (rs6432860: \( P = 2.2 \times 10^{-16} \)) and SCN2A (rs3769955: \( P = 3.1 \times 10^{-10} \)), a TMEM16 family gene (ANO3; rs11444506: \( P = 3.7 \times 10^{-20} \)) and a region associated with magnesium levels (12q21.33; rs11105468: \( P = 3.4 \times 10^{-11} \)). Finally, we show the functional relevance of ANO3 (TMEM16C) with electrophysiological experiments in wild-type and knockout rats.

Vaccination is one of the most effective public health interventions, and modern vaccines have an excellent safety record. However, on rare occasions, some individuals experience serious adverse events. Investigating the underlying causes of such events is essential to maintain public confidence in vaccination and may help improve vaccine safety. Fever is a common reaction to immunization, and febrile seizures occasionally occur after vaccination, especially with live-virus vaccines such as the MMR vaccine. Although generally well tolerated, MMR vaccination almost triples the risk of febrile seizures in the second week after vaccination, resulting in an estimated 3 to 16 additional febrile seizure cases per 10,000 vaccinated children 1,2. Overall, febrile seizures occur in 2–5% of children of European ancestry before 5 years of age 3, often induced by fever from viral infections 4.

Genetic studies of epileptic disorders with concomitant febrile seizures have identified a number of risk variants, particularly in ion channel genes 5,6. However, the vast majority of children with febrile seizures do not develop epilepsy 7, and, although family and twin studies suggest a strong genetic component to isolated febrile seizures 8–10, little is known about specific genetic risk variants. It is also unknown whether distinct variants influence the risk of febrile seizures occurring as an adverse effect of MMR vaccination or the MMR vaccine is just one of many possible stimuli that may trigger febrile seizures in susceptible individuals.

Here we address these questions using a series of genome-wide association scans and replication genotyping, cell-based overexpression assays and electrophysiological recordings of brain slices from wild-type and Ano3-null rats.

RESULTS

Study design

Our study design is illustrated in Supplementary Figure 1. In the discovery stage, we conducted four genome-wide association scans: (i) MMR-related febrile seizures versus controls; (ii) MMR-related febrile seizures versus MMR-unrelated febrile seizures; (iii) MMR-unrelated febrile seizures versus controls; and (iv) febrile seizures overall versus controls. Sample characteristics and inclusion criteria are given in Supplementary Table 1. After imputation based on reference data from the 1000 Genomes Project, we included approximately 8.1 million variants in each of the 4 association scans. Genomic inflation factors were 1.01, 1.00, 1.02 and 1.03 for the four scans, respectively, indicating minimal population stratification. Quantile-quantile and Manhattan plots are shown in Supplementary Figure 2. On the basis of the discovery-stage results, we selected 23 SNPs representing 16 loci for replication-stage genotyping (Supplementary Fig. 3). Furthermore, we conducted analyses conditioning on the selected SNPs, but we identified no additional SNPs fulfilling the selection criteria.
### Table 1 Discovery, replication and combined results for six loci associated with febrile seizures following MMR vaccination and overall febrile seizures

| Loci for MMR-related febrile seizures | Discovery (MMR-related FS, n = 929; MMR-unrelated FS, n = 1,070; FS overall, n = 1,999; controls, n = 4,118) | Replication (MMR-unrelated FS, n = 1,030–1,034; FS overall, n = 1,435–1,442; controls, n = 1,625–1,645) | Combined (MMR-unrelated FS, n = 1,334–1,337; MMR-unrelated FS, n = 2,100–2,104; FS overall, n = 3,434–3,441; controls, n = 5,743–5,863) |
|--------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| **Chromosome Position (bp)** | **SNP (effect/alternate allele)** | **Effect allele frequency** | **OR** | **Effect allele frequency** | **OR** | **OR** |
| 1 | 79093818 rs273259 (A/G) | MMR+ vs. controls | 0.767 | 0.702 | 1.40 | 1.4 | 10^{-8} | 0.754 | 0.683 | 1.42 | 9.2 | 10^{-5} | 1.41 | 1.28–1.55 | 10^{-12} |
|  | | MMR+ vs. MMR- | 0.767 | 0.694 | 1.46 | 2.0 | 10^{-7} | 0.754 | 0.692 | 1.36 | 0.0096 | 1.36 | 10^{-9} | 1.42 | 1.27–1.59 | 0.57 |
|  | | MMR- vs. controls | 0.694 | 0.702 | 0.97 | 0.52 | 0.692 | 0.683 | 1.04 | 0.53 | (0.92–1.17) | 1.00 | (0.92–1.08) | 0.95 | 0.36 |
|  | | All FS vs. controls | 0.728 | 0.702 | 1.14 | 0.0027 | 0.709 | 0.683 | 1.13 | 0.028 | (1.01–1.26) | 1.14 | (1.06–1.21) | 0.0002 | 0.91 |
|  | 208014922 rs1318653 (T/C) | MMR+ vs. controls | 0.828 | 0.774 | 1.41 | 2.1 | 10^{-7} | 0.831 | 0.767 | 1.49 | 8.8 | 10^{-5} | 1.43 | 1.28–1.59 | 9.6 | 10^{-11} |
|  | | MMR+ vs. MMR- | 0.828 | 0.771 | 1.44 | 5.9 | 10^{-6} | 0.831 | 0.761 | 1.55 | 4.1 | 10^{-5} | 1.48 | 1.30–1.67 | 1.6 | 10^{-9} |
|  | | MMR- vs. controls | 0.771 | 0.774 | 0.98 | 0.74 | 0.761 | 0.767 | 0.96 | 0.58 | (0.85–1.10) | 0.97 | (0.89–1.06) | 0.54 | 0.84 |
|  | | All FS vs. controls | 0.797 | 0.774 | 1.15 | 0.0037 | 0.781 | 0.767 | 1.08 | 0.22 | (0.96–1.22) | 1.12 | (1.04–1.21) | 0.0023 | 0.41 |

**Results with P < 1.25 × 10^{-8} are marked in bold. Controls in the MMR-related versus MMR-unrelated febrile seizure analyses are febrile seizure cases unrelated to MMR vaccination. OR, odds ratio; CI, confidence interval; MMR+, MMR-related febrile seizure cases; MMR-, MMR-unrelated febrile seizure cases; FS, febrile seizures; $P_i$, heterogeneity estimate. $P_{het}, P$ value from the Cochran Q test of heterogeneity.**
We applied a genome-wide significance threshold of $P < 1.25 \times 10^{-8}$, as four association scans were conducted. Six independent genetic loci were replicated and reached genome-wide significance in one or more of the combined analyses (Table 1 and Supplementary Table 2).

