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Journal

Science Advances, 11(16)

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Publication Date

2025-04-18

DOI

10.1126/sciadv.adr9986

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IMMUNOLOGY

Permissive central tolerance plus defective peripheral checkpoints license pathogenic memory B cells in CASPR2-antibody encephalitis

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Autoantibody-mediated diseases targeting one autoantigen provide a unique opportunity to comprehensively understand the development of disease-causing B cells and autoantibodies. Convention suggests that such autoreactivities are generated during germinal center reactions. Here, we explore earlier immune checkpoints, focusing on patients with contactin-associated protein-like 2 (CASPR2)-autoantibody encephalitis. In both disease and health, high (~0.5%) frequencies of unmutated CASPR2-reactive naïve B cells were identified. By contrast, CASPR2-reactive memory B cells were exclusive to patients, and their B cell receptors demonstrated affinity-enhancing somatic mutations with pathogenic effects in neuronal cultures and mice. The unmutated, precursor memory B cell receptors showed a distinctive balance between strong CASPR2 reactivity and very limited binding across the remaining human proteome. Our results identify permissive central tolerance, defective peripheral tolerance, and autoantigen-specific tolerance thresholds in humans as sequential steps that license CASPR2-directed pathology. By leveraging the basic immunobiology, we rationally direct tolerance-restoring approaches, with an experimental paradigm applicable across autoimmunity.

INTRODUCTION

Across autoimmunity, few diseases are proven to be mediated by autoantibodies that target a single antigen (1). A comprehensive understanding of the developmental autoantigen-reactive B cell lineage, and their escape from immune checkpoints, is likely sufficient to fully explain the pathway to disease causation (2, 3). Hence,

prototypical autoantibody-mediated conditions provide unique biological and translational opportunities.

The recent discovery of several causative autoantibodies that target cell surface neuronal proteins has revolutionized the diagnosis of multiple neurological conditions, most notably forms of autoimmune encephalitis (AE) (4). One such protein is contactin-associated protein-like 2 (CASPR2). Autoantibodies against the extracellular domain of CASPR2 associate with a common form of AE (CASPR2-Ab-E) which presents with memory loss, behavioral disturbances, seizures, cerebellar dysfunction, and neuropathic pain, consistent with the expression of CASPR2 in both central and peripheral nervous systems (5–8). The direct pathogenicity of CASPR2 antibodies is supported by the passive transfer of polyclonal patient serum immunoglobulin G (IgG) to rodents, which reproduces core clinical features observed in patients with CASPR2-Ab-E (9, 10). Despite some improvements with immunotherapies, nearly all patients with CASPR2-Ab-E remain disabled by multiple residual neuropsychiatric deficits or persistent neuropathic pain (5–8, 11). Furthermore, around 40% of patients relapse despite immunotherapies (6, 7). Current treatment options remain limited to broad-acting immunotherapies including corticosteroids, rituximab, and intravenous Igs (5, 7, 11–13). Precise immunotherapeutic paradigms for AE are needed to prevent the accumulation of irreversible neurologic dysfunction and to mitigate adverse effects commonly encountered with available immunotherapies.

To this end, a better understanding is required of the underlying immunological mechanisms driving CASPR2-Ab-E. The origins and inadvertent escape of autoreactive B cells form the fundamental

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pathway to pathogenic autoantibody production. In this process, key immune checkpoints need to be traversed by autoreactive B cells (14). These include a “central tolerance” checkpoint, governing bone marrow exit and entry to the circulating naïve B cell (NBC) compartment (15). Thereafter, several peripheral checkpoints likely oversee entry into the later NBC, memory B cell (MBC), and plasma cell repertoires (15–17). Examples of how autoantigen-specific B cells are tolerized in these processes are limited, with no mechanistic exploration in neurological disorders to date.

Nevertheless, a few clues have emerged. Paradigms from nonneurological and neurological autoantibody-mediated diseases suggest that the autoantigen specificity of MBC-derived B cell receptors (BCRs) is lost when BCR somatic hypermutations are reverted to their unmutated common germline ancestors (UCAs) (18–20). Although not a universal finding (21), this observation strongly implicates germinal centers as key sites that generate higher-affinity, pathogenic autoantigen-reactive BCRs. In contrast, more recent evidence in autoantibody-mediated neurological diseases suggests a more prominent role for autoantigen-specific NBCs. NBC BCRs recognize their cognate autoantigen aquaporin-4 in patients with neuromyelitis optica spectrum disorder (22), and autoantigen-specific unmutated BCRs with pathogenic potential have been detected in the cerebrospinal fluid of patients with *N*-methyl-D-aspartate receptor (NMDAR) antibody encephalitis (23). These findings led us to hypothesize that early loss of B cell tolerance may represent an underappreciated phenomenon in autoantibody-mediated neurological conditions. Furthermore, as CASPR2-antibodies have been reported in sera from healthy individuals and disease controls (24), we reasoned that CASPR2-Ab-E represents an elegant paradigm to evaluate how B cells traverse immune checkpoints in both health and disease.

In this study, we isolated 37 CASPR2-reactive BCRs from both NBCs and MBCs across patients with CASPR2-Ab-E and healthy controls (HCs) and compared their frequencies, biophysical characteristics, and functional properties. Our findings describe the thresholds of autoantigen reactivities in human BCRs that facilitate escape from key checkpoints. Hence, we inform the earliest fundamental events in the development of causative pathogenic CASPR2-reactive BCRs and establish mechanisms underlying dysregulated B cell tolerance as a plausible rationale for tolerance-restoring therapeutics (25).

RESULTS

CASPR2-reactive tolerance defects

To investigate the integrity of pre- and postgerminal center tolerance to CASPR2-reactive BCRs in both health and disease, either 10^5 NBCs ($CD19^+IgD^+CD27^-$) or MBCs ($CD19^+IgD^-CD27^+$) were isolated from six patients with CASPR2-Ab-E, eight patients with NMDAR- and leucine-rich glioma inactivated 1 (LGI1)-antibody encephalitis, plus six HCs, and bulk cultured under conditions that promote IgM and IgG secretion (Fig. 1A and table S1). Culture supernatants were screened for human CASPR2 reactivity using a live cell-based assay, with human embryonic kidney (HEK) 293T cells surface expressing full-length human CASPR2. Bulk NBC supernatants from patients with CASPR2-Ab-E, age-gender-matched disease controls, and all HCs contained IgMs that bound the extracellular domain of CASPR2 (Fig. 1B). In contrast, only MBC supernatants from patients contained CASPR2-IgG. Supernatants displayed no reactivity toward two other central nervous system autoantigens, including LGI1 and aquaporin-4 (fig. S1A).

To enumerate this CASPR2 reactivity at the individual B cell level and, in parallel, isolate the corresponding cognate-paired heavy- and light-chain BCR sequences, we performed single B cell cultures (26). Twelve thousand one hundred fifty-eight single NBCs or MBCs from the blood of two untreated patients with CASPR2-Ab-E and two HCs were sorted (fig. S1B) and cultured, and supernatants were screened for CASPR2 reactivities (Fig. 1A and table S1). Aligned with the bulk culture findings, CASPR2-IgMs were detected in both patient and healthy donor NBC supernatants at similar frequencies [16 of 4512 (0.4%) versus 11 of 1920 (0.6%); $P = 0.21$], while CASPR2-IgG was detected exclusively in patient MBCs [10 of 3806 (0.26%) versus 0 of 1920; $P = 0.037$, Fisher's exact test; Fig. 1C]. After sequencing, two clonal populations were observed within the MBCs (Fig. 1C and table S2). Next, CASPR2 monoclonal antibodies (mAbs) were generated from all culture wells with CASPR2-reactive supernatants by cloning cognate-paired heavy- and light-chain BCR sequences into expression plasmids. As expected, all mAbs bound the extracellular domain of human CASPR2 (Fig. 1A).

These concordant results across bulk and single B cell cultures suggested that ~0.5% of NBCs harbor CASPR2-reactive BCRs in both health and disease. However, from memory compartments, CASPR2-reactive BCRs were exclusively detected in CASPR2-Ab-E patients and included clonal expansions.

Origins of CASPR2 autoreactivity

Further BCR sequence analyses revealed that all NBC heavy- and light-chain variable regions, in both patients and HCs, contained no or very few mutations when compared to reported ancestral BCR gene segments (median, 0 nucleotides; range, 0 to 2; Fig. 2A and table S2). In distinction, memory BCRs were all mutated, often highly (median, 22 nucleotides; range 5 to 38, for heavy chains; median, 17; range 4 to 29, for light chains).

To explore how somatic hypermutation affected CASPR2 reactivity, memory BCRs were reverted to their corresponding UCAs. mAb affinity was determined by binding the extracellular domain of human CASPR2 to protein A-immobilized mAbs, using surface plasmon resonance (SPR). Memory mAb affinities varied by 10^6 -fold and included some in the “high affinity” picomolar range [measured affinity (K_D): 171 μ M to 152 pM]; their on and off rates were similarly heterogeneous (Fig. 2B and table S3). When expressed as UCAs, two mAbs (H01 and H02) lost detectable binding to CASPR2, both in IgG and pentameric IgM formats (Fig. 2B). In addition, one mAb (E04) lost reactivity as an IgG, but binding was detectable as an IgM. Overall, by comparison to memory counterparts, all UCAs showed a mean worsening of 29-fold in K_D (range, 1.4 μ M to 3.33 nM; Fig. 2C, left). K_D did not correlate with either heavy- or light-chain absolute mutation counts (Fig. 2C, middle and right).

Together, and consistent with the *ex vivo* isolation of naïve CASPR2-reactive BCRs, unmutated germline ancestors derived from MBCs usually retained detectable binding to CASPR2. However, somatic hypermutation conferred both improved CASPR2 reactivity and affinities, indicating the importance of mutations in generating the highest affinity CASPR2 binders.

