Human CASPR2 Antibodies Reversibly Alter Memory and the CASPR2 Protein Complex

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Objective: The encephalitis associated with antibodies against contactin-associated proteinlike 2 (CASPR2) is presumably antibody-mediated, but the antibody effects and whether they cause behavioral alterations are not well known. Here, we used a mouse model of patients' immunoglobulin G (IgG) transfer and super-resolution microscopy to demonstrate the antibody pathogenicity.

Methods: IgG from patients with anti-CASPR2 encephalitis or healthy controls was infused into the cerebroventricular system of mice. The levels and colocalization of CASPR2 with transient axonal glycoprotein 1 (TAG1) were determined with stimulated emission depletion microscopy (40–70μm lateral resolution). Hippocampal clusters of Kv1.1 voltage-gated potassium channels (VGKCs) and GluA1-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) were quantified with confocal microscopy. Behavioral alterations were assessed with standard behavioral paradigms. Cultured neurons were used to determine the levels of intracellular CASPR2 and TAG1 after exposure to patients' IgG.

Results: Infusion of patients' IgG, but not controls' IgG, caused memory impairment along with hippocampal reduction of surface CASPR2 clusters and decreased CASPR2/TAG1 colocalization. In cultured neurons, patients' IgG led to an increase of intracellular CASPR2 without affecting TAG1, suggesting selective CASPR2 internalization. Additionally, mice infused with patients' IgG showed decreased levels of Kv1.1 and GluA1 (two CASPR2-regulated proteins). All these alterations and the memory deficit reverted to normal after removing patients' IgG.

Interpretation: IgG from patients with anti-CASPR2 encephalitis causes reversible memory impairment, inhibits the interaction of CASPR2/TAG1, and decreases the levels of CASPR2 and related proteins (VGKC, AMPAR). These findings fulfill the postulates of antibody-mediated disease and provide a biological basis for antibody-removing treatment approaches.

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Antibodies to contactin-associated proteinlike 2 (CASPR2) are usually associated with autoimmune encephalitis, neuromyotonia, or Morvan syndrome.[1] In the central nervous system (CNS), CASPR2 is predominantly expressed in the hippocampus, cerebellum, and cortex, whereas at the cellular level it is found in the neuronal soma, dendritic spines, axonal initial segment, and juxtaparanodal regions.[2] The interaction of CASPR2

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with transient axonal glycoprotein 1 (TAG1) is critical for the recruitment of Kv1 shaker-type voltage-gated potassium channels (VGKCs) at the juxtaparanodal sites and it may play a role in the trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs).[3–5]

Patients with anti-CASPR2 encephalitis frequently respond to immunotherapy, suggesting that the antibodies are pathogenic.[1,6] However, previous studies on the antibody effects have focused only on the levels of CASPR2 or single components of the CASPR2 protein complex, sometimes with conflicting results.[5,7–10] Using cellular models and a solid phase assay, the most consistent finding was an antibody-mediated inhibition of the CASPR2/TAG1 interaction, but this was not modeled in animals.[7] Only 2 studies have examined the antibody effects in mouse brain, showing discordant results,[5,9] and neither of them demonstrated memory or behavioral changes that could be attributed to alterations of CASPR2 or related proteins.

CASPR2 and TAG1 are densely packed along neurites, and their interaction (or colocalization at the nanoscale level) is below the resolution of confocal microscopy (~250μm in lateral resolution).[11] This and the type of IgG subclass (most anti-CASPR2 encephalitis patients have both IgG1 and IgG4) may explain some of the indicated discrepancies among studies. Here, we applied confocal and super-resolution microscopy (stimulated emission depletion [STED], 40–70μm lateral resolution[12]) to a mouse model of cerebroventricular transfer of patients' IgG to determine the effect of the antibodies on memory and behavior, and whether this effect was associated with changes in the interaction of CASPR2/TAG1 or modified the surface levels of CASPR2 and related proteins (TAG1, VGKC, AMPAR). Finally, we also investigated whether any of these changes reversed after discontinuing the infusion of patients' antibodies. The findings are important because show extensive alterations of the CASPR2 protein complex that are accompanied by reversible memory impairment.

Subjects and Methods

Patients' Selection and IgG Purification

Sera from 6 patients with definite criteria for anti-CASPR2–associated autoimmune encephalitis[13] were pooled, and the IgG (patients' IgG) was isolated using agarose bead columns (#20423; Pierce Biotechnology, Waltham, MA), as reported.[14] Sera from 6 antibody-negative healthy blood donors were pooled, and the IgG was similarly isolated (controls' IgG). Analysis of CASPR2 IgG subclasses was performed by flow cytometry, as reported.[15] After IgG isolation, samples were dialyzed, normalized to a concentration of 1mg/ml using Amicon Ultra Centrifugal filters 30 K (#UFC903024; Sigma-Aldrich, St Louis, MO), filtered, and kept at −80°C until use. The specificity of patients' IgG preparation was assessed by immunoabsorption with HEK293 cells expressing CASPR2, which showed abrogation of reactivity with brain tissue and live neurons (Fig 1A, B).[16] In contrast, immunoabsorption with HEK293 cells that did not express CASPR2 did not abrogate the reactivity of patients' IgG (see Fig 1C, D).

Mice, Placement of Osmotic Pumps, and Tissue Sampling

Fifty-five male C57BL6/J mice (Charles River, Wilmington, MA), 8 to 10 weeks old (25–30g), were used for behavior and brain tissue studies. Bilateral catheters were placed in the lateral ventricles and connected to subcutaneous osmotic pumps (Alzet, Cupertino, CA; volume = 100μl, flow rate = 0.25μl/h for 14 days), as reported.[17] In a previous study, we showed that this procedure allows free flow of methylene blue across the ventricular system.[17] Subsets of mice were euthanized at serial time points (days 3, 18, and 28), and their brains were sagittally split. One hemisphere was mildly fixed in 4% paraformaldehyde (PFA) for 1 hour at 4°C and frozen, as reported,[17] and the other hemisphere was kept for hippocampus dissection. Experiments were performed in accordance with ARRIVE guidelines for reporting animal research.[18] All procedures were approved by the local ethics committee of the University of Barcelona following European (2010/63/UE) and Spanish (RD 53/2013) regulations about the use and care of experimental animals.