**Distinct associations for MMR-related febrile seizures**

Four loci reached genome-wide significance in the analysis of MMR-related febrile seizures versus controls. Out of these loci, two also reached genome-wide significance in the analysis of MMR-related febrile seizures versus MMR-unrelated febrile seizures while not showing any effect in the analysis of MMR-unrelated febrile seizures versus controls (Table 1). In agreement with these findings, a genetic risk score based on these two loci showed no association in a logistic regression analysis of MMR-unrelated febrile seizures versus controls ($P = 0.42$), whereas it was highly significant in comparisons of MMR-related febrile seizures versus controls ($P < 2 \times 10^{-16}$) and MMR-related febrile seizures versus MMR-unrelated febrile seizures ($P < 2 \times 10^{-16}$). Both loci were thus distinctly associated with febrile seizures following MMR vaccination. We found no evidence of interaction between the two top SNPs. There was also no interaction between either of the two SNPs and the four SNPs for febrile seizures overall listed in Table 1, and their effect estimates were not changed by conditioning on the four top SNPs for febrile seizures overall (data not shown). We considered all 48 genotyped or imputed variants (SNPs and indels) with association $P < 1 \times 10^{-3}$ at these two loci and searched for functional predictions. These variants were all in linkage disequilibrium (LD) with the top SNP at the given locus ($r^2 = 0.47–1$; Supplementary Table 3).

At the first locus for MMR-related febrile seizures on chromosome 1p31.1, the associated SNPs fell in a sharply defined 45-kb LD block containing the gene IFI44L (Fig. 1a). Of the 25 variants with association $P < 1 \times 10^{-5}$, 2 were missense variants (Supplementary Table 3). One of these, rs273259 (c.218A>G; p.His73Arg; Ensembl transcript ENST00000370751), ranked among the markers with the lowest $P$ value at the locus and was selected for replication genotyping. This variant showed genome-wide significant association in MMR-related febrile seizures versus controls (odds ratio (OR) = 1.41, 95% confidence interval (CI) = 1.28–1.55; $P = 5.9 \times 10^{-12}$) and in MMR-related febrile seizures versus MMR-unrelated febrile seizures (OR = 1.42, 95% CI = 1.27–1.59; $P = 1.2 \times 10^{-9}$). It was not predicted to be damaging by MutationTaster or PolyPhen-2 but appears to affect the relative levels of the spliced isoforms. The risk allele (rs273259[AG]) for MMR-related febrile seizures corresponds to decreased expression of exon 2 (ENST00000370751 transcript), in which it resides, in lymphoblastoid cell lines and corresponds with decreased expression of the IFI44L-001 transcript (ENST00000370751) and increased expression of the IFI44L-002 transcript (ENST00000486882) relative to other transcripts. In peripheral blood, rs273259[AG] is associated with decreased expression of the neighboring IFI44 gene and IFI44L and IFI44 belong to the group of interferon-stimulated genes (ISGs) and are both transcriptionally induced by type I interferon signaling. The expression of IFI44L (in dendritic cells) is significantly upregulated after infection with measles virus. In a large-scale antiviral screen of ISGs, IFI44L modestly inhibited hepatitis C virus replication. We tested whether IFI44L affected the replication of a recombinant measles virus expressing green fluorescent protein (GFP). Using a lentiviral ectopic expression assay, three tested IFI44L variants had no effect on measles virus replication in immortalized human fibroblasts lacking STAT1 (Supplementary Fig. 4). Under these experimental conditions, IFI44L variants are not sufficient to confer direct antiviral protection against measles virus. Other cellular backgrounds or host factors might be required for a functional antiviral phenotype.

The most significant SNP at the second locus on chromosome 1q32.2, rs1318653 (OR = 1.43, 95% CI = 1.28–1.59; $P = 9.6 \times 10^{-11}$) versus controls and OR = 1.48, 95% CI = 1.30–1.67; $P = 1.6 \times 10^{-9}$

### Figure 1

**Discovery-stage results from the MMR-related febrile seizures versus controls scan.** (a) Regional association plots for the 1p31.1 locus (a) and the 1q32.2 locus (b). SNPs are plotted by chromosomal location ($x$ axis) and disease association ($-\log_10 P$ value; left $y$ axis). The colors reflect the LD of each SNP with the top SNP at the locus, and recombination rates (from HapMap; right $y$ axis) are shown to reflect the local LD structure. The $P$ value for the top SNP in the combined analysis is represented by a purple diamond, and that from the discovery-stage analysis is represented by a purple circle.
versus MMR-unrelated febrile seizures), was located between CD46 and CD34 (Fig. 1b). None of the 23 variants with association $P < 1 \times 10^{-5}$ at the locus were coding, nor were they reported in the genome-wide association study (GWAS) catalog or as expression quantitative trait loci (eQTLs) (Supplementary Table 3). However, one of these variants, rs2724384, which is intronic in CD46 and is highly correlated with rs1318653 ($r^2 = 0.95$), has been reported in candidate gene studies to associate with the immune response after MMR 15,16 and measles virus17 vaccination. The rs2724384 variant was therefore also genotyped in the replication stage and reached genome-wide significance in comparisons of MMR-related febrile seizures to both controls and MMR-unrelated febrile seizures (Supplementary Table 2). The risk allele (rs2724384[A]) for MMR-related febrile seizures corresponds to increased measles-specific IgG antibody levels15–17 and reduced interleukin (IL)-6, interferon (IFN)-$\alpha$ and tumor necrosis factor (TNF)-$\alpha$ secretion after stimulation with vaccine-strain measles virus 18. Furthermore, rs2724384[A] is associated with increased expression of exons 7 and 8 of CD46 (ENST00000358170 transcript) in lymphoblastoid cell lines as well as increased expression of the CD46-004 transcript (ENST00000367042) relative to other transcripts and increased overall expression of the gene11.