Discrete conformationally dependent domain binding on native CASPR2

Having established binding kinetics, we determined other core antigenic properties that affect the binding of potentially pathogenic memory mAbs. First, to understand their preferred conformation

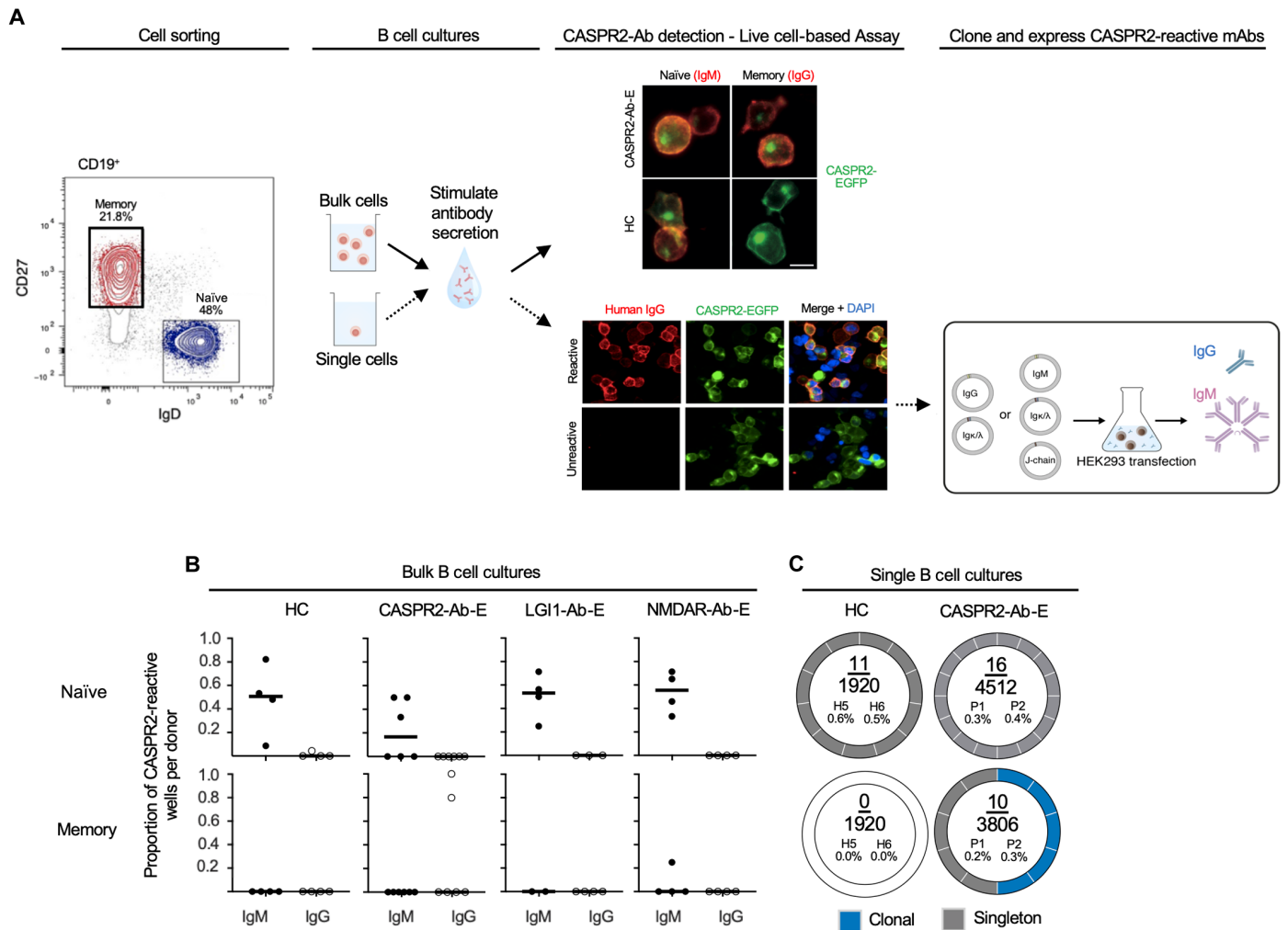


Fig. 1. Central and peripheral immune tolerance in CASPR2-antibody encephalitis and HCs. (A) Left: Representative flow cytometry cell gating strategy to isolate MBCs (red) and NBCs (blue) for bulk (top) and single (bottom, dotted line) B cell cultures. Representative fluorescence microscopy images of culture supernatant detection of secreted CASPR2-reactive IgG or IgM using a live cell-based assay with CASPR2-enhanced green fluorescent protein (EGFP) expressing HEK293T cells. 4',6-Diamidino-2-phenylindole (DAPI), nuclear stain. Scale bar, 10 μ m. Right: mRNA was extracted from CASPR2-reactive single B cell cultures to amplify and clone paired heavy- and light-chain BCR sequences. These were expressed in HEK293S cells to secrete CASPR2-reactive IgG (blue) or IgM (pink). (B) The proportion of bulk B cell culture wells containing CASPR2-reactive IgM or IgG from patients (CASPR2-Ab-E, $n = 6$; LGI1-Ab-E, $n = 4$; NMDAR-Ab-E, $n = 4$) and HCs ($n = 5$). Black bars depict the median value. (C) Donut plots visualizing the frequency and clonality of CASPR2-reactive BCRs in single-cell cultures. Numerator, total number of CASPR2-reactive BCRs; denominator, total number of cells screened. The absolute percentage of CASPR2-reactive BCRs is then shown for two patients (P1 and P2) and two HCs (H5 and H6).

for CASPR2, mAbs were used to immunoprecipitate linearized 49-mer peptides tiled across the full length of CASPR2 (Fig. 3A) (27, 28). None showed greater binding than isotype control mAbs. Furthermore, from Western blotting, only one of seven bound to denatured and reduced CASPR2 at high mAb concentrations (fig. S2A). Hence, these mAbs did not bind linearized or short regions of CASPR2. Rather, and consistent with their preference for native CASPR2, binding was observed upon their application to lightly fixed and unfixed neuronal substrates that represent the most relevant anatomical localizations of symptoms in patients with CASPR2-Ab-E: hippocampus, cerebellum, dorsal root ganglia, and peripheral sensory neurons (Fig. 3B and fig. S2, B and C). Four of seven memory mAbs bound mouse dorsal root ganglia and brain sections, and three of seven bound the surface of live rat hippocampal neurons and live human inhibitory postsynaptic current (iPSC)-derived sensory

neurons. Binding was abolished upon immunostaining CASPR2^{-/-} (knockout) brain sections, confirming exclusive CASPR2 specificity on this tissue. No NBC-derived CASPR2-reactive mAbs bound these substrates (fig. S2C).

As tissue-binding mAbs were not consistently those with higher affinities, we explored whether species differences in CASPR2 structure influenced mAb binding. A direct comparison of binding to the extracellular domains of surface expressed human versus mouse CASPR2 revealed distinctive patterns: three MBC mAbs with markedly reduced binding to mouse CASPR2, two with 10-fold increases, and two with no differences in endpoint dilutions (Fig. 3C). We hypothesized these differences related to epitopes. To investigate this, we first identified individual domains preferentially targeted by the mAbs using membrane-expressing fusion constructs engineered with six single-domain substitutions by “knocking-in” the structurally

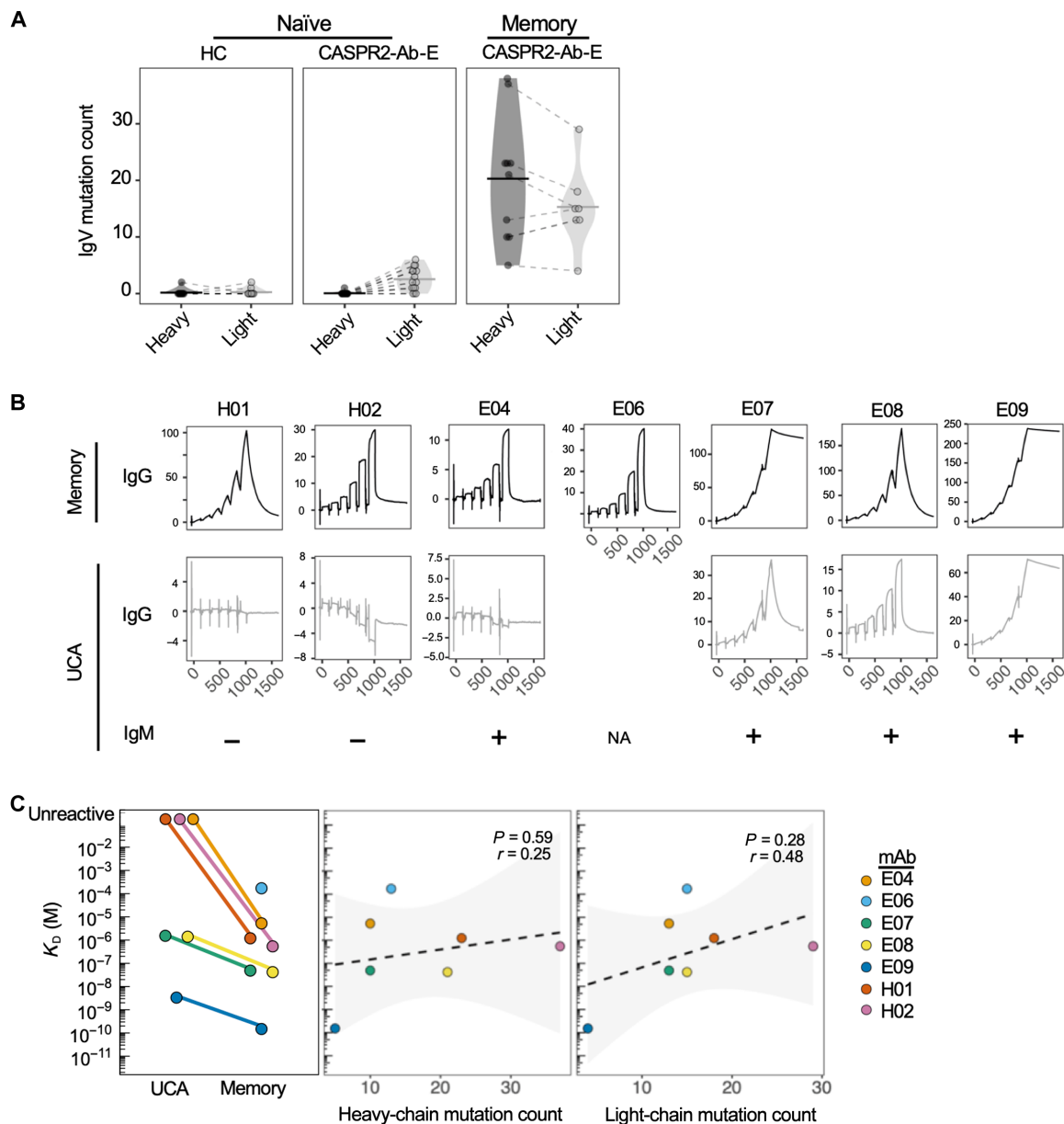


Fig. 2. CASPR2 autoreactivity is enhanced by somatic hypermutation. (A) Ig heavy- and light-chain variable region mutation counts across B cell subsets in both patients (CASPR2-Ab-E) and HCs. (B) Raw surface plasmon resonance (SPR) traces representing the soluble extracellular domain of human CASPR2 binding to immobilized CASPR2 memory mAbs (top row) and their corresponding UCAs (middle row). E06 UCA mAb did not express. UCA binding as an IgM was tested via a live cell-based assay with “+” indicating CASPR2 reactivity (bottom row). NA, not applicable. (C) K_D (M) quantification of mAbs via SPR (left). Nonsignificant K_D Pearson’s correlations with heavy (middle)– and light (right)–chain mutation count.