Immunoprecipitation of CASPR2 Bound to Human IgG

The CASPR2 specificity of the human IgG bound to the brain of mice was demonstrated by immunoprecipitation. In brief, hippocampus of infused mice was dissected under a stereomicroscope (Stemi 2000; Carl Zeiss, Jena, Germany), washed with cold phosphate-buffered saline (PBS), homogenized in laurylamidopropyl dimethylamine oxide 0.1% lysis buffer containing protease inhibitors (1:50, #P8340, Sigma-Aldrich), and centrifuged (15,000 × *g*) for 15 minutes to remove the debris. Then, the supernatant was ultracentrifuged (200,000 × *g*) for 1 hour at 4°C. The pellet was then resuspended in lysis buffer and incubated overnight with protein A/G beads, under agitation. The beads were then centrifuged (5 minutes, 16,000 × *g*), washed 4 times to remove unbound IgG, and resuspended in loading buffer for immunoblot studies using a rabbit CASPR2 antibody (ab33994; Abcam, Cambridge, UK), as described elsewhere.[19]

Behavioral Tasks

Animals selected for behavioral studies were habituated to the experimental room for 1 week before starting the tests. These included novel object location, locomotor activity, sucrose preference, and open field, as reported.[17,20] Additional paradigms studied included anxiety with the black and white test,[16] motor function and coordination with the rotarod test,[21] sensitivity to pain with the Hargreaves and Von Frey tests,[22] and ataxia with analysis of the footprint after inking the paws.[23]

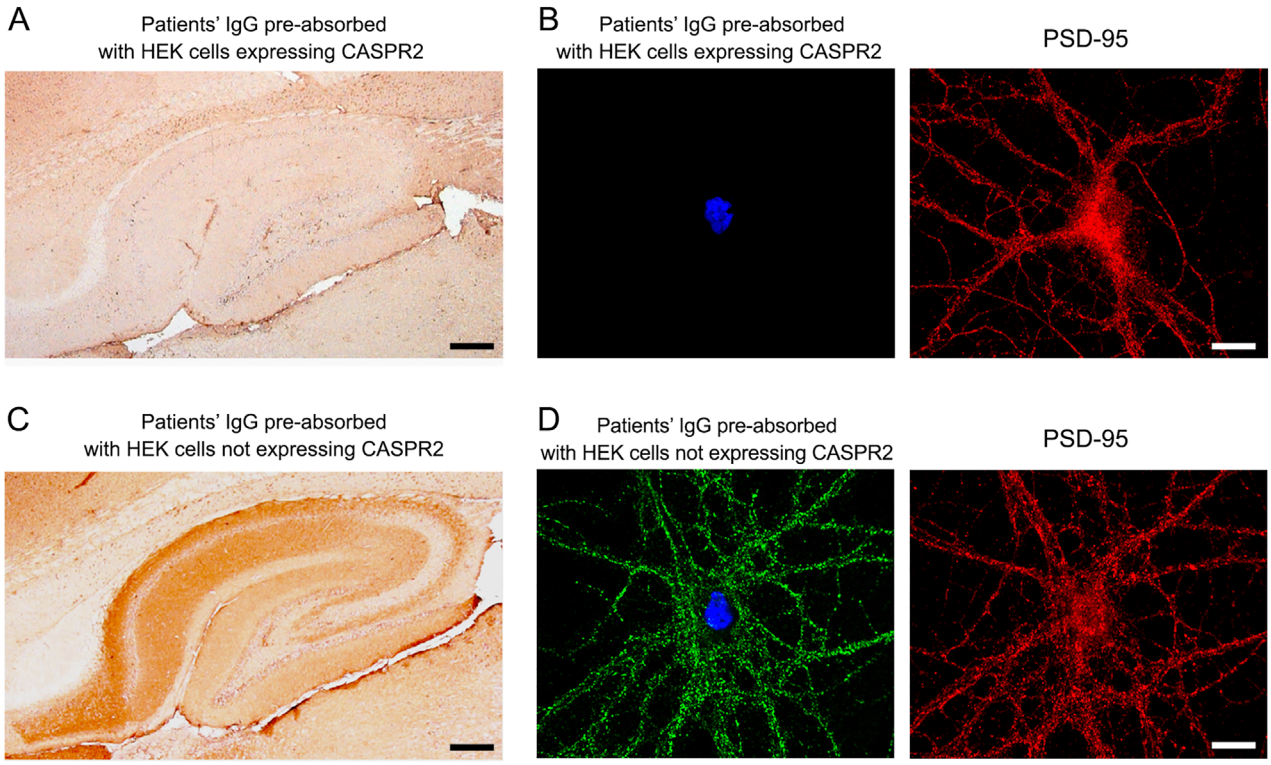


FIGURE 1: CASPR2 specificity of patients' immunoglobulin G (IgG). Immunostaining is shown on sections of rat hippocampus (A, C) and live neurons (B, D) with pooled patients' IgG preabsorbed (A, B) or not preabsorbed (C, D) with CASPR2. The preabsorptions were done with HEK293 cells expressing or not expressing CASPR2. Preabsorbed patients' IgG no longer reacted with rat brain and live neurons (A, B), indicating that the reactivity was caused by CASPR2 antibodies. The same neurons immunolabeled with a commercial PSD-95 antibody (red) are shown in panels labeled "PSD-95." Scale bars = 200μm (A, C), 20μm (B, D).

Immunofluorescence and Confocal Microscopy with Brain Tissue

Nonpermeabilized 5μm-thick cryostat brain sections were incubated sequentially with either a rabbit antibody against Kv1.1 (1:100, #PA5-77654; Invitrogen, Waltham, MA) or a guinea pig antibody against GluA1 (1:200, #AGP009; Alomone, Jerusalem, Israel) for 2 hours at room temperature. Sections were then washed and permeabilized with PBS containing 0.3% Triton X, and incubated with either a mouse antibody against the presynaptic marker Bassoon (1:250, #SAP7F407; Enzo Life Sciences, Lausen, Switzerland) or a rabbit antibody against the postsynaptic marker PSD-95 (1:250, #ab18258, Abcam), overnight at 4°C. Secondary antibodies included goat antirabbit Alexa Fluor 488, goat antimouse Alexa Fluor 594, goat anti–guinea pig Alexa Fluor 488, or goat antirabbit Alexa Fluor 594 (Invitrogen; #A11008, #A11005, #A11073, and #A11012, respectively; all 1:1,000). Kv1.1 and GluA1 clusters that colocalized respectively with Bassoon or PSD-95 were defined as synaptic. Extrasynaptic and synaptic clusters of the indicated proteins were visualized and quantified by confocal imaging (LSM710, Carl Zeiss) using Imaris suite 7.6.4 (Oxford Instruments, Abingdon, UK), as reported.[17]