CD46 encodes a type I membrane protein that is a regulatory part of the complement system, induces the proliferation...
and differentiation of regulatory T cells\textsuperscript{19}, and acts as a cellular receptor for measles virus\textsuperscript{20,21}, primarily vaccine-strain virus\textsuperscript{22}.

**Associations for febrile seizures in general**

Variants at four loci reached genome-wide significance in the analysis of febrile seizures overall versus controls, and none of these differed between MMR-related febrile seizures and MMR-unrelated febrile seizures (Table 1 and Supplementary Table 2). A genetic risk score based on these four loci thus showed no effect in a logistic regression analysis of MMR-related febrile seizures versus MMR-unrelated febrile seizures ($P = 0.22$) but was highly significant in comparisons of MMR-related febrile seizures, MMR-unrelated febrile seizures and febrile seizures overall versus controls ($P < 2 \times 10^{-16}$ in all three analyses). In the febrile seizures overall versus controls analysis, the 10% of children with the highest genetic risk scores were at almost four times higher risk than the 10% of children with the lowest risk scores ($OR = 3.73, 95\% CI = 3.06–4.56$). We found no evidence of interaction between the four top SNPs, and their effect estimates were also not changed by conditioning on the two SNPs for MMR-related febrile seizures listed in Table 1 (data not shown). The loci that were genome-wide significant in the febrile seizures overall versus controls analysis were also selected for genotyping in an auxiliary replication set of individuals with febrile seizures with 25 or more years of follow-up whose children did not have any epilepsy diagnosis. Three of the four sets of individuals with febrile seizures with 25 or more years of follow-up were also selected for genotyping in an auxiliary replication set. In the febrile seizures overall versus controls analysis, the 10% of children with the highest genetic risk scores were at almost four times higher risk than the 10% of children with the lowest risk scores ($OR = 3.73, 95\% CI = 3.06–4.56$). We found no evidence of interaction between the four top SNPs, and their effect estimates were also not changed by conditioning on the two SNPs for MMR-related febrile seizures listed in Table 1 (data not shown). The loci that were genome-wide significant in the febrile seizures overall versus controls analysis were also selected for genotyping in an auxiliary replication set of individuals with febrile seizures with 25 or more years of follow-up whose children did not have any epilepsy diagnosis. Three of the four sets of individuals with febrile seizures with 25 or more years of follow-up were also selected for genotyping in an auxiliary replication set.

The second locus was also on chromosome 2q24.3, in a region containing $SCN1A$, $TTT21B$ and the noncoding transcripts $LCO100506124$ and $TTT21B-AS1$ (Fig. 2b and Supplementary Table 6). Four SNPs at the loci were genotyped in the replication stage, all reaching genome-wide significance (Supplementary Table 2). The lowest $P$ value was seen for rs6432860 ($OR = 1.34, 95\% CI = 1.25–1.43$; $P = 2.2 \times 10^{-16}$), a synonymous SNP in $SCN1A$ and an eQTL for $TTT21B$ in the liver\textsuperscript{26}. Among the 238 variants with association $P < 1 \times 10^{-5}$ at the locus, rs5787026 was reported to associate with mesial temporal lobe epilepsy with hippocampal sclerosis and febrile seizures\textsuperscript{27} in a recent GWAS meta-analysis, with the reported risk allele (rs5787026[A]) corresponding to increased risk of febrile seizures in our data (Supplementary Table 6). Another associated SNP at the locus, rs3812718, affects alternative splicing of $SCN1A$ in brain tissue\textsuperscript{27,28} and was significantly associated with febrile seizures in general in two relatively small sample sets but not in a third\textsuperscript{27}. Again, the reported risk allele (rs3812718[A]) corresponded to increased risk of febrile seizures in our data (Supplementary Table 6). $SCN1A$ encodes the voltage-gated $Na^+$ channel $\alpha$ subunit Nav1.1, which is expressed predominantly in the axon initial segment of inhibitory interneurons\textsuperscript{29}. Rare mutations in $SCN1A$ cause a wide spectrum of epilepsy syndromes, including genetic epilepsy with febrile seizures plus (GEFS+) and Dravet syndrome (also known as severe myoclonic epilepsy of infancy$^5$), depending on the nature of the mutation and possible genetic modifiers in other genes\textsuperscript{30}.

In the larger region encompassing both loci, rs3769955 and rs6432860 are 660 kb apart in different LD blocks, with $r^2 = 0.02$ and $D' = 0.24$ between the two SNPs on the basis of the replication-stage genotypes. Conditioning on either SNP left little residual association signal in its own LD block while only mildly attenuating the signal in the other block (Supplementary Fig. 5a,b). In an