similar CASPR4 domain to closely preserve the native conformations of the remaining CASPR2 domains (Fig. 3D). These constructs resolved binding of five mAbs to the discoidin domain and the remaining two of seven to the laminin G-like 3 domains. As the latter two mAbs were also the two that displayed similar binding to human and mouse CASPR2, we predicted that these bound interspecies conserved regions of the laminin G-like 3 domain (Fig. 3E). In contrast, the former mAbs likely bound nonconserved amino acids on the discoidin domain (Fig. 3E and fig. S2D) (29). Direct cross-competition of pairs of mAbs against human CASPR2 using fluorophore-conjugated and fluorophore-unconjugated mAbs refined binding to

three epitope pockets: two within the discoidin domain and one within the laminin G-like 3 domain (Fig. 3F). These epitopes corresponded closely to the cross-species binding differences, with consistencies across the two patients. Hence, patient-derived MBC-derived mAbs with diverse kinetics show preferential binding to the extracellular domain of natively expressed CASPR2 and principally target three regions within two domains.

Diverse mAb pathogenic potentials

Next, to understand whether these varied binding characteristics translated to functional heterogeneity, the individual relative pathogenic

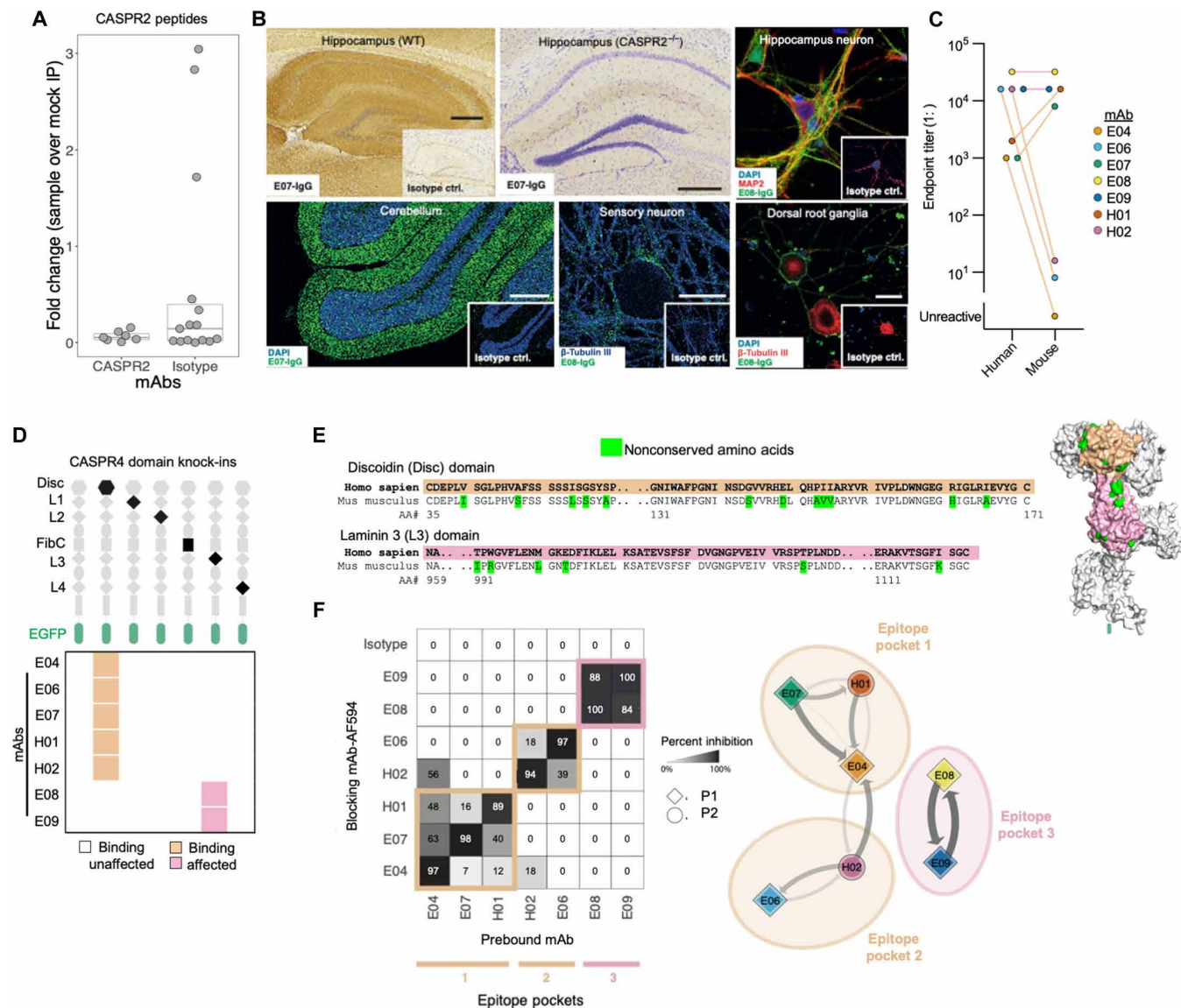


Fig. 3. CASPR2 mAbs bind distinct conformational epitopes in native tissues. (A) No differences in the number of CASPR2 peptides after immunoprecipitation (IP) by peptide phage display versus isotype control mAb. (B) Representative immunohistochemistry staining (all inlays = isotype control mAb) of CASPR2 mAbs on fixed murine hippocampal brain tissue with hippocampus visualized (top left; mAb, E07) in wild-type (WT) and CASPR2 knockout (−/−; top middle) tissue. Representative immunofluorescent staining using E08 on live hippocampal neurons (top right) fixed rat cerebellum (lower left; DAPI, nuclear counterstain), live human iPSC-derived sensory neurons (bottom middle; mAb, E08), and live mouse dorsal root ganglia (bottom right; mAb, E08). Costaining markers to identify cell types include microtubule-associated protein 2 (MAP2) and β-tubulin III. Scale bars, 500 μm (brain tissue) and 10 μm (all others). (C) Endpoint titrations (1: dilutions) of binding across human and mouse CASPR2 live cell-based assays. mAbs are colored as in (F). (D) Cartoon representation of CASPR4 single domains knocked into full-length human CASPR2-EGFP (top). Heatmap depicts CASPR4 knock-in domains that abrogated mAb binding (bottom). FibC, Fibrinogen C. (E) Discoidin (Disc) and laminin3 (L3) domain amino acid sequences from human and mouse, showing nonconserved amino acids in green. The tan and pink highlight Disc and L3 domains throughout the figure, respectively. Predicted CASPR2 protein structure (right; AlphaFold ID 5Y4M) showing nonconserved amino acids (green), Disc, and L3 domains. (F) Binding competition map (left) demonstrating the displacement of a prebound mAb (x axis) by a competing mAb preconjugated with Alexa Fluor 594 (AF594; y axis). Percent inhibition is defined as percentage reduction of fluorescence intensity of the respective mAb compared to isotype control and represented by a forced directed network of epitope binning (right). Arrowhead indicates the direction of binding competition and line thickness, and intensity denotes percentage inhibition. Shapes denote patient sample.

effects of mAbs were directly studied with a focus on published works using polyclonal human CASPR2-antibody sera: namely, CASPR2 internalization (30, 31), modulation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) expression and function (32), and altered rodent behaviors (9).

First, CASPR2 MBC-derived mAbs were labeled with pHrodo, a dye that fluoresces upon entry to acidic endophagosomes. After 4 hours of mAb incubation with HEK293 cells expressing the human CASPR2-intracellular enhanced green fluorescent protein (EGFP) fusion construct, all mAbs showed varied magnitudes of pHrodo signal that consistently colocalized with EGFP, representing consistent yet differential cointernalization of the autoantibody-autoantigen

complex (Fig. 4A). No internalization was observed with an isotype control, and all mAbs showed reduced internalization after pharmacologic inhibition of dynamin. The magnitude of internalization did not correlate with K_D , K_{on} , or K_{off} but more closely associated with the three established epitope pockets (Fig. 3F), with mAbs directed against pocket 3 showing greatest internalization (Fig. 4B).