STED Microscopy

We conjugated commercial antibodies against CASPR2 (AF5145; R&D Systems, Minneapolis, MN) and TAG1 (AF4439, R&D Systems) with NHS Alexa Fluor 488 (A20000; Thermo Fisher Scientific, Waltham, MA), and NHS Abberior STAR 635P (07679, Sigma-Aldrich) fluorophores, as reported.[12] The conjugated antibodies were incubated overnight at 4°C (1:10 anti-CASPR2 and 1:5 anti-TAG1) on nonpermeabilized 5μm-thick brain sections, previously blocked with 5% goat serum. After incubation, the slides were washed and mounted with ProLong Gold (P36930; Molecular Probes, Eugene, OR) and scanned under a gated-STED microscope (TCS-SP8 STED 3X; Leica Microsystems, Wetzlar, Germany). For CASPR2-AF488 and TAG1-AS635P, we used excitation lines of 488 and 635nm, and depletion lines (STED) of 592 and 775nm, respectively. Fluorescence light was collected with HyD SMD molecule detectors on an HC PL APO CS2 100×/1.40 OIL objective (Leica Microsystems). For each animal, we scanned the cornu ammonis 1 (CA1), Schaffer collateral, and dentate gyrus areas (13 regions total) at 400Hz with a final pixel size of 19nm (resolution = 1,024 × 1,024 pixels). All generated images were deconvolved using the Lightning GPU-Based Deconvolution package (Leica Microsystems). Mean density of surface clusters of CASPR2 and TAG1 were obtained using a spot detection algorithm for Imaris suite 8.3 (Oxford Instruments). Colocalization of CASPR2 and TAG1 clusters was determined applying the colocalized spot algorithm implemented in Imaris. For each experimental group, the mean cluster densities of CASPR2 and TAG1 were normalized with the corresponding values in control animals.

Determination of Intracellular CASPR2

Primary cultures of hippocampal neurons were grown as reported.[20] At 17 days in vitro, the cells were treated for 24 hours with patients' IgG or controls' IgG. After washing with culture medium, surface CASPR2 or TAG1 was saturated with excess concentration of sheep CASPR2 antibodies (1:10, AF5145, R&D Systems) or goat TAG1 antibodies (1:10, AF4439, R&D Systems) followed by red fluorescent secondary antibodies (donkey antisheep Alexa 594, A-11016, Thermo Fisher Scientific, 1:1,000; or donkey antigoat Alexa 594, A-11058, Thermo Fisher Scientific, 1:1,000). Neurons were then fixed with 4% PFA for 5 minutes and permeabilized with 1% Triton X, and intracellular CASPR2 or TAG1 were labeled with the same primary antibodies (diluted 1:100) followed by green fluorescent secondary antibodies (donkey antisheep Alexa 488 antibody, A-11015, Thermo Fisher Scientific, 1:1,000; or donkey antigoat Alexa 488 antibody, A32814, Thermo Fisher Scientific, 1:1,000). Slides were then mounted, and results were scanned by confocal LSM710 microscope (Carl Zeiss with EC-Plan NEOFLUAR CS 100×/1.3 NA oil objective). Images were deconvolved, and the mean density of CASPR2 or TAG1 clusters was obtained using a spot detection algorithm from Imaris suite 7.7.1 (Oxford Instruments) and expressed as cluster/length (μm). Green fluorescent clusters were considered intracellular CASPR2 or TAG1, and were quantified. To confirm that the initial secondary antibodies saturated all surface CASPR2 or TAG1, preventing the binding of the second secondary antibodies to the cell surface, a similar immunohistochemical experiment was conducted in which the neurons were not permeabilized.

Statistical Analyses

Confocal cluster densities of GluA1, Kv1.1, PSD-95, or Bassoon, and behavioral tests were analyzed using 2-way analysis of variance. Post hoc Bonferroni testing was used to calculate multiplicity-adjusted p values. STED cluster densities of CASPR2 and TAG1 from different brain regions between the two experimental groups were analyzed with the Mann–Whitney U test as non-normally distributed parameters. For the measurements of intracellular clusters densities of CASPR2 and TAG1, we used an unpaired t test. Experiments were assessed for outliers and tested for normality of data. The alpha level used to determine significance was $p \leq 0.05$. The tests were performed using Prism v8 (GraphPad Software, San Diego, CA) and RStudio v1.4 1717 (http://www.rstudio.com/).

Results

Patients' Clinical Features and IgG Subclasses

All 6 patients with anti-CASPR2 encephalitis were men, with a median age of 69.5 years (range = 62–70) and symptoms of encephalitis that included memory loss, confusion, seizures, or behavioral alterations (Table 1). Three of 6 patients

TABLE 1. Clinical Features of Patients with Encephalitis and CASPR2 Antibodies

Case	Age, yr	Sex	Symptoms	Brain MRI	EEG	CASPR2 Serum Antibody Titer[a]
1	62	M	Seizures postsurgery for lung cancer; episodes of paroxysmal unsteadiness, memory loss	Normal	n/a	1:25,600
2	70	M	Generalized seizures, memory loss, abnormal behavior, unintelligible language, myoclonic jerks, hallucinations	Bilateral medial temporal lobe increased FLAIR signal	Generalized slow activity, no epileptic activity	1:12,800
3	70	M	New onset seizures, confusion	Normal	Slow diffuse activity, no epileptic activity	1:12,800
4	68	M	New onset short-term memory loss, confusion	Normal	Normal	1:400
5	69	M	Generalized seizures, confusion, delusions, agitation	Increased FLAIR signal, edema in right medial temporal lobe	Generalized theta and delta activity; nonconvulsive status epilepticus	1:6,400
6	70	M	New onset short-term memory loss, anxiety	MRI normal; FDG-PET increased uptake in left frontotemporoparietal regions	Bilateral temporal epileptic activity	1:3,200

[a]Obtained with serial dilution of serum and brain tissue immunohistochemistry.
EEG, electroencephalogram; FDG-PET = fluorodeoxyglucose positron emission tomography; FLAIR = fluid-attenuated inversion recovery; M = male; MRI = magnetic resonance imaging; n/a = not available.

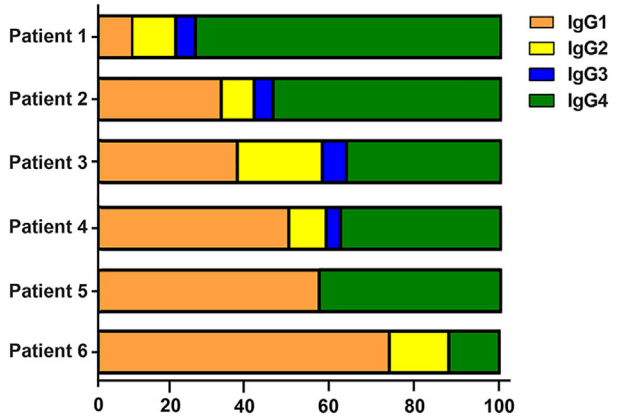


FIGURE 2: Analysis of CASPR2 immunoglobulin G (IgG) subclasses in patients' serum. For each patient's serum, the graph demonstrates the distribution of the indicated CASPR2-specific IgG subclasses measured with flow cytometry. Of note, the figure does not compensate for the differential affinity or signal strength of antihuman IgG subclass antibodies, but it does reflect the wide range of CASPR2 IgG subclasses among patients.

had predominant temporal lobe involvement in magnetic resonance imaging (MRI) or fluorodeoxyglucose positron emission tomography studies, and the other 3 had normal MRI. The electroencephalogram was abnormal in 4 of 5 patients, demonstrating temporal or diffuse epileptic or slow activity. The antibody titers (median = 1:9,200, range = 1:400 to 1:25,600) were assessed with rat brain immunohistochemistry and serial dilution of sera. Each patient harbored IgG1 and IgG4 CASPR2 antibodies, the percentages of which assessed by flow cytometry are shown in Figure 2.