![Figure 3](image-url)  
**Figure 3** ANO3 is involved in the temperature response of hypothalamic neurons. Neurons in the AHN were classified on the basis of their temperature responses as heat sensitive (red), cold sensitive (blue), temperature insensitive (yellow) or silent (white). (a,b) The proportion of each type of neuron in AHN from wild-type (WT) (a) and Ano3-null (b) rats. (c,d) The firing frequencies of individual neurons from wild-type (c) and Ano3-null (d) rats recorded at 33 °C, 36.5 °C and 40 °C. A lower proportion of heat-sensitive neurons was detected in Ano3-null slices ($n = 30$ recorded neurons) in comparison with wild-type slices ($n = 30$ recorded neurons; $P = 0.005$, Fisher’s exact test).
Figure 4  Hippocampal CA1 pyramidal neurons exhibit hyperexcitability in the absence of ANO3. (a–d) Basic membrane properties, namely, resting membrane potential (\(V_m\)) (a), input resistance (\(R_i\)) (b), membrane capacitance (\(C_m\)) (c) and time constant (\(\tau\)) (d) at 36.5 °C and 40 °C for wild-type (WT) versus ANO3-null neurons (\(n = 7–8\)). *P < 0.05, two-way ANOVA followed by HSD (honestly significant difference) test. (e) Sample traces of neuronal responses to current injections of 40, 80 and 120 pA in wild-type versus ANO3-null CA1 pyramidal neurons at 36.5 °C. (f) Stepwise increases in current elicit more action potentials in ANO3-null neurons than in wild-type controls (\(n = 7–8\); **P < 0.01, *P < 0.05, wild type versus ANO3 null, two-way ANOVA). All error bars, s.e.m.

analysis conditional on both top SNPs, no SNP in the region achieved \(P < 5 \times 10^{-4}\) (Supplementary Fig. 5c).

Large-effect variants at ANO3

The most significant SNP at the third locus for febrile seizures overall on chromosome 11p14.2, rs114444506 (OR = 2.09, 95% CI = 1.79–2.44; \(P = 3.7 \times 10^{-20}\)), mapped to the first intron of the ANO3 (also known as TMEM16C) splice variant ANO3-201 (ENST00000537978) (Fig. 2c). None of the 30 variants with association \(P < 1 \times 10^{-5}\) at the locus were coding (Supplementary Table 6), nor were they reported in the GWAS catalog or as eQTLs. ANO3 belongs to the TMEM16 (anocyst) protein family, a group of ten homologous transmembrane proteins that includes at least two \(\text{Ca}^{2+}\)-activated chloride channels and other members about which less is known. Rare ANO3 missense mutations have been found to segregate with autosomal dominant craniofacial dysostosis, and high expression of the gene in human striatum, hippocampus and cortex has been documented.

It was recently reported that Ano3-null rats exhibit hyperexcitability of nociceptive neurons and a decreased threshold for pain. Below, we investigate the potential role of ANO3 in seizure genesis through electrophysiological recordings in brain slices from wild-type and Ano3-null rats.

A locus associated with serum magnesium levels

At the fourth locus for febrile seizures overall, the top SNP, rs11105468 (OR = 1.25, 95% CI = 1.17–1.33; \(P = 3.4 \times 10^{-11}\)), was located in an intergenic region on chromosome 12q21.33 (Fig. 2d). All 38 variants with association \(P < 1 \times 10^{-5}\) at the locus were intergenic (Supplementary Table 6); none were eQTLs, but several were reported in a GWAS of serum magnesium levels, with \(P = 3.8 \times 10^{-12}\) for rs11105468. For these SNPs, the allele associated with lower magnesium levels was associated with increased risk of febrile seizures in our data. It is well established that magnesium deprivation can lead to seizures in laboratory animals and humans, and in vitro experiments have shown that magnesium deficiency results in spontaneous epileptiform discharges in rat hippocampal brain slices. At the molecular level, magnesium ions block the channel pore of excitatory N-methyl-D-aspartate (NMDA) receptors under basal conditions. The magnesium blockade is relieved by cellular depolarization, thus allowing calcium and sodium to enter the postsynaptic neuron as potassium exits. To explore the role of other variants associated with magnesium levels, we looked up the top SNP at all nine confirmed and suggestive loci for serum magnesium levels, but, apart from the 12q21.33 locus, these loci were not associated with febrile seizures (Supplementary Table 7).

Electrophysiology of Ano3-null rats

We performed electrophysiological recording in brain slices from wild-type and Ano3-null rats to investigate potential mechanisms involving ANO3 in febrile seizure genesis. Given the role of the anterior hypothalamic nucleus (AHN) in thermoregulation, we first performed whole-cell patch-clamp recordings of AHN neurons to determine the effect of ANO3 on spontaneous action potential (SAP) firing patterns at different temperatures. Recordings were made in slices from male rats on postnatal day (P) 10–12 at 33 °C, 36.5 °C and 40 °C, and we found a significantly lower proportion of heat-sensitive neurons (increased SAP firing with increasing local brain temperature) in Ano3-null rats in comparison to wild-type rats (Fisher’s exact test, \(P = 0.005\); \(n = 30\) neurons for each group; Fig. 3; see Supplementary Fig. 6 for the distribution and comparable membrane properties of AHN neurons from wild-type and Ano3-null rats).

The hippocampus is often the focus of seizures; hence, we next examined whether ANO3 influenced hippocampal neuronal excitability. We performed whole-cell current–clamp recordings of hippocampal pyramidal neurons from P14 male wild-type and Ano3-null rats at different temperatures. Slice recordings from CA1 pyramidal neurons showed that the resting membrane potential (\(V_m\)) was more depolarized by 4–5 mV in Ano3-null rats than in wild-type controls at room temperature (Student’s \(t\) test, \(P < 0.05\); \(n = 9–11\) neurons per group; Supplementary Fig. 7a). Furthermore, stepwise injections of current showed that neurons from Ano3-null rats fired more action potentials than wild-type neurons with the same amount of injected current (Supplementary Fig. 7c.f). To mimic shifts in body temperature during fever, we performed similar experiments at 36.5 °C and 40 °C and found that hippocampal neurons without Ano3 displayed increased excitability at both temperatures (two-way ANOVA, \(P < 0.01\); Fig. 4).