To model functional effects in a more disease-relevant system, HEK293T cells were substituted for rodent neuronal cultures. CASPR2 expression, glutamatergic AMPAR expression, and synaptic currents were assessed (Fig. 4, C to G, and fig. S3) (32), molecular alterations which may account for the seizures, amnesia, and psychiatric features observed in CASPR2-Ab-E. In addition, as patient CASPR2-IgGs

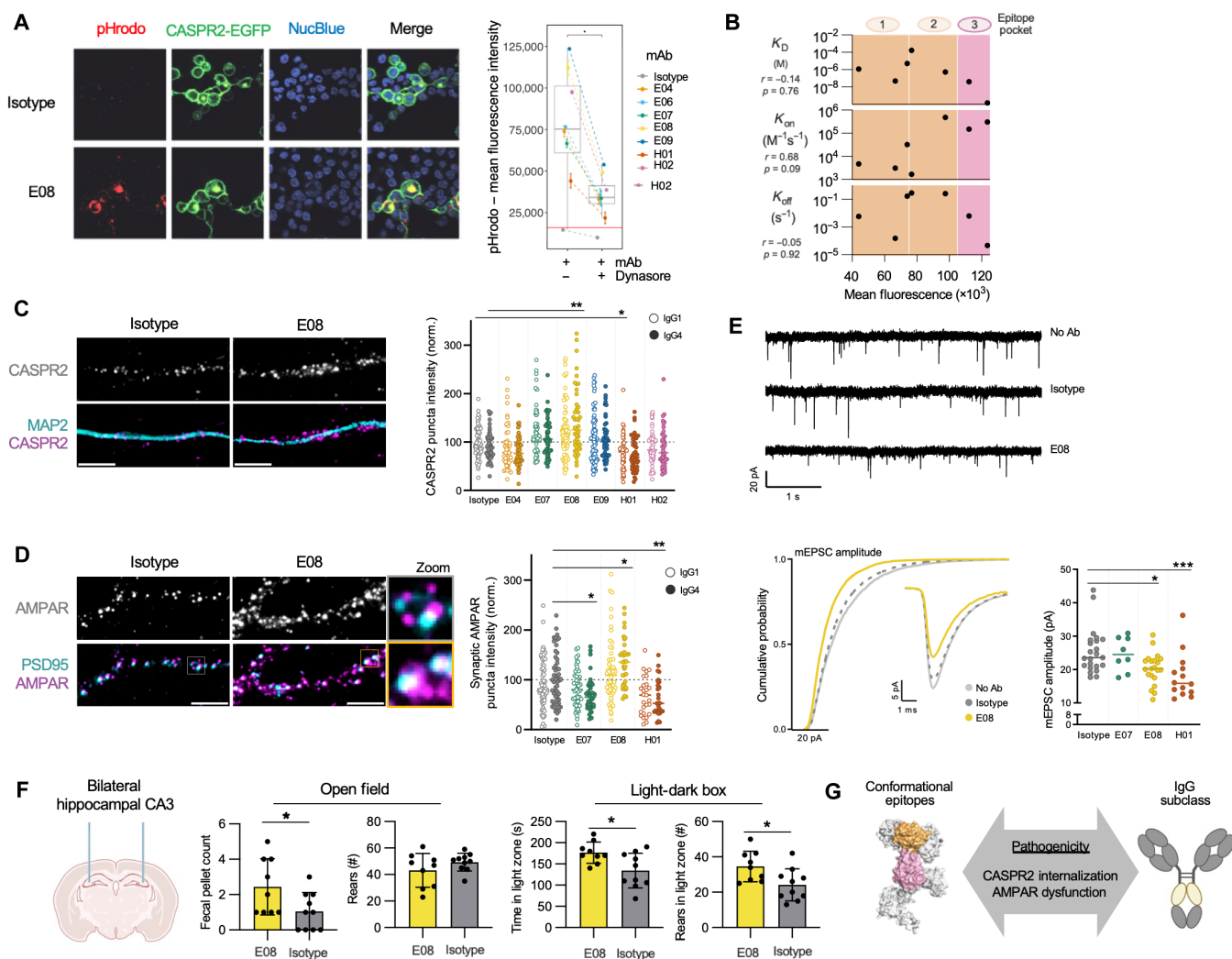


Fig. 4. Diverse pathogenic potentials of CASPR2 mAbs. (A) Representative images used to calculate colocalization of pHrodo-conjugated IgG4 mAbs and CASPR2-EGFP, reflecting receptor internalization by CASPR2-expressing HEK293T cells (left). Internalization quantified by mean pHrodo fluorescence intensity mAbs in the presence and absence of dynamin inhibition (dynasore; right). (B) Pearson's correlation of receptor internalization to mAb binding parameters; bottom x-axis label denotes internalization. Background colors indicate the three epitope pockets as presented in Fig. 3; top x-axis label annotates epitope. (C) Representative images of CASPR2 and MAP2 expression after a 4-hour application of IgG1 (open circles, left) or IgG4 mAbs (closed circles). Scale bars, 5 μ m. CASPR2 puncta intensity summarized on the right with $*P < 0.05$, $**P < 0.01$, nonparametric Kruskal-Wallis with Dunn's multiple comparisons post hoc test. mAbs colored as in (A). (D) Representative images (left) and quantification (right) of synaptic AMPAR and PSD95 expression similar to (C). (E) Representative tracings (top), single average event and cumulative probability (middle), and amplitude quantifications (bottom) of AMPAR-mediated mEPSC recordings of pyramidal neuronal cultures. $*P < 0.05$, $***P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparisons post hoc test. (F) Cartoon depiction of intracerebral mAb injection into bilateral hippocampal CA3 regions (left). Open-field (middle) and light-dark box (right) behavioral test performance was assessed at 6 and 9 hours postinjection, respectively. $*P < 0.05$, t test. (G) Summary model cartoon.

are often IgG1s or IgG4s (8, 13), the antibody subclass dependence of mAb effects was studied.

Two memory mAbs modulated neuronal CASPR2 expression, with dependence on IgG subclass (Fig. 4C and fig. S3): E08 up-regulated CASPR2 as an IgG4 ($P = 0.019$), whereas H01 down-regulated CASPR2 as an IgG1 ($P = 0.002$). Both mAbs not only influenced CASPR2 expression but also altered the intensity of synaptic AMPAR puncta [colocalizing with postsynaptic density 95 (PSD95)], with the same directionality as CASPR2 modulation (Fig. 4D), and additionally both decreased the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs; Fig. 4E). Further highlighting their overall diversity, another mAb (E07) showed no effect on CASPR2 expression but reduced AMPAR expression as an IgG4 without an effect on mEPSCs (Fig. 4, C to E). As a proof of concept towards in vivo pathogenicity, E08 was stereotactically injected into the CA3 region of rat hippocampi. After only 6 to 9 hours, by comparison to isotype control mAbs, E08-injected rats showed increased defecation, more time spent, and rears performed in the light zone of a light-dark box (Fig. 4F), behavioral phenotypes broadly consistent with affective aspects of patients with CASPR2-Ab-E (8, 9).

Collectively, these functional assays show that individual CASPR2 mAbs derived from the memory compartment of patients with CASPR2-Ab-E can differentially induce rapid molecular-, cellular-, and system-level alterations including AMPAR dysfunction, synaptic reorganization, and behavioral alterations. Effects of these disease-relevant BCRs are associated with select mAb characteristics, including epitopes and IgG subclasses (Fig. 4G).

Contrasting CASPR2 NBC receptor sequences

Next, we aimed to identify BCR features facilitating the escape of these disease-relevant MBC-derived mAbs from the patient NBC compartment. As NBC selection into the memory compartment likely relates to BCR signaling strength (33), we hypothesized that naïve CASPR2-reactive BCR sequence characteristics in patients would differ from the effectively tolerized CASPR2-reactive NBCs isolated from HCs.

First, compared to anticipated VH family distributions (Fig. 5A) (34, 35), all CASPR2-reactive B cell populations in health and disease favored VH3 family use [population usage, 43.1% versus 63.6% in HC ($P = 0.0003$), 62.5% in CASPR2-Ab-E NBCs, and 71.4% MBCs (both $P < 0.0001$, chi-square)]. Despite this shared VH3 preferential usage, specific V genes and combinations of paired heavy-chain V-J genes were distinct between NBCs isolated from HCs and patients with CASPR2-Ab-E ($P < 0.0001$, Fisher's exact test; Fig. 5B). Only two BCRs with overlapping V-J genes were observed, and these differed in their paired light chains (table S2). Therefore, despite their equivalent frequencies (Fig. 1C), no CASPR2-reactive NBCs across patients and HCs showed identical paired sequences. Within MBCs (whose V-J gene combinations are, by definition, identical to those in the corresponding UCAs), one V-J gene pair overlapped with the CASPR2-Ab-E NBCs (again, with a different light chain) but not with any V-J combinations from HC NBCs (Fig. 5B). Moreover, affected patients preferentially used two V gene segments (VL 1-39 and 3-1), and only one light-chain V-J combination overlapped between HC and CASPR2-Ab-E NBCs. In contrast, three MBC V-J combinations overlapped with the NBC population from patients with CASPR2-Ab-E but not those of HCs. More traditional correlates of autoreactivity including HCDR3 length, HCDR3 charge, and κ/λ ratio (14, 16) showed no differences between health and disease (Fig. 5, C to E). Hence,

fundamentally different CASPR2-reactive BCRs escape from central tolerance into the circulating NBC compartment in health versus disease, and only the naïve BCRs from patients with CASPR2-Ab-E show sequence-based similarities to those from MBCs.

Unmutated CASPR2-reactive BCR properties

Last, to interrogate the fundamental basis of B cell tolerance, we hypothesized that these divergent BCR repertoires determine sequences that confer their relative potential to escape immune checkpoints. Hence, we assessed unmutated mAb binding strengths to an array of human autoantigens, in particular CASPR2, focusing on a comparison between those known to have entered the MBC compartment—the UCAs—with those ex vivo-derived naïve BCRs from patients with CASPR2-Ab-E and HCs that likely never entered, and were not detected within, the MBC population.

First, relative avidity to CASPR2 was determined by live cell-based assay: While all NBCs bound as recombinant IgMs, none of 16 CASPR2-Ab-E-derived NBC mAbs compared to 5 of 11 healthy NBC-derived mAbs ($P = 0.0057$, Fisher's exact test) and all four CASPR2-reactive disease UCAs ($P = 0.0002$, Fisher's exact test) bound as either IgG or Fabs (Fig. 6A). This trend was also reflected in endpoint dilutions: By comparison to those from patients with CASPR2-Ab-E, NBC mAbs from HCs remained bound to CASPR2 at lower quantities ($P = 0.06$, Kruskal-Wallis with Dunn's test for multiple comparisons; Fig. 6B). Most notably, of all three unmutated populations, the UCAs bound CASPR2 at by far the lowest observed endpoint dilutions ($P = 0.001$ versus UCA and $P = 0.04$ versus HC naïve).