Patients' IgG Binds CASPR2 in the Brain of Infused Mice

Brains from mice infused with patients' IgG sacrificed at day 18 postsurgery (4 days after the infusion of IgG was stopped) showed robust deposits of human IgG with a predominant distribution in the hippocampus and anterior cortex (Fig 3A, B), whereas the presence of human

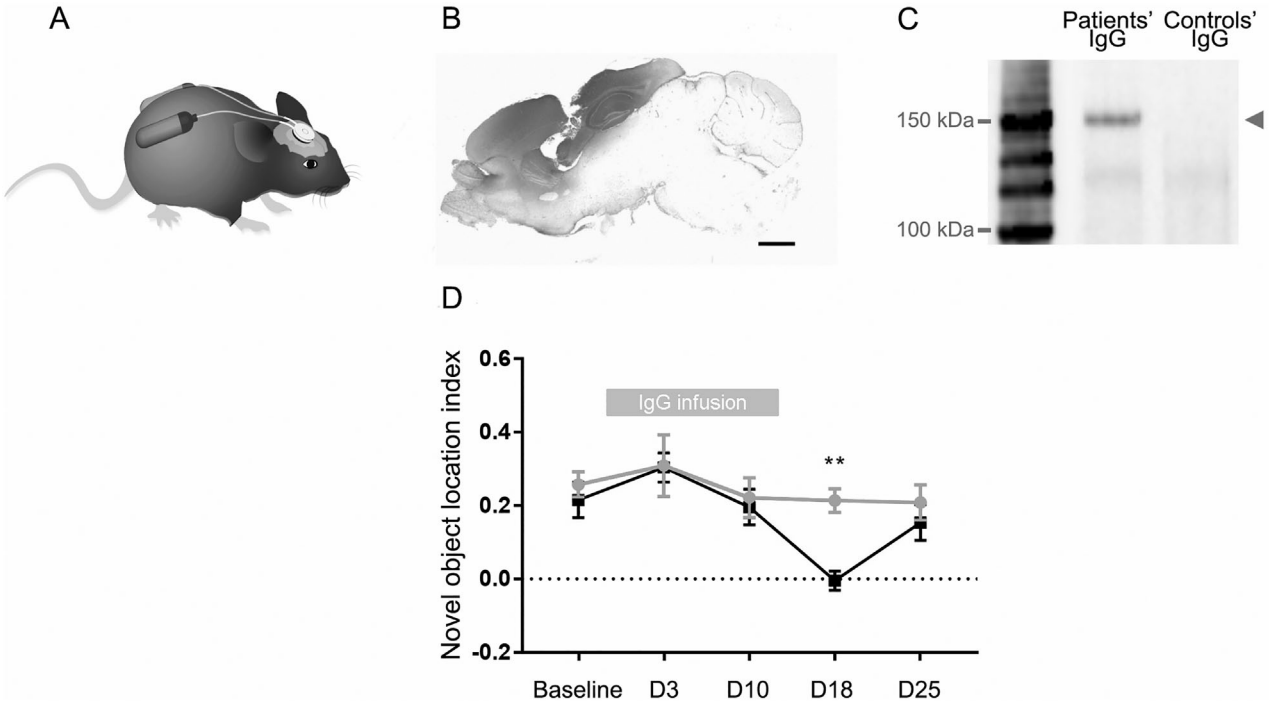
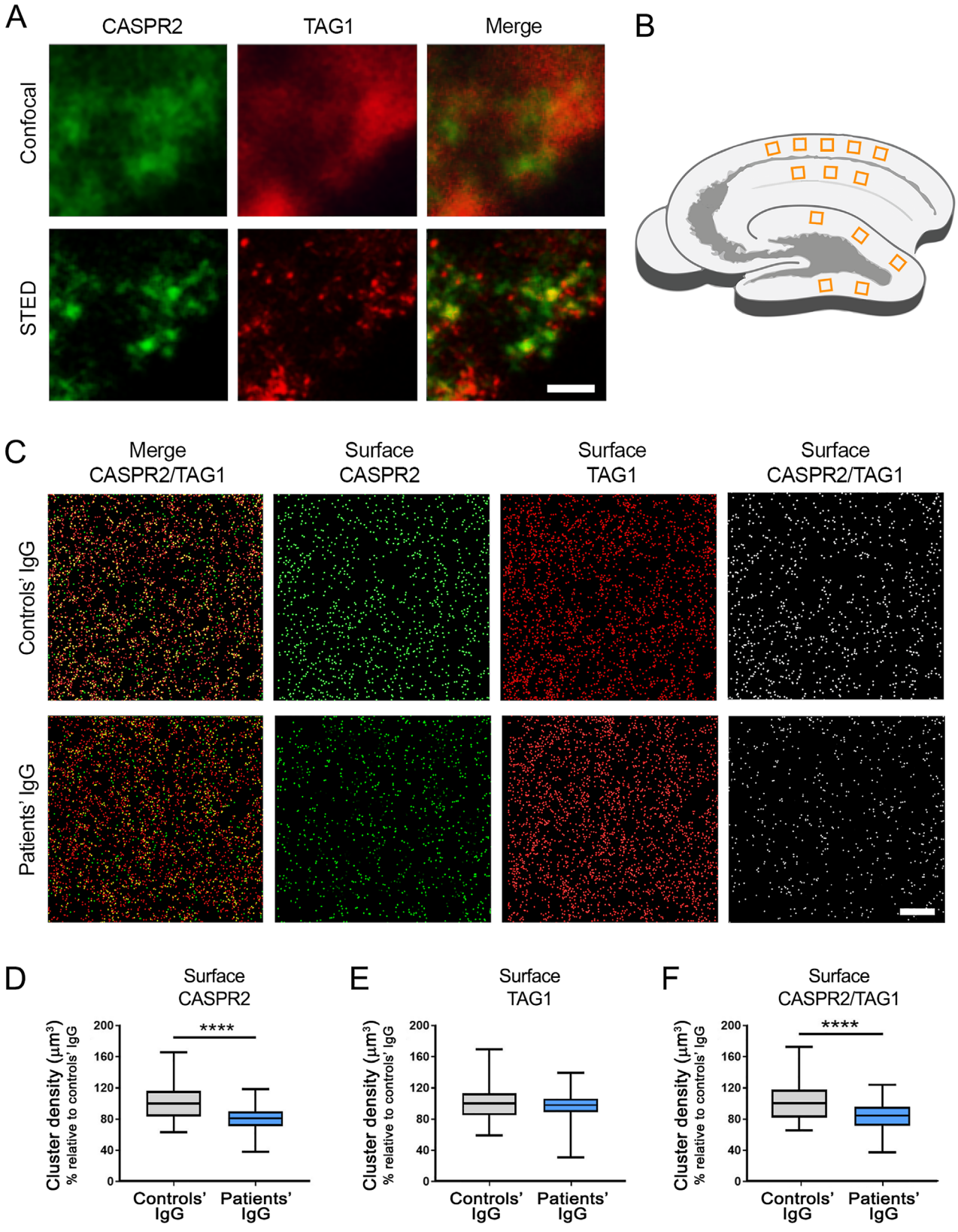