DISCUSSION

In this work, designed to investigate both the genetics of an adverse effect of vaccination and febrile seizures, we found that two loci were distinctly associated with febrile seizures as an adverse event following MMR vaccination and that four additional loci were associated
with febrile seizures in general. Further, in the absence of ANO3, hypothalamic neurons were less responsive to heat, which could lead to impaired homeostatic control when body temperature rises, and hippocampal neurons became hyperexcitable, which could possibly contribute to febrile seizure genesis.

Our findings, implicating loci harboring the innate immune system genes IFI44L and CD46, represent a first step in understanding the biological mechanisms underlying febrile seizures as an adverse effect of MMR vaccination. An important next step will be to elucidate the pathways by which the identified variants influence the immune response and contribute to the development of fever, seizures or both. One possibility might be that the pathogenic mechanism of MMR-related febrile seizures involves two independent steps: febrile response influenced by the distinct MMR-related febrile seizure variants and, then, given fever, seizure response influenced by the general febrile seizure variants. A genetic study of children with detailed information about febrile response after MMR vaccination would be needed to determine whether the IFI44L and CD46 variants are also associated with specific fever patterns in individuals who are not susceptible to febrile seizures. Other future investigations are required to identify the precise identity of the causal variants at the loci and to determine whether the variants are associated with response to other vaccines or to live-virus infections. Eventually, such knowledge may translate into improved vaccine design or personalized vaccination strategies.

With respect to febrile seizures in general, SCN1A and SCN2A are strong functional candidates at the two independent 2q24.3 loci, as variants in these genes have been linked to a range of epilepsy syndromes, some involving febrile seizures. Some observations are worth noting. First, variants affecting SCN2A function are likely to show age-dependent changes in effect because Na\textsubscript{v}1.2 channels are expressed early in development in the axon initial segments of principal neurons but are gradually replaced by Na\textsubscript{v}1.6 channels during maturation. In mice, this transition occurs gradually from about P15 onward; in humans, it has been suggested as a possible explanation for the age-dependent remission of seizures in BFNIS\textsuperscript{25} and might also have a role in the spontaneous remission of febrile seizures around 6 years of age, if a causal link with SCN2A function underlies the febrile seizure association seen in our data. Second, given the predominant expression of the Na\textsubscript{v}1.1 and Na\textsubscript{v}1.2 channels in the axon initial segments of inhibitory interneurons and excitatory pyramidal neurons, respectively, it is conceivable that the SCN1A variants affect risk of febrile seizures through decreased activity of the inhibitory circuitry, whereas the SCN2A variants act directly by increasing the activity of excitatory neurons. Third, rare SCN1A missense mutations are commonly found in Dravet syndrome and GEFS+, two epilepsy syndromes that include febrile seizures as part of the clinical presentation. Mice heterozygous for Scn1la loss-of-function mutations show a severe phenotype resembling Dravet syndrome\textsuperscript{29,40}, whereas mice heterozygous for the Scn1la missense mutations seen in GEFS+ only have partial loss of channel function and show a much less severe phenotype\textsuperscript{40,41}. In line with this pattern, we expect future investigations to uncover more subtle effects of the SCN1A variants identified here on susceptibility to febrile seizures, for example, involving decreased gene expression or altered regulation of alternative splicing.

The 12q21.33 association indicates that revived research into the role of magnesium deficiency in seizure susceptibility is warranted. In clinical practice, magnesium sulfate has long been used as an effective treatment for the seizures of neonatal tetany\textsuperscript{42} and eclampsia\textsuperscript{43}, and oral magnesium supplementation has been suggested as an adjunct therapy in patients with drug-resistant epileptic seizures\textsuperscript{44,45}. We note that other previously reported magnesium-related loci were not associated with febrile seizures. However, the previous findings were based on serum concentrations measured in adult participants\textsuperscript{34}, and different loci may regulate distinct aspects of magnesium metabolism, such as tissue-specific bioavailability, over a lifetime.

The implication of ANO3 variants in susceptibility to general febrile seizures opens novel avenues for future research in the field of seizure disorders. In comparison to typical GWAS findings in other complex diseases, the OR estimate of 2.09 is unusually high and, together with the supportive electrophysiological results, underlines the importance of ANO3 as a target for further inquiry. In nociceptive dorsal root ganglion neurons, ANO3 acts indirectly by modulating the properties of the sodium-activated potassium (KNa) channel KCNT1 (SLACK)\textsuperscript{33}, but it is unclear whether this is also the case in central neurons, for example, hippocampal and hypothalamic neurons. Rare KCNT1 mutations have been reported in two early-onset epileptic disorders\textsuperscript{16,47}, and it will be interesting to determine whether the mechanism underlying the association with febrile seizures reported here involves altered cellular excitability through interaction between ANO3 and KCNT1.

Given the occurrence of febrile seizures in several epilepsy syndromes, one might speculate whether our association findings for general febrile seizures could be driven by the inclusion of infants who would later develop epilepsy, for example, GEFS+ or Dravet syndrome. We consider this scenario to be highly unlikely, as only a small fraction of febrile seizure cases are expected to later develop epilepsy\textsuperscript{2}. Thus, an epilepsy-associated variant would need to have an extremely large effect in this set and be in strong LD with the top SNP at one of the loci for general febrile seizures to drive the association. However, among the common SNPs at the four loci, the effect size for the only previously reported genome-wide significant epilepsy-related SNP, rs75870206, was modest (OR = 1.42)\textsuperscript{27}, and it is implausible that rare large-effect variants in SCN1A and SCN2A known to cause familial epilepsies can explain the associations for the common SNPs (risk allele frequencies of >0.4) reported here\textsuperscript{48}. Furthermore, we found that the results did not change when we excluded febrile seizure cases who later developed epilepsy (Supplementary Table 5) and that three of the four loci replicated when using an auxiliary set of febrile seizure cases with more than 25 years of follow-up without any records of epilepsy (Supplementary Table 4); the association signal for rs376995 at the fourth locus was consistent with the replication-stage results in the main analysis (Table 1).