Next, to mimic other autoantigens likely available to NBCs in germinal centers, we assessed broader self-reactivities using two platforms: (i) a limited series of canonical autoantigens representing polyreactive (double-stranded DNA, insulin, and lipopolysaccharide) or autoreactive [human epithelial type 2 (HEp-2) cell] antigen substrates (fig. S4) (14, 36) and (ii) a phage-expressed array of self-antigens tiled across the entire human proteome (Fig. 6C) (27). When NBCs from patients with CASPR2-Ab-E were compared to NBCs from HCs, rates of polyreactivity trended [3 of 16 (18.8%) versus 4 of 11 (36.4%); Fisher's exact test, $P = 0.39$], and autoreactivity rates were significantly lower [2 of 16 (12.5%) versus 6 of 11 (54.5%); Fisher's exact test, $P = 0.033$; fig. S4]. UCAs showed reactivities akin to the NBCs from patients with CASPR2-Ab-E.

More quantitatively, from the phage display, higher rates of self-reactivities were also observed from HC NBCs compared to those from patients with CASPR2-Ab-E [1657 ± 157 versus 1360 ± 141 , respectively; ordinary one-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons, $P < 0.0001$; Fig. 6C]. Yet, and by contrast to CASPR2 binding strengths, the lowest self-reactivities were observed in UCAs ($P = 0.0001$), similar to MBC characteristics.

In summary, when compared to those originating from patients with CASPR2-Ab-E, the CASPR2-reactive naïve BCRs from HCs exhibited stronger binding to both CASPR2 and multiple other autoantigens. Most notably, UCAs showed around three logs higher affinity for CASPR2 accompanied by very limited self-reactivity toward a comprehensive array of other human autoantigens, both similar to the MBC BCRs. These findings suggest that a balance between specifically binding CASPR2 while ignorance towards other autoantigens may help determine selection versus elimination of autoreactive BCRs at peripheral immune checkpoints in humans (Fig. 6D).

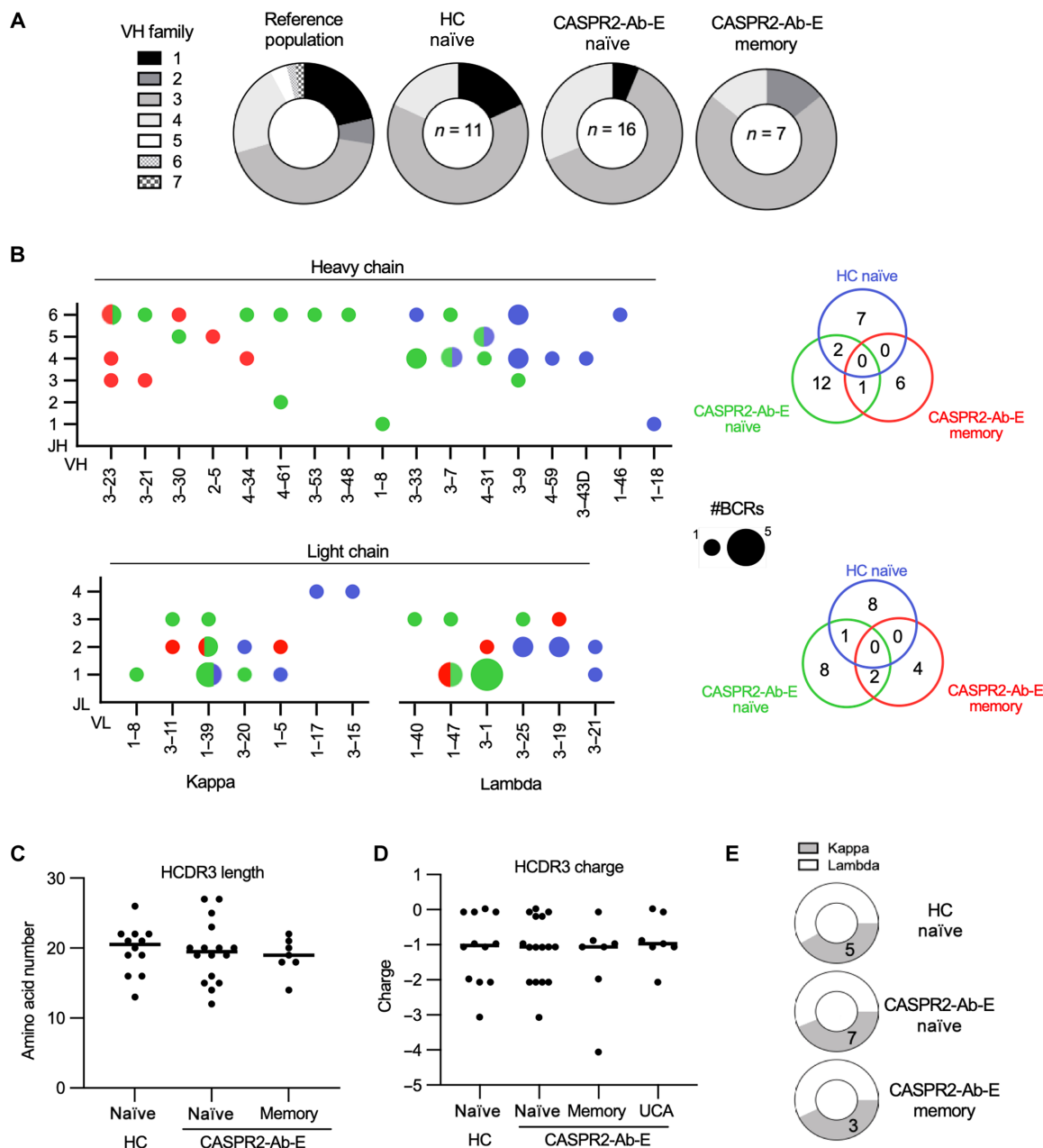


Fig. 5. Distinct CASPR2-reactive BCRs mature in affected patients. (A) Pie charts depicting the percentage of VH family usage in each labeled population. (B) Heavy-chain germline VH and JH usage (top) and light-chain germline VL and JL (bottom) usage within the indicated B cell populations. Circle size corresponds to frequency. Venn diagrams (right) depict the absolute number of unique V-J pairs by population; $P < 0.0001$, 2×2 contingency analysis comparing naïve groups. (C and D) Heavy-chain CDR3 amino acid length (C) and CDR3 net charge (D) did not statistically differ (ANOVA, $P > 0.05$). (E) Pie charts demonstrating similar ratios of kappa and lambda light-chain usage by population.

DISCUSSION

This study leverages CASPR2-Ab-E, a prototypical single autoantigen-directed condition, as an opportunity to molecularly dissect the integrity and thresholds of autoantigen-specific B cell checkpoints in both health and disease. Our observations construct a “multihit” model of immunopathogenesis which begins with promiscuous early central tolerance checkpoints that release unmutated CASPR2-reactive BCRs at comparable frequencies in both health and disease states but with different BCR sequences. UCA studies revealed that the combination of strong reactivity for native CASPR2 together with

a lack of other proteome-wide self-reactivities most effectively facilitates the escape of CASPR2-reactive BCRs into the memory compartment. Although this was strictly observed in patients, memory compartment access alone was insufficient to confer pathogenicity. The rapidly induced neuronal dysfunction appeared influenced by other BCR properties, including IgG subclass and epitope preferences. Together, these multimodal findings both highlight basic human immunological tolerance mechanisms and, simultaneously, aid the rational design of autoantigen-specific immunotherapies in CASPR2-Ab-E. We anticipate that this comprehensive approach

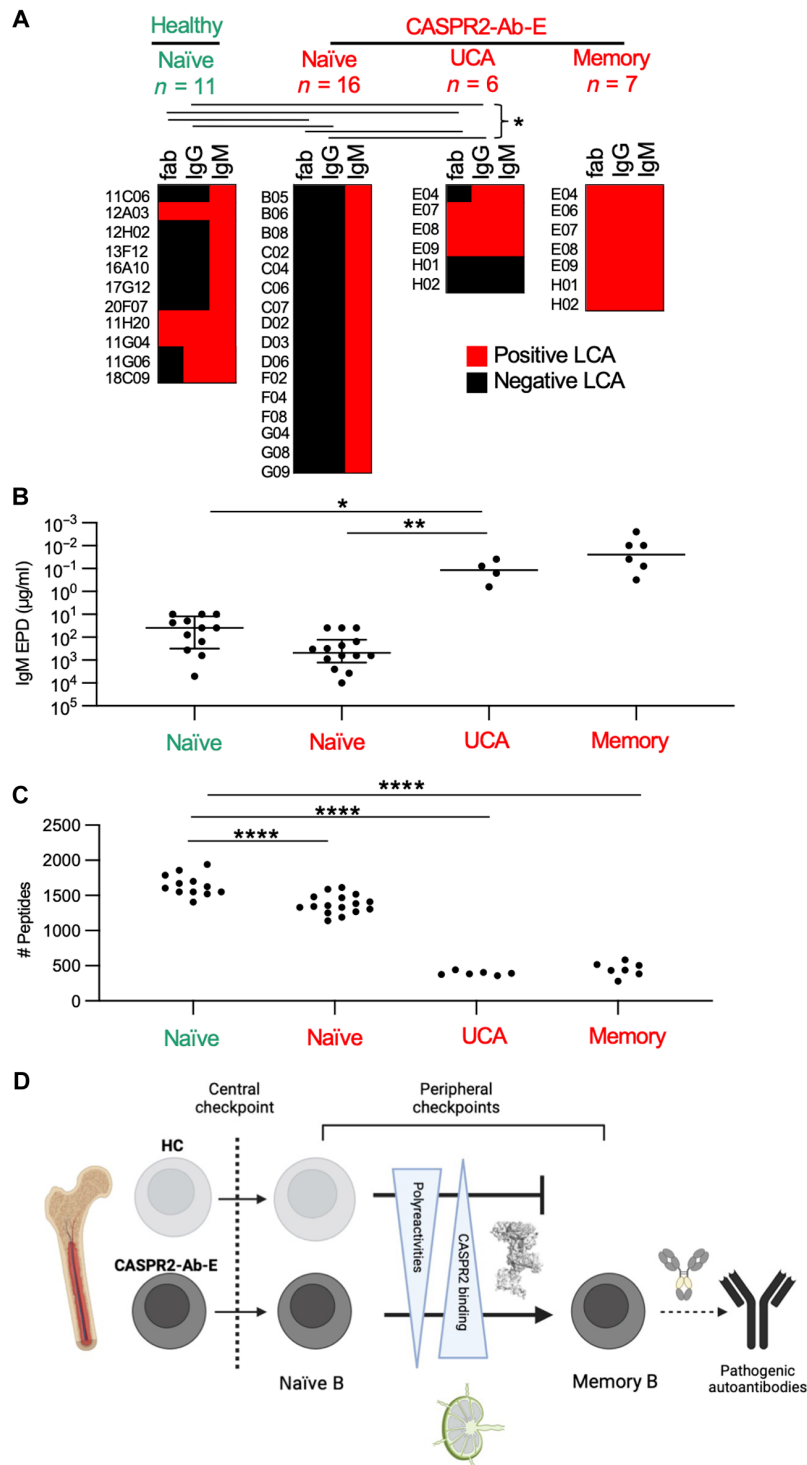


Fig. 6. High CASPR2 avidity and otherwise low self-autoreactivity facilitate clonal escape. (A) Heatmaps depicting binding by CASPR2-reactive fab segments, -IgG or -IgM; red, positive binding by live cell based assay (LCA). $*P < 0.05$ for fab and IgG frequency distributions. Populations labeled in green depict HC, and populations labeled in red depict CASPR2-Ab-E throughout the figure. (B) CASPR2-IgM mAb endpoint dilution (EPD) in a live cell-based assay. All mAbs expressed as class IgM to prevent class confound; $*P < 0.05$ and $**P < 0.005$. (C) Quantification of the number of peptides enriched in phage immunoprecipitation; $****P < 0.0001$, Wilcoxon unpaired. (D) Summary cartoon model.