FIGURE 3: Cerebroventricular infusion of patients' immunoglobulin G (IgG), binding to CASPR2, and reversible memory deficit. (A) Schematic representation of the model that uses osmotic minipumps to infuse patients' or controls' IgG to the lateral ventricles of mice. (B) Immunostaining of human IgG present in the brain of a representative mouse at day 18 (4 days after the cerebroventricular infusion of patients' IgG had stopped). There is a predominant IgG distribution in the hippocampus and frontal regions. Scale bar = 2mm. (C) Precipitation of the human IgG from lysates of hippocampus coprecipitated CASPR2 (~150kDa), confirming the in vivo binding of patients' IgG to mouse CASPR2; the lane on the left corresponds to the molecular weight markers. (D) Mice infused with patients' IgG (*black line*) showed a significant reduction of the novel object location (NOL) index at day 18. This memory deficit reverted to values similar to those of controls at day 25. No significant memory changes were noted at other time points or in the group of mice infused with controls' IgG (*gray line*). The total time of exploration of the two objects during the NOL test was similar in the two experimental groups, ruling out the presence of a motor deficit as potential cause of the reduced NOL index, and showing normal exploratory behavior in all animals (data not shown). Number of animals infused with patients' IgG, $n = 14$; and infused with controls' IgG, n = 11. Significance of assessment was performed by two-way analysis of variance (ANOVA; $p < 0.0001$) with Bonferroni post hoc correction, $^{**}p < 0.01$.

IgG was milder and inconsistently found in mice infused with controls' IgG (not shown). In mice infused with patients' IgG, but not in those infused with controls' IgG, the precipitation of IgG bound to hippocampus coprecipitated CASPR2, confirming the in vivo binding of patients' IgG to CASPR2 (see Fig 3C).



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Patients' IgG Causes Transient Memory Impairment in Mice

Compared to mice infused with controls' IgG, those infused with patients' IgG showed a decrease in the novel object location (NOL) index at day 18, indicating a spatial memory deficit (see Fig 3D).[24] The NOL index was not affected at days 3 or 10, and the memory deficit detected at day 18 subsequently reverted to normal values, so that by day 25 (11 days after stopping the infusion of patients' IgG), the NOL index was similar to that of controls. For all time points examined, the total time that mice infused with patients' IgG spent exploring the two objects was similar to that of mice infused with controls' IgG, indicating that the transient decrease of the NOL index was not due to motor deficits interfering with their exploratory behavior.[24] Anxiety-related behavior, motor and cerebellar functions, anhedonia, and pain sensitivity thresholds were normal in both experimental groups. Overall, the findings suggest that hippocampus-independent behavioral tasks were not affected in this model. No difference of body weight was identified between groups.

Patients' IgG Reduces CASPR2 Levels and Its Colocalization with TAG1

We next examined whether the specific binding of patients' IgG to CASPR2 in mouse hippocampus was associated with a reduction of cell-surface CASPR2 clusters or a disruption of the normal physical interaction between CASPR2 and TAG1. For these studies, we used tissue of mice sacrificed at day 18, and STED microscopy to obtain images with resolution at the nanoscale level; otherwise, the resolution with standard confocal microscopy was suboptimal for quantification of CASPR2/TAG1 colocalization. The level of resolution of both types of microscopy is illustrated in Figure 4. Compared with mice infused with controls' IgG, those infused with patients' IgG showed a reduction of surface CASPR2 clusters ($p < 0.0001$), and a decrease of the level of colocalization with TAG1 ($p < 0.0001$), whereas the density of surface TAG1 clusters was not affected. These findings suggest that patients' IgG not only causes a reduction of surface CASPR2 but also disrupts the crosstalk of CASPR2/TAG1.

Patients' IgG Internalizes CASPR2 in Cultured Neurons

To further characterize whether CASPR2 IgG is able to internalize CASPR2, we quantified the levels of intracellular CASPR2 or TAG1 in primary cultures of hippocampal neurons following a short treatment (24 hours) with patients' or controls' IgG. Compared with neurons treated with controls' IgG, those exposed to patients' IgG showed a significant increase of intracellular CASPR2 levels ($p < 0.0001$) without the levels of TAG1 being affected (Fig 5). The selective intracellular labeling was confirmed by demonstrating that after saturating the surface clusters of CASPR2 or TAG1 with specific antibodies (red clusters in Fig 5A, C), no additional labeling of these proteins was observed (data not shown) unless the neurons were permeabilized (green clusters in Fig 5A, C, corresponding to intracellular CASPR2 and TAG1, respectively). An antibody-mediated reduction of surface CASPR2 clusters without significant change of surface TAG1, similar to the changes demonstrated in tissue (see Fig 4D), was also identified in cultured neurons (not shown). Altogether, patients' IgG caused an increase of the levels of intracellular CASPR2 (and decrease of the cell-surface levels, as shown in Fig 4D) without changing the levels of TAG1, suggesting selective internalization of CASPR2.