Our study was restricted to individuals of Danish descent, and further studies are needed to examine the effects of the identified variants in populations of different ancestry. Several of the six SNPs in Table 1 show substantial differences in allele frequency, particularly between East Asians and other populations (Supplementary Fig. 8). The incidence of febrile seizures varies considerably in different populations across the world. In Japan, 6–9% of children experience febrile seizures in comparison to 2–5% of children of European descent\textsuperscript{3,49}, and genetic studies in East Asians or other populations might identify different loci for febrile seizures. Further studies are also required to identify the functionally relevant variants at each locus and to examine their effects in thoroughly characterized febrile seizure samples across the entire phenotypic spectrum, from isolated febrile seizures (simple or complex) to febrile seizures occurring in specific epilepsy syndromes such as GEFS+ or Dravet syndrome.

In conclusion, using detailed health register information on vaccinations and febrile seizure episodes, we identified common variants at two loci associated with febrile seizures as an adverse event following MMR vaccination.
vaccination. From a public health perspective, it is essential to study the underlying cause of any serious adverse event of the MMR vaccine, a preventive pharmaceutical product given to millions of children each year, and our findings provide important leads for further research in the fields of immunogenetics and vaccinology. Concomitantly, we identified four loci associated with febrile seizures in general, which, together with supporting evidence from electrophysiological experiments, underline the importance of altered ion channel function in this common childhood disorder. Further functional studies will illuminate the biological mechanisms behind the associations reported here and might also provide more general insights into mechanisms of epileptogenesis and neuronal hyperexcitability.

URLs. Danish National Biobank, http://www.biobankdenmark.dk/; 1000 Genomes Project, http://www.1000genomes.org/; International HapMap Project, http://www.hapmap.org/; Ensembl browser, http://www.ensembl.org/; GWAS catalog, http://www.genome.gov/gwastudies/; NCBI Genotype-Tissue Expression (GTEx) eQTL database and browser, http://www.ncbi.nlm.nih.gov/projects/gap/eqtli/index.cgi; Blood eQTL browser, http://www.broadinstitute.orgalandoucet/eqtli/browser/; GEUVADIS data browser, http://www.ebi.ac.uk/tools/geuvadis-das/; R software, http://www.r-project.org/.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.F., B.P., F.G. and M.M. carried out the project and designed the manuscript. B.P., H.S., M.V. and A.H. planned and performed register data acquisition, informatics and phenotypic characterization. B.F., F.G. and L.C. carried out the statistical genetics and bioinformatics analyses. M.V.H. and D.M.H. performed sampling, whole-genome amplification and genotyping. J.E.E. and L.W.S. performed the electrophysiology experiments and analyzed the data. T.W., F.H. and L.Y. designed and performed the electrophysiology experiments and analyzed the data. All authors contributed to the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Subjects. The cases for both the discovery and replication stages were identified from the Danish National Patient Register, which includes individual-level information from all hospitals in Denmark, including physician-assigned diagnoses and dates of hospital contact. The register includes information on all inpatient admissions since 1977 and all emergency and outpatient hospital contacts since 1995, with diagnostic information coded according to the International Classification of Diseases (ICD; version 8 through 1993 and version 10 from 1994 on). The positive predictive value of a diagnosis of febrile seizures (ICD-8 and ICD-10) recorded in the register is 93% (ref. 51).

Information on other medical conditions was similarly obtained from the Danish National Patient Register. Data on gestational age at birth were derived from the Danish Medical Birth Register, which records detailed information on all births in the country. Information on vaccination status and date of vaccination was obtained from the Childhood Vaccination Database at Statens Serum Institut. Two brands of MMR vaccine have been in use in Denmark over the period during which cases were recruited to the study. MMR II (Sanofi Pasteur MSD (in the United States, Merck)) was used through 17 October 2008; this vaccine contains the Edmonston measles strain, the Jeryl Lynn mumps strain and the Wistar RA27/3 rubella strain. From 18 October 2008 on, Priorix (GlaxoSmithKline Biologicals) has been used; this vaccine contains the Schwarz measles strain, the Jeryl Lynn mumps strain and the Wistar RA27/3 rubella strain. Varicella immunization is not included in the national vaccination program in Denmark. Deidentified information was linked between these sources of data, which all have nationwide coverage, through the use of unique personal identifiers.

Cases for the discovery stage were identified from a background population of children born in Denmark between 1 January 1991 and 31 December 2008, with follow-up for an index event of febrile seizures between 1 January 1992 and 1 January 2010. After the identification of febrile seizure cases associated with MMR vaccination, cases of febrile seizures with no association with MMR vaccination were matched according to the calendar year in which the index event occurred. Cases for the replication stage were identified from a background population of children born in Denmark between 1 January 1991 and 30 September 2011, with follow-up for an index event of febrile seizures between 1 January 1992 and 30 September 2012. ICD-8 code 78021 and ICD-10 code R560 were used for case identification. A vaccine-associated case was defined as a case of febrile seizures that occurred in a risk window of 9 to 14 days after the date of MMR vaccination. A febrile seizure case with no association with MMR vaccination was defined as a case that occurred 6 weeks or more after vaccination or in an infant with no vaccine exposure (the risk of febrile seizures transiently increases after MMR vaccination and is back to baseline risk by 4 weeks after vaccination); our definition of cases with no association with vaccination was thus conservative with regard to the time window after vaccination). All cases were required to be between 1 and 2 years of age at the index date of the febrile seizure event and were allowed to have experienced additional febrile seizure events either before 1 year of age or after the index event. Follow-up information from the Danish National Patient Register was available through 11 January 2014. At the end of the follow-up period, the febrile seizure cases were between 5.1 and 23.0 years old (median of 15.1 years) in the discovery stage and between 2.6 and 23.0 years old (median of 10.0 years) in the replication stage. As an analysis for sensitivity, we conducted association testing excluding all febrile seizure cases with an epilepsy or non–febrile seizure diagnosis code during follow-up. Furthermore, an independent set of febrile seizure cases with 25 or more years of follow-up without any epilepsy diagnosis were included in an additional replication-stage analysis.