could be applied toward mechanistic tolerance insights across additional human autoantigen-specific diseases, to draw both parallels and distinctions in disease-specific characteristics.

A notable aspect of our study is the direct isolation of autoantigen-reactive NBCs. Overall, a substantial fraction of NBCs displayed CASPR2 reactivity (~0.5%), an impressively large subset dedicated to CASPR2 given that ~20,000 nonmodified proteins exist in the human proteome. This permissive central tolerance to CASPR2 was reinforced by the observation that most UCAs bound CASPR2. Furthermore, its fundamental basis showed differences in HCs versus patients based on Ig gene usage. Overall, the frequent tendency to early-lineage CASPR2 reactivity in association with VDJ recombination differences suggests that the immunological origins of CASPR2-Ab-E begin before the central B cell tolerance checkpoint. This may reflect the almost exclusive neuronally restricted expression of CASPR2, limiting its availability for bone marrow B cell tolerization (15).

Downstream in B cell development, the stringent deletion of bone marrow released CASPR2-reactive BCRs in both relevant disease controls and HCs suggests that later peripheral checkpoints fail to effectively maintain tolerance against CASPR2 exclusively in patients with the CASPR2-antibody disease. However, in patients, BCRs which definitively entered the MBC compartment (studied as UCAs) appeared distinctively “tuned” to balance strong native CASPR2 reactivity with minimal binding to other autoantigens. This may reflect their preferential ability to exclusively acquire CASPR2-reactive T cell help. Conversely, the broad polyreactivity combined with limited CASPR2 reactivity—as observed in most CASPR2-reactive NBCs—may confer a higher likelihood of being more readily sensed and tolerized and hence strictly excluded from the memory compartment. This concept is consistent with rodent studies which suggest that BCR affinity shapes downstream memory compartments (37) and provides a rare mechanistic insight into the properties of human tolerance thresholds.

Within MBCs, knockout and proteome-wide screens showed that CASPR2 reactivities were highly specific for native CASPR2 conformations and exclusive to patients. These memory BCRs showed small clonal expansions and carried mutations which conferred substantial increments in binding strength to human CASPR2. Hence, it is likely conformationally native CASPR2 is presented in germinal centers. Peripherally, we hypothesize that these reside in cervical lymph nodes (38), which are thought to drain the CNS (26, 39). Yet, the highest affinity mAb with the greatest internalization capacity had acquired only five variable region mutations, not precluding a role for extrafollicular responses (40). Nevertheless, this peripherally dominant process of autoantibody production is consistent with the far higher levels of CASPR2 autoantibodies in serum versus cerebrospinal fluid (2), and the relatively low rate of intrathecally generated somatic hypermutations observed in patient cerebrospinal fluid CASPR2-reactive B cells (41).

Yet, the entry of mutated CASPR2-reactive B cells into the MBC compartment does not appear sufficient for pathogenesis. Many MBC-derived BCRs had limited effects *in vitro* and, overall, showed notable diversity across multiple parameters including kinetics of CASPR2 binding and their relative capacities to induce correlates of pathogenicity: CASPR2 internalization and modulation, AMPAR clustering, and altered synaptic kinetics. Also, their pathogenicity in individual assays varied on the basis of subclass and epitope. Hence, we propose that only some mAbs confer direct pathogenic potential. This

conclusion may explain why overall levels of serum CASPR2-IgG do not correlate with disease status and help reconcile functional differences between previous experiments which studied polyclonal serum IgGs (30, 31). By extension, we hypothesize that a detailed deconvolution of mAb properties within polyclonal samples may more accurately correlate with clinical and functional features. Furthermore, these observed functional molecular alterations offer future targets to potentially modify symptoms of CASPR2-Ab-E.

An important outstanding question raised by our model is whether the observed loss of both central and peripheral tolerance is continually present in affected patients, or instead represents a “one-time” dysregulation. Probing checkpoint function longitudinally may provide further insights and guide therapeutic strategies that not only limit the potential for adverse effects but also inform the natural history of the disorder. These could be complemented with measures of germinal center activity, such as CXCL13 levels and antigen-specific IgMs (26, 39), to redefine composite prognostic biomarkers. Such approaches may improve relapse prediction and guide preventative therapeutic strategies.

In addition, our observations help rationally direct future therapeutic considerations. For example, restoration of peripheral tolerance with regulatory T cells may represent a logical approach to effectively treat CASPR2-Ab-E, as these cells can limit autoreactive B cell accumulation (42). Central tolerance defects may also be combated using approaches that modulate this checkpoint (15). As elimination of both CASPR2-reactive memory and NBCs may be required for long-lasting effective treatment, broad B cell lineage depletion (for example, with CD19 rather than CD20 directed therapeutic antibodies) may hold most promise (43). Also, our neurobiological findings inform more downstream therapeutics by highlighting AMPAR up-regulation as a goal for symptomatic benefit.

Our data also speak to diagnostic paradigms. The high frequencies of naïve CASPR2-reactive BCRs may explain the observed false-positive rates of serum CASPR2 antibodies (24, 44). Furthermore, to reduce false-negative results, differences in cross-species reactivities should encourage the use of human-based substrates in diagnostic laboratories rather than rodent brain sections and cultured neurons (45).

Limitations of our work include the low numbers of patients and HCs studied at the single-cell level. Yet, our focus was on the detailed characterization of >12,000 B cells and 37 mAbs from untreated patients, who are difficult to recruit in a rare disease. Also, we did not examine B cells from cervical lymph nodes or cerebrospinal fluid, the site closest to the neuronal dysfunction where autoantigen-reactive plasmablasts have been detected in neurological conditions (26, 39, 41, 46, 47). Nevertheless, our observations suggest that substantial affinity maturation and loss of tolerance begin in the periphery, consistent with independent observations from cerebrospinal fluid (41, 47). Also, the absence of clonal overlaps between NBC and MBC compartments necessitated use of UCAs to study the evolution of individual BCRs and indicates substantial CASPR2-reactive NBC diversity, which should be quantified and investigated in future studies. While UCAs are widely used to infer germline characteristics, they may fundamentally fail to capture the junctional diversity observed in native NBCs (20). Also, we did not formally map the new emigrant to mature naïve peripheral checkpoint but rather studied all NBCs (17). Last, our experiments did not examine factors responsible for a faulty peripheral checkpoint in patients, such as polymorphisms in key checkpoint molecules and the presence of

CASPR2-reactive T cells (48). Future experiments should study these in addition to the relative importance of other end-effector mechanisms including complement fixation, Fc receptor binding, and Fab-Fab arm exchange.

In summary, our data reveal key aspects of the therapeutically tractable immunobiology and neurobiology underlying CASPR2-Ab-E and present a roadmap to systematically dissect how sequential, comparative studies of autoantigen-specific B cell inform our understanding of how and where immunological tolerance is lost in human autoimmune diseases.

MATERIALS AND METHODS

Ethics

All human investigations were reviewed and approved by the University of Oxford, ethical approvals REC16/YH/0013 and REC16/ES/0048. All experiments involving animals were reviewed and approved under project license P996B4A4E and personal license I11739608 at the University of Oxford or Órgão de Bem-Estar e Ética Animal and Direção Geral da Alimentação e Veterinária at the University of Coimbra.

Participants and samples

Patients with CASPR2-antibody encephalitis ($n = 6$), LGI1-antibody encephalitis ($n = 4$), NMDAR-antibody encephalitis ($n = 4$), and healthy participants ($n = 5$) provided written informed consent. Available clinical information is summarized in table S1; researchers were completely anonymized to further details of three of the HCs (NCI donor blood bank). Donated peripheral blood mononuclear cells (PBMCs) were cryopreserved in liquid nitrogen until use.

Fluorescence-activated cell sorting with bulk and single-cell lymphocyte cultures

PBMCs were thawed, labeled with antibodies against CD3, CD14, CD19, CD27, and IgD, and subsequently fluorescence-activated cell sorted for NBCs ($CD19^+IgD^+CD27^-$) and class-switched MBCs ($CD19^+IgD^-CD27^+$) from $CD3^+CD14^-DAPI^-$ lymphocytes. A complete list of all primary and secondary antibodies used in this study is provided in table S5.

Conditions for 13-day bulk (22, 39) and 22-day single-cell cultures (26) are reported. Briefly, for bulk cultures, 10,000 of each B cell subset were sorted and cultured in complete B cell media (RPMI with 5% IgG-depleted fetal calf serum) with R848 (2.5 μ g/ml; Enzo Life Sciences), soluble CD40L (50 ng/ml; R&D Systems), interleukin-2 (50 ng/ml; PeproTech), interleukin-1 β (1 ng/ml), interleukin-21 (50 ng/ml; PeproTech), interleukin-6 (10 ng/ml; R&D Systems), and tumor necrosis factor- α (1 ng/ml; PeproTech). For single-cell cultures, single sorted B cells were cultured for 22 days with MS40L^{low} cells (gift from G. Kelsoe) (1, 2), supplemented with interleukin-2, interleukin-21, and interleukin-4.