Patients' IgG Decreases the Surface Levels of Kv1.1 VGKC and GluA1-Containing AMPAR

Because CASPR2 indirectly interacts with the presynaptic Kv1.1 VGKC and the postsynaptic AMPAR, we next examined whether the antibody-mediated reduction of CASPR2 and the disruption of the CASPR2/TAG1 interaction had effects on the surface levels of Kv1.1 and AMPAR. Compared with animals infused with controls' IgG, those infused with patients' IgG showed a significant reduction of synaptic and extrasynaptic Kv1.1 surface clusters at day 18 (both, $p < 0.0001$) that reverted to normal values at day 28 (Fig 6B, C). Similar results were obtained for GluA1-containing AMPAR; mice infused with patients' IgG had a significant reduction of synaptic and extrasynaptic clusters of GluA1 at day 18 ($p < 0.0001$ and $p = 0.0001$, respectively), which reverted to normal values

FIGURE 4: Patients' immunoglobulin G (IgG) cause a reduction of surface CASPR2 and disrupt its interaction with TAG1 in mouse hippocampus. (A) Comparative images of CASPR2 and TAG1 immunolabeling using conventional confocal microscopy (3 upper panels) and stimulated emission depletion (STED) microscopy (3 lower panels). Scale bar = 500nm. (B) Schematic representation of 13 hippocampal regions examined for each animal. (C) Representative hippocampal region of an animal infused with controls' IgG (4 upper panels) or an animal infused with patients' IgG (4 lower panels) immunolabeled with CASPR2 or TAG1 antibodies and analyzed with STED. Studies in all panels correspond to animals sacrificed at day 18. Scale bar = 3μm. (D) Compared with animals infused with controls' IgG, those infused with patients' IgG showed a reduction of surface clusters of CASPR2, without change in the number of clusters of TAG1, and a reduction of CASPR2/TAG1 colocalization. Number of animals per each experimental group, $n = 5$. Box plots show the median and 25th and 75th percentiles; whiskers indicate the 10th and 90th percentiles. Significance of treatment effect was assessed by unpaired *t* test, ****$p < 0.0001$.

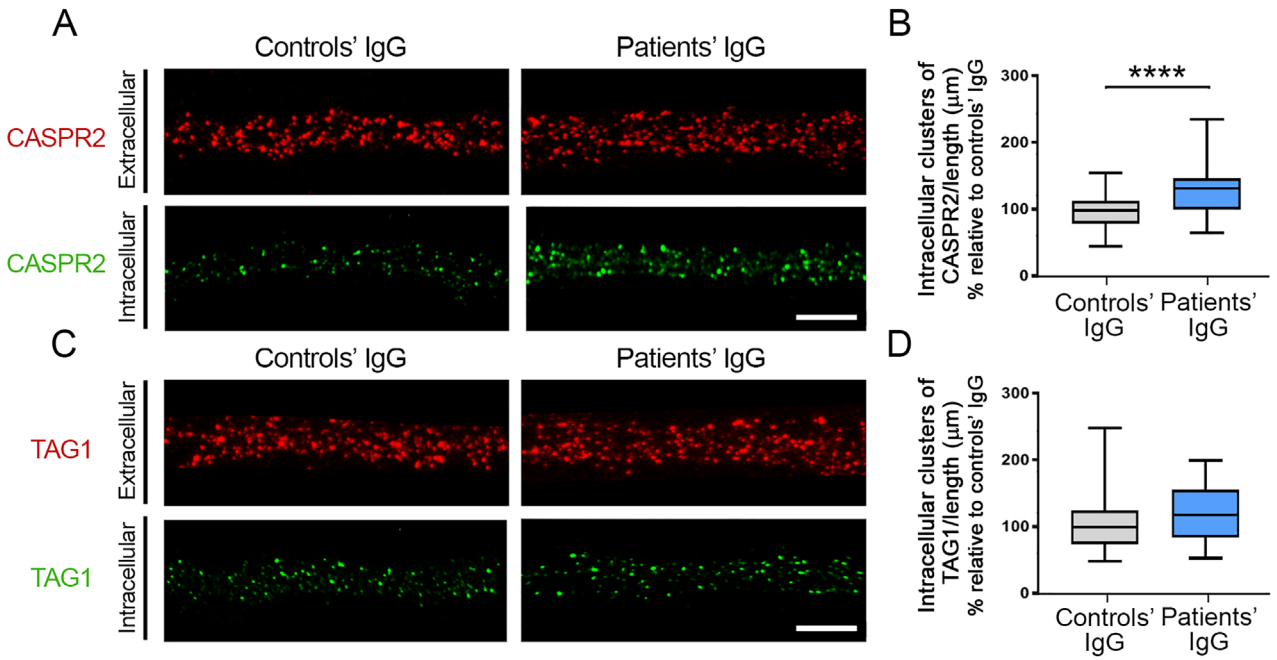


FIGURE 5: Treatment of neurons with patients' immunoglobulin G (IgG) leads to an increase of intracellular CASPR2 clusters. (A, C) Representative dendrites from rat hippocampal cultured neurons exposed to patients' or controls' IgG. The top rows in A and C (red clusters) show the surface clusters of CASPR2 or TAG1 immunolabeled with an excess of the corresponding commercial antibodies (saturated immunolabeling). The lower panels (green clusters) show the corresponding intracellular clusters of CASPR2 and TAG1 after cell permeabilization. Note the visible increase of intracellular CASPR2 after incubation with patients' IgG. In contrast, no visible increase of TAG1 is observed. Scale bars = 5µm. (B, D) Quantitation of the intracellular clusters of CASPR2 and TAG1 after incubation with patients' or controls' IgG. Only CASPR2 is increased in the intracellular compartment. Number of dendrites per condition, $n = 15$, 3 independent experiments. Box plots show the median and 25th and 75th percentiles; whiskers indicate the 10th and 90th percentiles. Significance of treatment effect was assessed by unpaired *t* test, ****$p < 0.0001$.