Population controls (n = 4,118) for the discovery stage were selected from individuals with GWAS data from various Illumina Omni arrays generated in other research projects at Statens Serum Institut, excluding individuals with febrile seizures or epilepsy diagnosis codes in the Danish National Patient Register. Controls for the replication stage were randomly selected among children from the Danish National Birth Cohort who had participated in all surveys, including the 11-year follow-up investigation, and who did not have any febrile seizures or epilepsy diagnosis code. Sample characteristics and inclusion criteria for cases and controls are shown in Supplementary Table 1.

To ensure a high degree of genetic homogeneity in the genotyped sample, we obtained birthplace information from the Civil Registration System and only included subjects who were born in Denmark and whose parents and grandparents were not born outside of northwestern Europe. The study was approved by the Scientific Ethics Committee for the Capital City Region (Copenhagen) and the Danish Data Protection Agency. The Scientific Ethics Committee also granted exemption from obtaining informed consent from participants (H-3-2010-003) as the study was based on biobank material.

Sampling, amplification and genotyping. All samples were drawn from the Danish Newborn Screening Biobank and the Danish National Birth Cohort biobank, both of which are part of the Danish National Biobank. All cases and controls were sampled using two 3-mm punches from dried blood spot samples. Genomic DNA was extracted using the Extract-N-Amp kit (Sigma-Aldrich) and subjected to whole-genome amplification in triplicate using the Repli-g kit (Qiagen) at Statens Serum Institut as previously described. All 6,117 samples in the discovery stage of the GWAS were genotyped with Illumina Omni Bead arrays and GenomStudio software. Febrile seizure cases (n = 1,999) were genotyped using the HumanOmniExpressExome-8 v1.1 array; controls were genotyped using the HumanOmniExpressExome-8 v1.1 array (n = 1,931), the HumanOmniExpress-12v1_H array (n = 1,173) or the HumanOmni-Quad v1.0 array (n = 1,014). For the replication stage, we sampled 408 cases with febrile seizures after MMR vaccination, 1,035 febrile seizure cases unrelated to MMR vaccination, 1,647 controls and 515 febrile seizure cases with 25 years of follow-up without any epilepsy diagnosis. Genomic DNA was extracted from punches of dried blood spot samples and amplified using the same protocol as in the discovery stage. Genotyping for the selected replication-stage SNPs was performed using competitive allele-specific PCR (KASP) chemistry (LG Genomics).

Data cleaning and imputation. The data cleaning process was initiated by aligning all genotypes to the forward strand and restricting the data to the 615,786 SNPs that were available on all 3 of the different Omni arrays used in the study. Next, we excluded individuals that (i) had more than 4% missing genotypes, (ii) had an autosomal heterozygosity rate deviating by more than 2.5 s.d. from the mean, (iii) had discordant sex information or (iv) were more than 6 s.d. away from the mean of any of the first five principal components in a principal-component analysis. We then excluded SNPs on the basis of a missing rate of >2%, a MAF of <0.01 and deviations from Hardy-Weinberg equilibrium (P < 1 × 10−8). Finally, we excluded SNPs that showed differential missingness between arrays, differences in allele frequencies between arrays or differences in allele frequencies between male and female subjects. The remaining 548,642 SNPs were used for imputation. We employed a two-step procedure to impute unobserved genotypes using phased haplotypes from the integrated Phase I release of the 1000 Genomes Project. In a first prephasing step, we used SHAPEIT to estimate the haplotypes for our study samples. In a second step, we imputed the missing alleles for additional SNPs directly onto these phased haplotypes using IMPUTE2 (ref. 59). We chose imputed SNPs or indels with MAFs of >1% and SNPTEST value of >0.8 for further analyses. Depending on the analysis, this filtering yielded 8,129,533 (febrile seizures overall versus controls), 8,129,524 (MMR-related febrile seizures versus controls), 8,129,384 (MMR-unrelated febrile seizures versus controls) or 8,129,288 (MMR-related febrile seizures versus MMR-unrelated febrile seizures) imputed genetic variants. To further assess the imputation accuracy of the six genome-wide significant SNPs in Table 1, these SNPs were genotyped in a subset of 762 discovery-stage samples (181 MMR-related febrile seizure cases, 202 MMR-unrelated febrile seizure cases and 379 controls) using KASP assays. The concordance between observed allele counts and imputed allele dosages was high (all six SNPs had r2 > 0.96), indicating that imputation was accurate for these SNPs.

Association analysis. We used logistic regression to test for differences in allele dosage between cases and controls under an additive genetic model. We carried out combined analysis of the discovery- and replication-stage data using the inverse variance method, applying genomic control to the discovery-stage results. The genomic inflation factors were 1.01, 1.00, 1.02 and 1.03 for the four scans (MMR-related febrile seizures versus controls, MMR-related
febrile seizures versus MMR-unrelated febrile seizures, MMR-unrelated febrile seizures versus controls and febrile seizures overall, respectively), indicating minimal population stratification. In line with this observation, association results were essentially unchanged when adjusting for the first five principal components from our principal-components analysis. We therefore report results where test statistics were scaled by genomic control using the genomic inflation factors but where no further adjustment was made on the basis of principal components. We estimated heterogeneity between the discovery and replication results using the $\Phi$ statistic. To explore possible allelic heterogeneity, we conducted analyses conditioning on the top SNP at each of the selected loci. Using the combined discovery and replication data, we tested for interaction effects between the two loci associated with febrile seizures after MMR vaccination and also between the four loci associated with febrile seizures in general by including risk allele count at each locus in a logistic regression model together with pairwise interaction terms. We evaluated the combined impact of the associated loci by constructing genetic risk scores for all individuals in the discovery and replication samples. For each SNP, a weight (log(OR)) was multiplied by the number (or dosage) of risk alleles. The genetic risk scores were then calculated by summation over the two SNPs associated with MMR-related febrile seizures or by summation over the four SNPs associated with febrile seizures overall. We used the weighted risk scores in logistic regression analyses. The association analyses were conducted using SNPTEST, METAL, and R software.