Culture supernatant screening and live cell-based assays

Well-described live cell-based assays (5, 6) were used to test for the presence of CASPR2-reactive IgM/IgGs in 50 μ l of the culture supernatant or quantify mAb antigen specificity and endpoint dilutions (starting at 10 μ g/ml). Briefly, live HEK293T cells transiently transfected with either a human or mouse CASPR2-EGFP intracellular C terminus fusion construct were exposed to the supernatant or mAbs for 45 min and subsequently washed and fixed. Bound antibodies were detected with secondary antibodies against either human IgG or human IgM.

Generation of recombinant CASPR2-reactive antibodies

RNAasin plus (12.8 μ l/ml; Promega) in Tris-EDTA buffer (pH 8) (Bioultra) was added to CASPR2-antibody-positive wells and flash frozen. Single-cell RNA was made into cDNA from which heavy- and light-chain Ig variable region genes were amplified as described (table S4) (49) and validated with Sanger sequencing. Sequence annotation and analyses (including germline VDJ usage, mutation count, HCDR3 length, and charge) were performed using international ImMunoGeneTics (IMGT) High-Vquest (which reports established polymorphisms) and spectral clustering for clone partitioning. Validated variable domain genes were then cloned into expression vectors containing mu, gamma 1, or gamma 4 constant region Ig domains or hexahis-tagged Fab fragments.

For production, HEK293F cells cultured in Freestyle expression medium (Thermo Fisher Scientific) were polyethylenimine (PEI)-transfected with vectors encoding cognate-paired heavy- and light-chain sequences, at a 1:1 vector ratio. For IgG1/IgG4 mAbs, the supernatant was harvested after 5 days, and IgGs were purified on a single AKTA pure chromatography system. For IgM mAbs, a J-chain expression plasmid was cotransfected, and IgMs were purified using centrifugal size exclusion. Hexahis-tagged FAB fragments were purified using nickel affinity chromatography.

UCA reversion

Somatically hypermutated variable region sequences were aligned with IMGT-VQUEST to identify the ancestral germline gene fragments (<https://imgt.org>). Replacement mutations were reverted to the respective nucleotides from the best matched germline gene. Nontemplated nucleotides at V-D, D-J, and VL-JL junctions were unmodified. Last, using the IMGT junctional tool, CDR3 regions of the heavy chains were also back-mutated to the best aligned D-gene. Reverted sequences were ordered as gene fragments with flanking cloning sites and inserted into IgG, IgM, and FAB expression vectors.

Surface plasmon resonance

A Biacore 8k (Cytiva) K_D , and kinetics (association and dissociation constants: k_{on} and k_{off}) of immobilized mAb binding to the C-terminally biotinylated purified extracellular domain of human CASPR2. Approximately 200 response units of IgG was captured onto a Protein A Series S Sensor Chip (Cytiva). Single-cycle kinetic analysis of binding was undertaken, using twofold serial dilutions of CASPR2 in HBS-P+ buffer (Cytiva) and six dilutions per cycle. The CASPR2 starting concentrations varied from 20 to 1000 nM. All measurements were made with injection times of 120 s (30 μ l/min) and dissociation times of 600 s (30 μ l/ml) at 37°C. Regeneration of the sensor chip was performed with 10 mM glycine-HCl (pH 1.7) for 30 s (30 μ l/min) between experiments. For analysis, the sensorgrams were double reference subtracted and fitted with a 1:1 binding model using the Biacore Insight Evaluation Software version 2.0.15.12933 (Cytiva). For all 1:1 binding fits, the t_c value was set at 1×10 .

Antibody binding to self- and nonself-substrates

Immunoblot analysis was performed per the manufacturer's instruction of electrophoresis and Western blotting system (Life Technologies). Briefly, purified extracellular domain of CASPR2 or HEK cell lysate (generated by adding radioimmunoprecipitation assay buffer containing protease inhibitor cocktail over ice; Thermo Fisher Scientific) was protein sources. CASPR2 protein was denatured with SDS buffer

containing β -mercaptoethanol. Samples were separated by 10% bis-tris precast gels and transferred to the polyvinylidene fluoride membrane. Nonspecific binding was blocked by 5% skim milk (Sigma-Aldrich) in tris-buffered saline/0.05% Tween 20 (Life Technologies) at room temperature. The blot was probed with antibodies of interest at 4°C overnight. Commercial polyclonal rabbit anti-human CASPR2 antibody (Novus, A45565) was used as an isotype control for detecting denatured CASPR2. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako) were used for subsequent incubation. Enhanced chemiluminescence solution (Thermo Fisher Scientific) was used for chemiluminescence. Signals were detected by a ChemiDoc MP imaging system (Bio-Rad). The exposure time was determined automatically when saturated signal was detected.

Methods to determine mAb binding to neuronal substrates (starting dilution, 20 μ g/ml) expressing CASPR2 have been described previously: Briefly, 10- μ m-thick rat brain sections were lightly fixed in 4% paraformaldehyde and stained with diaminobenzidine (6), live rat dorsal root ganglion neurons (10), live primary cultured rat hippocampal neurons (5, 41), 4% sucrose/paraformaldehyde-fixed rat cortical neurons (32), and live human sensory neuron IPSCs myelinated by rat Schwann cells (50).

Polyreactivity was assessed against double-stranded DNA (20 μ g/ml), lipopolysaccharide (10 μ g/ml), or recombinant human insulin (15 μ g/ml; all from Sigma-Aldrich), as described (14). The highly poly-reactive antibody ED38 served as a positive control. For the Hep-2 enzyme-linked immunosorbent assay (ELISA), mAbs were applied to HEp-2 (HELA cells) cell lysates (INOVA ELISA kit) and developed with HRP (Bio-Rad). Plates were read out with the Epoch (BioTek), and positivity was designated optical density of >0.7 (405 nm).

Programmable phage immunoprecipitation and sequencing investigated the conformational nature of CASPR2 epitopes as well as broader self-reactive potential. Briefly, IgG1 mAbs were incubated with 10^{10} plaque-forming units/ml of a phage display library composed of overlapping 49-mer peptides arrayed across the human proteome or CASPR2 protein. Antibody-bound phage particles were isolated by protein G immunoprecipitation and underwent MiSeq next-generation sequencing to identify putative human antigens (27).

CASPR2-CASPR4 domain swaps and epitope binning

Gene sequences corresponding to CASPR4 protein domains discoïdin, laminin G-like domains 1 to 4, and fibrinogen C terminus were identified using <https://uniprot.org>. Complementary overhangs matching flanking 5' and 3' regions of CASPR2 were added. CASPR4 domain "knock-in" constructs were generated using these full gene constructs (IDT) via overlap extension PCR. The constructs were used in live cell assays as above.

For competitive binding experiments, CASPR2-expressing HEK293T cells were first saturated with an excess of a single unlabeled mAb (100 μ g/ml). After 60 min, mAbs (10 μ g/ml; expressed as IgG1s to avoid potential Fab-arm exchange) preincubated with Alexa Fluor 594 (AF594; Thermo Fisher Scientific, #A20185) were added for 30-min incubation. Wells were washed, and AF594 fluorescence was detected on a BMG Omega Fluostar fluorescence plate reader (excitation, 560 nm; detection, 610 nm). Percentage inhibition was defined as the percentage reduction from maximal binding.

Cross-species protein structures

CASPR2 mouse and human protein sequences were obtained from <https://uniprot.org>. The predicted crystal structure of CASPR2 (structure ID 5Y4M, AlphaFold.ebi.ac.uk) was modeled using PyMOL.

pHrodo internalization and image quantification

pHrodo-conjugated mAbs (20 μ g/ml) were incubated overnight with CASPR2-transfected HEK293T cells, and mean fluorescence was quantified at regular intervals using BMG OMEGA FLUOstar. For some wells, pretreatment with 50 μ M dynasore was performed 1 hour before mAb incubation. pHrodo mean fluorescence quantification was preprocessed in FIJI and analyzed in R, using packages *rstatix* (<https://github.com/kassambara/rstatix>) and *RKcolocal* (<https://github.com/lakerwsl/RKColocal>). In FIJI, 12 regions of interest were selected, and the mean fluorescence intensities for each region were exported for analysis in R.

Primary cultures of cortical neurons

Primary cultures of rat cortical neurons were prepared from the cortices of embryonic day 17/18 (E17/18) Wistar rat embryos, as previously described (32). Briefly, after dissection, tissue was treated for 10 min at 37°C with trypsin (0.06%, Gibco Invitrogen), in Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution (HBSS; 5.36 mM KCl, 0.44 mM KH_2PO_4 , 137 mM NaCl, 4.16 mM NaHCO_3 , 0.34 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, and 0.001% phenol red). Cells were then washed six times in HBSS and mechanically dissociated. Cells were plated in neuronal plating medium (minimum essential medium supplemented with 10% horse serum, 0.6% glucose, and 1 mM pyruvic acid) onto poly-D-lysine-coated (0.1 mg/ml) coverslips in 60-mm culture dishes, at the desired density. For imaging purposes, cells were plated at a final density of 2.5×10^5 cells per dish; for electrophysiology experiments, cells were plated at a density of 11×10^5 cells per dish. After 2 to 4 hours, coverslips were flipped over an astroglial feeder layer in neurobasal medium [supplemented with SM1 neuronal supplement (STEMCELL Technologies, Grenoble, France), 0.5 mM glutamine, and gentamycin (0.12 mg/ml)]. Wax dots on the neuronal side of the coverslips allowed the physical separation of neurons from the glia, despite neurons growing face down over the feeder layer. To further prevent glia overgrowth, neuron cultures were treated with 10 μ M 5-Fluoro-2'-deoxyuridine (Sigma-Aldrich) after 3 days in vitro (DIV). All cultures were maintained at 37°C in a humidified incubator of 5% CO_2 /95% air, until DIV14.