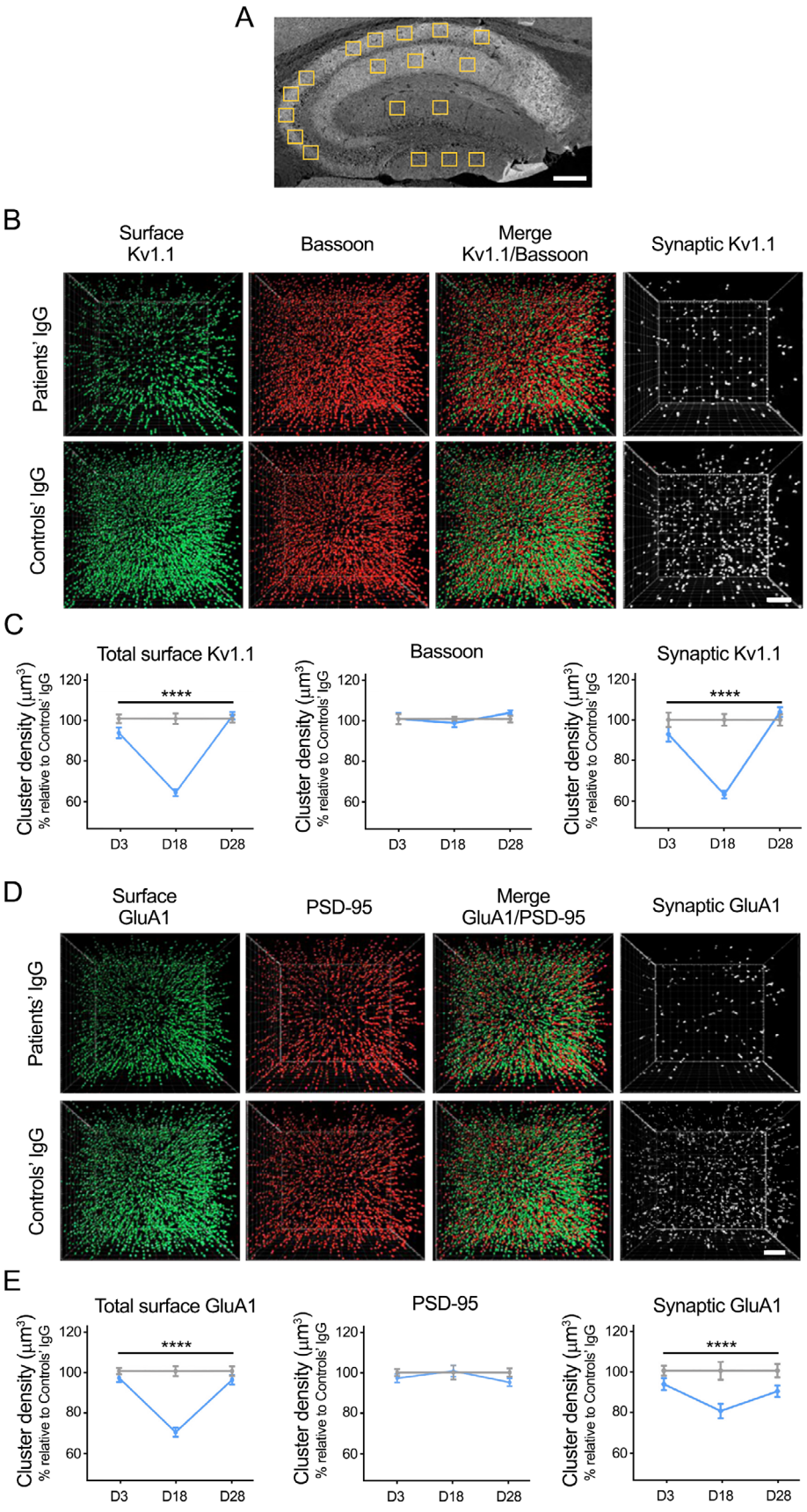
at day 28 (see Fig 6D, E). In contrast, patients' IgG did not alter the levels of the pre- or postsynaptic markers Bassoon or PSD-95 at any time point (see Fig 6B–E), suggesting that the reduction of Kv1.1 and AMPAR clusters was specifically related to the antibody-mediated decrease of surface CASPR2. No detectable changes were observed in mice infused for 3 or 10 days with patients' IgG, suggesting the need of a longer exposure or higher antibody concentration (eg, 14-day infusion) to observe the indicated changes in the CASPR2 protein complex. The time course of these protein changes (maximal alterations on day 18; reversion to normal on day 25) mirrored the memory deficits in the animals (see above).

Discussion

We show that the IgG from patients with anti-CASPR2 encephalitis causes memory deficits accompanied by inhibition of CASPR2/TAG1 interaction and a reduction of the surface levels of CASPR2, Kv1.1, and AMPAR. In addition, we found that after discontinuing the infusion or patients' IgG, all these alterations reversed to values similar to those of the control group. These findings fulfill the Witebsky criteria for autoimmune disease and provide robust evidence of the antibody pathogenicity.[25]

For these studies, we used a homogeneous group of patients with CNS-restricted symptoms along with concurrent IgG1 and IgG4 CASPR2 antibodies, which is the most common IgG subclass composition in this disease.[1] In addition to these features, two known autoantigen characteristics of CASPR2, the occurrence of epitopes along the multiple protein domains[26] and its broad distribution and complex interactions at the cellular level,[2] made more difficult the assessment of antibody effects compared with other autoimmune encephalitis. For example, in anti–N-methyl-D-aspartate receptor (NMDAR) encephalitis, the indicated paradigms are better delimited and easier to investigate (predominant IgG1 subclass, restricted epitope region, and main location of NMDAR at the postsynaptic site).[27,28]

These features and limitations are important to consider when assessing previous reports on CASPR2 antibody effects (Table 2). For example, in two animal models examining the antibody effects on the CNS,[5,9] only serum from a single patient was used to isolate the experimental CASPR2 IgG; in both instances, the IgG



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was composed of IgG1, IgG2, and IgG4. One of the studies included experiments with cultured neurons that showed antibody-mediated decrease of surface levels of CASPR2 and AMPAR.[5] The levels of these proteins were not examined in brain tissue, but AMPAR-mediated currents were found to be decreased after injection of patients' IgG in the visual cortex.[5] In the other study,[9] mice received intraperitoneal injections of patients' IgG followed by opening of the blood–brain barrier with injections of lipopolysaccharide, which by itself can cause neuroinflammation, microglial activation, and behavioral changes.[29] Animals developed microglial and astrocytic activation, with increased complement C3 on astrocytes, associated with impairment of working memory and social interaction.[9] However, it was unclear whether any of these behavioral alterations were caused by patient's CASPR2 IgG, because no significant changes in the levels of CASPR2 or any component of the CASPR2 complex were demonstrated in these animals. The reversibility of the findings was not determined.[9]

Another model examined the effect of systemic CASPR2 IgG administration on the peripheral nervous system.[10] A reduction of CASPR2 and Kv1.1 was found at the juxtaparanodal region, although no IgG binding was detected (this was only found in the dorsal root ganglia). These changes were accompanied by mechanical pain-related hypersensitivity. The reversibility of the effects, IgG subclasses, and changes in CASPR2/TAG1 interaction or AMPAR levels were not investigated.

Two studies with cellular models, one using a solid phase assay and cultured neurons,[7] and the other using HEK cells and cultured neurons,[8] showed that patients' IgG inhibited the interaction of TAG1/CASPR2. Neither of the studies detected changes in the levels of CASPR2, but in one of them,[8] the levels of Kv1.2 were found to be elevated in HEK cells and cultured neurons. The potential reversibility of the antibody effects was not investigated.