Power analysis. For each of the four scans, we estimated the power of the discovery sample at a significance threshold of $P < 1 \times 10^{-6}$ (Supplementary Table 8), as this threshold was used to select SNPs for replication genotyping (Supplementary Fig. 3). Power estimates are presented at representative and relevant ORs (OR = 1.25, OR = 1.4, OR = 1.5 and OR = 2.0) and risk allele frequencies (0.05, 0.20, 0.30, 0.40 and 0.70). The power analyses were performed using the Genetic Power Calculator.

Bioinformatics analysis. For each locus with genome-wide significant SNPs, we explored possible functional effects of the associations by considering all genotyped or imputed variants with association $P < 1 \times 10^{-8}$ at the locus. We searched the National Human Genome Research Institute (NHGRI) GWAS catalog and the NCBI Genotype-Tissue Expression (GTEx) database for previously reported trait or eQTL associations for these variants using $P$-value thresholds of $5 \times 10^{-8}$ and $1 \times 10^{-8}$, respectively. Furthermore, we searched the Blood eQTL browser for cis- and trans-eQTL associations in peripheral blood and the GEUVADIS data browser for exom- and transcript-level eQTL associations in lymphoblastoid cell lines. Ensembl (release 74) IDs were used for the annotation of transcripts. MutationTaster and PolyPhen-2 were used to predict the deleteriousness of missense mutations.

Cell-based assays. Assays to assess the impact of ectopic ISG expression on virus infection have been described previously. Briefly, SCFPSY lentiviral vectors (provided by P. Bieniasz (Rockefeller University)) were used to express the IFI44L variants or an empty cassette as control. Lentiviral-transduced STAT1−/− fibroblasts (originally from the laboratory of J.-L. Casanova (Rockefeller University)) were infected at a multiplicity of infection (MOI) of 1.0 with Edmonston strain measles–GFP (provided by J.-L. Casanova (Rockefeller University)) were infected at a multiplicity of infection (MOI) of 1.0 with Edmonston strain measles–GFP (provided by J.-L. Casanova (Rockefeller University)).

Brain slice preparation. Ano3-null rats and their wild-type littermates were bred at the University of California, San Francisco (UCSF), as reported previously, and used for whole-cell patch-clamp recordings; they were maintained under a 12-h light/12-h dark schedule and consumed food and water ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at UCSF and are fully compliant with US National Institutes of Health (NIH) guidelines for the humane treatment of animals.

Rats at P10−P14 were anesthetized with isoflurane and decapitated. Brains were removed and submerged in ice-cold sucrose slicing solution (2.5 mM KCl, 10 mM MgSO4, 7H2O, 0.5 mM CaCl2, 2H2O, 1.25 mM NaH2PO4, 26 mM NaHCO3 and 10 mM glucose, pH 7.2−7.4, saturated with 95% O2/5% CO2) at 37 °C for 20 min to allow the slices to recover from the treatment in ice-cold solution. Slices were further incubated for at least 1 h at room temperature before recordings were performed at various temperatures.

Electrophysiology. Whole-cell patch electrodes had pipette tip resistances of 4−6 MΩ and were filled with a solution containing 122 mM potassium gluconate, 13 mM KCl, 0.07 mM CaCl2, 1.0 mM MgCl2, 0.1 mM EGTA, 10.0 mM HEPES, 4.0 mM Na-ATP and 0.4 mM Na-GTP, pH 7.3, with an osmolality of 290−300 mOsm/l. Recordings were performed using a Multiclamp 700B amplifier (Molecular Devices). Signals were sampled at 10 kHz, subject to low-pass filtering at 10 kHz using a Digidata 1440 digitizer and stored on the computer for subsequent analyses using pClamp software (Molecular Devices). Liquid junction potential was corrected in reported results. In patch-clamp recordings, access resistance (<1 MΩ) was continuously monitored throughout each experiment. If the fluctuation deviated by more than 20% from baseline values, the cell was regarded as unhealthy or an unsuccessful patch and the recording was excluded. The investigators were blinded to the genotype of the rats from which the cells were obtained when performing electrophysiological recordings.

Whole-cell patch recording was initiated by breaking into the cell under current-clamp mode and then performing a stepwise increase in current (each step with a duration of 400 ms) from −100 pA to 120 pA, with a 20-pA increment; the basic membrane properties (at 36.5 °C for AHN neurons and at room temperature, 36.5 °C and 40 °C for hippocampal neurons), including resting membrane potential (Vm), input resistance (Rin), membrane capacitance (Cm) and the time constant (τ) were obtained. Specifically, Vm was obtained directly with a holding current of 0 pA. Rin was determined from the slope of the current-voltage (I-V) relationship with the 0-pA holding current by linear regression. τ was determined from the voltage response to a current injection of −20 pA by exponential regression and Cm was calculated as τ−Rin. In hippocampal recordings, we compared the frequency of the action potentials elicited by the injection of various amounts of current into wild-type and Ano3-null neurons, at room temperature, 36.5 °C and 40 °C. In hypothalamic recordings, the firing rate was monitored after shifts in bath temperature between 33 °C and 40 °C. The bath temperature was controlled using an inline heater (Warner, SC-20). SAPs were counted at the corresponding temperature, and neurons were classified on the basis of their responses: temperature-insensitive neurons had the same frequency of SAPs at 33 °C, 36.5 °C and 40 °C; heat-sensitive neurons exhibited a decrease in firing rate during cooling and an increase in the SAP firing rate during warming; cold-sensitive neurons showed the opposite temperature dependence; and silent neurons did not discharge SAPs during the duration of recording.
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