Immunocytochemistry, cell imaging, and quantitative fluorescence analysis

Primary cortical neurons at DIV14 were incubated for 2 hours at 37°C with 20 μ g/ml of each mAb. Cortical cells were then fixed for 15 min in 4% sucrose/4% paraformaldehyde in phosphate-buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 (pH 7.4)], permeabilized with 0.25% Triton X-100 in PBS for 5 min, and then incubated in 10% (w/v) bovine serum albumin (BSA) in PBS for 30 min, at 37°C, to block nonspecific staining. Cells were then incubated with primary antibodies against Caspr2, the neuronal dendritic marker microtubule-associated protein 2 (MAP2) or the glutamatergic synapse marker PSD95, diluted in 3% BSA in PBS (2 hours, 37°C or overnight, 4°C). Following several PBS washes, cells were incubated with the appropriate fluorophore-conjugated secondary antibodies (1 hour, 37°C), and coverslips were lastly washed and mounted using fluorescent mounting medium from Dako (Glostrup, Germany). To label cell surface AMPAR subunits, live neurons were incubated for 10 min at room temperature with a pan-antibody against extracellular epitopes in the N terminus of the GluA1 and GluA2 subunits, diluted in conditioned neuronal culture medium. Coverslips were then fixed and probed as described above.

Sets of cells that were cultured and stained simultaneously were imaged using identical acquisition settings on a Zeiss Axiovert 200M microscope with a 63×1.4 numerical aperture oil objective. Blind-to-condition quantification was performed in the image analysis software FIJI using an in-house developed macro to automatize quantification steps. The region of interest was randomly selected avoiding primary dendrites, and dendritic length was measured using MAP2 staining. Surface AMPAR and Caspr2 digital images were thresholded such that recognizable clusters were included in the analysis and measured for cluster intensity, number, and area for the selected region. Synaptic clusters of AMPAR were selected by their overlap with thresholded and dilated PSD95 signal. Measurements were performed in a minimum of three independent experiments, and at least 10 cells per condition were analyzed for each preparation.

Electrophysiology

Whole-cell patch-clamp recordings in voltage-clamp configuration were measured from 14 DIV cortical neurons plated on coverslips following 2-hour incubation with each mAb. The recording chamber was mounted in a fixed-stage inverted microscope (Zeiss Observer.A1) and perfused at a constant rate (2 to 3 ml/min) with extracellular solution [ECS; 140 mM NaCl, 2.4 mM KCl, 10 mM Hepes, 10 mM glucose, 4 mM CaCl_2 , and 4 mM MgCl_2 (pH 7.3); 300 to 310 mOsm], at room temperature ($\sim 23^\circ\text{C}$). AMPAR-mediated mEPSCs were pharmacologically isolated by adding 1 μM tetrodotoxin, 100 μM picrotoxin, and 50 μM (2R)-amino-5-phosphonovaleric acid to the ECS. Neurons were patched using a borosilicate glass recording pipette (tip resistance, 3 to 5 megohm) filled with a Cs-based internal solution [107 mM CsMeSO_3 , 10 mM CsCl, 3.7 mM NaCl, 5 mM TEA-Cl, 0.2 mM EGTA, 20 mM Hepes, 4 mM adenosine triphosphate magnesium salt, and 0.3 mM guanosine triphosphate sodium salt (pH 7.3); 295 to 300 mOsm], and recordings were initiated 2 to 3 min after break-in. The EPC 10 USB patch-clamp amplifier (HEKA Elektronik) was used for voltage-clamp recordings. Cells were held at -70 mV, and mEPSCs were recorded over a period of 5 min in a gap-free acquisition mode. Data was digitized at 25 kHz and acquired using the PatchMaster software (HEKA Elektronik), with a signal filter of 2.9 kHz. The Clampfit software (Axon Instruments) was used to analyze the acquired mEPSCs, using a template search method to detect events, as previously described (51). The template was generated by averaging approximately 30 events, and the template match threshold was set to 4. Recordings were excluded from analysis if the series resistance (R_s) was >25 megohm, the holding current was >250 pA, or if the R_s or holding current changed more than 20%. One hundred fifty consecutive events that met these criteria were analyzed from each cell.

Intracerebral mAb injection

Five-week-old female C57BL/6J mice (Charles River) were housed in cages of five in a room maintained at a controlled temperature (21°C) and humidity (5 to 10%) with illumination at 12-hour cycles; food and water were available ad libitum. All injections were performed during the light phase, and animals were habituated to the experimental room for 1 hour before beginning injections and 15 min before behavioral testing. Procedures were conducted in compliance with the Animal Scientific Procedures Act 1986, revised in 2012, and the European Directive 63/2010 on the protection of animals, as well as in accordance with the Institutional Animal Care and Use Committee (University of Oxford).

On the day of surgery, mice were anesthetized with isoflurane and placed in a stereotactic apparatus. A mid-sagittal incision was made to expose the cranium, and two burr holes were drilled over the hippocampi to the following coordinates from bregma: antero-posterior, -1.5 mm; lateral, ± 1.8 mm. A glass microcapillary containing the solution to be injected (E08 IgG4 mAb or an isotype control mAb) was lowered 1.5 mm ventral to bregma, and 1 μl of mAb at a concentration of 1 mg/ml was injected per hemisphere. The incision was then cleaned and closed with sutures. Ten mice were injected with the Caspr2 mAb and 10 with an isotype control mAb. Mice were allowed to recover postsurgery before behavioral testing. The open-field test was performed 6 hours postsurgery, and the light-dark box test was performed 9 hours postsurgery. Behavior was manually scored after video review.

Supplementary Materials

This PDF file includes:

Figs. S1 to S4

Tables S1 to S5

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Acknowledgments

Funding: This research was funded in whole or in part by (to S.R.I.) a senior clinical fellowship from the Medical Research Council (MR/V007173/1), Wellcome Trust Fellowship (104079/Z/14/Z), the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC) [The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health], an Association of British Neurologist Clinical Research Training Fellowship via the Patrick Berthoud Charitable Trust (2018-PBCT-1 to BS), R01MH122471 and Westridge Foundation grants (to M.R.W.), a DFG Research fellowship (FI 2471/1-1 to M.L.F.), Boehringer Ingelheim Fonds (BIF; to M.G.I.), COMPETE 2020–Operational Programme for Competitiveness and Internationalisation, and Portuguese national funds via FCT-Fundação para a Ciência e a Tecnologia, under project(s) POCI-01-0145-FEDER-029452, UIDB/04539/2020, UIDP/04539/2020, and LA/P/0058/2020 (to A.L.C.). **Author contributions:** B.S.: acquiring and analyzing data, writing the manuscript, and study design; D.F.: acquiring and analyzing data and study design; A.-K.K.: acquiring and analyzing data and study design; S.P.: acquiring and analyzing data; R.H.: acquiring and analyzing data; S.R.: acquiring and analyzing data; A.L.H.: acquiring and analyzing data and writing the manuscript; M.M.: acquiring and analyzing data; M.L.F.: acquiring and analyzing data; R.F.D.: acquiring and analyzing data; D.A.: acquiring and analyzing data; H.B.: acquiring and analyzing data; M.G.I.: acquiring and analyzing data; R.W.: acquiring and analyzing data; A.V.: acquiring and analyzing data; S.T.: acquiring and analyzing data; A.F.: acquiring and analyzing data; R.S.: acquiring and analyzing data; H.F.: acquiring and analyzing data; V.M.: acquiring and analyzing data; A.D.: acquiring and analyzing data; M.T.: acquiring and analyzing data; A.H.: data analysis and interpretation; M.K.: acquiring and analyzing data; M.Z.: acquiring and analyzing data; J.B.: data analysis and interpretation; R.B.-R.: data analysis and interpretation; J.V.P.: data analysis and interpretation; J.V.P. and R.D.: acquiring and analyzing the data; B.D.A.: data analysis and interpretation; L.B.D.: reagents and interpretation; S.R.: data analysis and interpretation; R.O.: data analysis and interpretation; D.A.: data analysis and interpretation; D.L.B.: data analysis and

interpretation; P.W.: data analysis, interpretation, and study design; S.J.D.: data analysis and interpretation; M.R.W.: data analysis and interpretation; K.C.O.: data analysis and interpretation; J.S.: acquiring and analyzing the data and writing the manuscript; A.L.C.: data analysis and interpretation; S.R.I.: data analysis and interpretation, study design, and writing the manuscript. **Competing interests:** S.R.I. has received honoraria/research support from UCB, Immunovant, MedImmun, Roche, Janssen, Cerebral therapeutics, ADC therapeutics, Brain, CSL Behring, and ONO Pharma, receives licensed royalties on patent application WO/2010/046716 entitled “Neurological autoimmune disorders,” and has filed two other patents entitled “Diagnostic method and therapy” (WO2019211633 and US-2021-0071249-A1; PCT application WO202189788A1) and “Biomarkers” (PCT/GB2022/050614 and WO202189788A1). M.R.W. receives unrelated research grant funding from Roche/Genentech and Novartis and received speaking honoraria from Genentech, Takeda, WebMD, and Novartis. K.C.O. is an equity shareholder of Cabaletta Bio. M.L.F. has received speaker’s honoraria by Alexion, received a SPIN award from Grifols (outside the submitted work), and is a member of the Alexion-Akademie since 2022. D.L.B. has acted as a consultant for 5 AM Ventures, AditumBio, Astra Zeneca Biogen Biointervene, Combigene, LatigoBio, GSK, Lonis, Lexicon Therapeutics, Neuvati, Novo Ventures, Olipass, Orion, Replay, SC Health Managers, Third Rock ventures, Vida Ventures, and Vertex on behalf of Oxford University Innovation. A.D. is named inventor on patent pending: “Immune cell therapy for nerve damage” (WO2020009437A1, US-2021-0121501-A1). R.B.-R. is a cofounder of Alchemab Therapeutics Ltd. and is a consultant for Alchemab Therapeutics Ltd., Roche, Enara Bio, UCB, and GSK. S.R. serves as a consultant on an advisory board for UCB and Limbic Neurology, has been an invited speaker for educational/research sessions coordinated by Biogen, Alexion, Novartis, Excemed, and Limbic Neurology, and is on the medical advisory board (nonremunerated positions) of The MOG Project and the Sumaira Foundation. The authors declare that they have no other competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Submitted 27 July 2024

Accepted 7 March 2025

Published 16 April 2025

10.1126/sciadv.adr9986