Thus, the current mouse model is the first to show a link between antibody-mediated alterations of CASPR2-related proteins and memory impairment. Other potential alterations such as ataxia cannot be addressed with this cerebroventricular transfer model, because the infused IgG preferentially diffuses to the hippocampus and frontal regions.[17] Together with previous studies,[7,8] the findings suggest that the antibody-mediated inhibition of the interaction of CASPR2/TAG1 plays a central pathogenic role. This has been consistently demonstrated in 3 studies using different experimental approaches (solid phase studies,[7] cell cultures,[8] and our current animal model). We postulate that the discrepancy of some studies regarding the surface levels of CASPR2 may be caused by the IgG subclass composition; for example, the presence of IgG1 is likely associated with a decrease of surface levels of CASPR2 (via antibody-mediated internalization), as shown here and in a study using cultured neurons,[5] whereas the presence of highly enriched IgG4 probably inhibits the CASPR2/TAG1 interaction but without substantial alteration of the surface levels of CASPR2, as in the study of Patterson et al.[7] These mechanisms, which should be the focus of future investigations, would be similar to those described in other autoimmune encephalitis (IgG1 for anti-NMDAR encephalitis,[30] IgG4 for anti–leucine-rich glioma-inactivated 1 [LGI1] encephalitis,[16] and coexistence of IgG1 and IgG4 in IgLON5[15]). Any type of disruption of the CASPR2/TAG1 interaction is expected to decrease the clustering and confocal detection of Kv1 VGKC,[31,32] as shown here and in a model examining the antibody effect on peripheral nerves[10]; thus, we have no clear explanation for the increased levels of Kv1.2 reported in a study that used in vitro experiments.[8]

The CASPR2 IgG-mediated reduction of Kv1 and AMPARs resembles the effects of LGI1 antibodies.[16,33] In both animal models, patients' antibodies cause reversible memory deficits linked to a decrease of surface levels of

FIGURE 6: Patients' immunoglobulin G (IgG) causes a reduction of Kv1.1 and GLuA1 in the hippocampus of mice. (A) Distribution of the 18 hippocampal regions (cornu ammonis 1 [CA1], CA3, and dentate gyrus) included in the studies of each animal. Scale bar = 200μm. (B) Three-dimensional projection of a representative hippocampal region (such as those shown in A) from an animal infused with patients' or controls' IgG at day 18, and analysis of the density of total cell surface clusters of Kv1.1, Bassoon, and clusters of synaptic Kv1.1 (defined as those that colocalized with Bassoon). Scale bar = 2μm. (C) Quantification of the densities of cell surface Kv1.1 and Bassoon in subsets of mice infused with patients' IgG (*blue line*) or controls' IgG (*gray line*) and sacrificed at the indicated time points (days 3, 18, or 28 postsurgery). Animals infused with patients' IgG showed a significant decrease of total cell surface and synaptic Kv1.1 at day 18, without affecting the levels of Bassoon. For each time point, 7 animals of each condition were analyzed. (D, E) Parallel studies to those described in B and C, using the same representative animals and hippocampal regions, show the effects of patients' IgG on the density of GluA1-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor or PSD-95. Synaptic clusters of GluA1 were defined as those that colocalized with PSD-95. At day 18, animals infused with patients' IgG (*blue line*) showed a significant decrease of total cell surface and synaptic GluA1 clusters compared with animals infused with controls' IgG (*gray line*). The levels of PSD-95 were unaffected. Scale bar in D = 2μm. For both analyses (C, E), the 14-day infusion of patients' IgG was identical in the subsets of mice euthanized at day 18 or 28, suggesting that after day 18 there was a progressive normalization of the levels of Kv1.1 and GluA1. Data are presented as mean ± standard error of mean. Significance of assessment was performed by repeated-measures two-way analysis of variance with Bonferroni post hoc correction, ****$p < 0.0001$.

TABLE 2. Previous Studies Examining the Pathogenic Effect of Antibodies from Patients with Anti-CASPR2 Encephalitis

Reference	Samples, n	IgG Subclass	Type of Model	Surface CASPR2 Levels	CASPR2/TAG1 Interaction	Surface AMPAR Levels	Surface Kv1 VGKC Levels	Behavioral Effects (reversibility)
7	6	4 IgG4; 2 IgG4 >> IgG1	Solid phase; cultured neurons	No effect	Inhibited	n/s	n/s	n/a
10	2 (1 with LGI1 abs)	n/s	Mouse, systemic injection	Decreased in JXP of peripheral nerves (no IgG bound)	n/s	n/s	Kv1.1 decreased in JXP of peripheral nerves (no IgG bound)	Mechanical pain-related hyper sensitivity (n/s)
8	4	n/s	HEK cells; cultured neurons	No effect on cluster numbers; cluster size increased	Inhibited	n/s	Increased Kv1.2 in HEK cells and neurons	n/a
5	1	IgG1, IgG2, IgG4	IgG injection to visual cortex of mouse; cultured neurons	n/s in mouse; decreased in cultured neurons	n/s	n/s in mouse but decreased AMPAR currents in visual cortex; decreased in cultured neurons	n/s	n/s
9	1	IgG1, IgG2, IgG4	Ip IgG administration to mice; LPS to open BBB	No effect	n/s	n/s	n/s	Impairment of WM and social interaction (n/s)
Current	6	IgG1, IgG2, IgG3, IgG4	Mouse, cerebroventricular infusion, and cultured neurons	Decreased in hippocampus	Inhibited	Decreased in hippocampus	Kv1.1 decreased in hippocampus	Memory loss (reversible)

AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BBB = blood–brain barrier; HEK = human embryonic kidney cell line; IgG, immunoglobulin G; Ip = intraperitoneal; JXP = juxtaparanodal region; LPS = lipopolysaccharide; n/a = not applicable; n/s = not studied; WM = working memory.

Kv1 and AMPAR.[16,34] These alterations probably lead to neuronal hyperexcitability along with an ineffective recruitment of postsynaptic AMPARs (as occur in the anti-LGI1 model[16]), in line with the observed reduction of AMPAR currents after local cortical injection of CASPR2 IgG.[5] A limitation of our study is that we have not included electrophysiological investigations; however, the comprehensive surface protein quantitation with nanoscale level microscopy resolution, and the observed memory alteration in this mouse model offer a framework for these investigations. It will also be important to determine whether enriched IgG4 CASPR2 (which represents ~30% of patients) may cause the same CASPR2/TAG1 inhibition and downstream effects on Kv1 VGKC and AMPARs, but without altering the surface levels of CASPR2.

Finally, the current experience, together with the variety of results from previous reports,[5,7–10] suggests that for a harmonious interpretation of future investigations on CASPR2 antibody pathogenicity, it is important to define well the patients' syndrome, the repertoire of antibody subclasses, the comprehensive assessment of CASPR2-related proteins, and the reversibility of potential alterations, as done here.

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Author Contributions

B.J., M.P.-P., J.P., M.R., and J.D. were responsible for conception and design of the study. B.J., M.P.-P., J.P., F.M., M.R., M.M., E.M., A.G.-S., E.A., A.A.-B., P.L.-A., L.S., and X.G. were responsible for acquisition and analysis of data. B.J., M.P.-P., M.R.R., J.P., and J.D. were responsible for drafting the text and preparing the figures.

Potential Conflicts of Interest

J.D. receives royalties from Athena Diagnostics for the use of Ma2 as an autoantibody test and from Euroimmun for the use of NMDA, GABAB receptor, GABAA receptor, DPPX, and IgLON5 as autoantibody tests.

Data Availability

Data supporting these findings are available upon reasonable request.

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