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Original Article

ORIGINAL ARTICLE

Disrupted AMPA Receptor Function upon Genetic- or Antibody-Mediated Loss of Autism-Associated CASPR2

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Abstract

Neuropsychiatric disorders share susceptibility genes, suggesting a common origin. One such gene is *CNTNAP2* encoding contactin-associated protein 2 (CASPR2), which harbours mutations associated to autism, schizophrenia, and intellectual disability. Antibodies targeting CASPR2 have also been recently described in patients with several neurological disorders, such as neuromyotonia, Morvan's syndrome, and limbic encephalitis. Despite the clear implication of *CNTNAP2* and CASPR2 in neuropsychiatric disorders, the pathogenic mechanisms associated with alterations in CASPR2 function are unknown. Here, we show that Caspr2 is expressed in excitatory synapses in the cortex, and that silencing its expression *in vitro* or *in vivo* decreases the synaptic expression of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors and the amplitude of AMPA receptor-mediated currents. Furthermore, Caspr2 loss of function blocks synaptic scaling *in vitro* and experience-dependent homoeostatic synaptic plasticity in the visual cortex. Patient CASPR2 antibodies decrease the dendritic levels of Caspr2 and synaptic AMPA receptor trafficking, and perturb excitatory transmission in the visual cortex. These results suggest that mutations in *CNTNAP2* may contribute to alterations in AMPA receptor function and homoeostatic plasticity, and indicate that antibodies from anti-CASPR2 encephalitis patients affect cortical excitatory transmission.

Key words: AMPA receptors, Autoantibodies, *CNTNAP2*/CASPR2, experience-dependent plasticity, homoeostatic synaptic scaling, neuropsychiatric disorders

Introduction

Psychiatric disorders are often associated with manifestations of cognitive dysfunction, abnormal thoughts, and behavioural deficits. Despite a complex aetiology, these disorders present common defective features in brain structure and wiring and share susceptibility risk genes, suggesting a similar neurodevelopmental

origin. One such gene is *CNTNAP2*, in which several disease-associated mutations have been identified (Verkerk et al. 2003; Strauss et al. 2006; Belloso et al. 2007; Alarcon et al. 2008; Arking et al. 2008; Bakkaloglu et al. 2008; Friedman et al. 2008; Vernes et al. 2008; Zweier et al. 2009; O'Dushlaine et al. 2011; Smogavec et al. 2016). Patients carrying *CNTNAP2* mutations display seizures, language impairments, intellectual disability, and varying autistic-core behaviours (Penagarikano and Geschwind 2012; Rodenas-Cuadrado et al. 2014; Poot 2017). *Cntnap2* knockout (KO) mice develop epileptic and autism-related behavioural phenotypes that parallel patients' symptoms (Penagarikano et al. 2011), thus emphasising the relevance of the *CNTNAP2* gene in brain function.

The *CNTNAP2*-encoded protein contactin-associated protein 2 (CASPR2) has been recently identified as an antigen in autoimmune synaptic encephalitis (Irani et al. 2010; Lancaster et al. 2011). Serum and cerebrospinal fluid autoantibodies targeting CASPR2 (CASPR2-Abs) have been associated with diverse neurological syndromes including neuromyotonia, Morvan's syndrome, and limbic encephalitis (Bastiaansen et al. 2017; van Sonderen et al. 2017). These syndromes frequently overlap, presenting with peripheral disturbances but also central nervous system dysfunctions such as sleep alterations, seizures, memory impairment, cognitive deficits, and psychosis (Irani et al. 2010; Lancaster et al. 2011; Somers et al. 2011; Klein et al. 2013; Sunwoo et al. 2015; Joubert et al. 2016; van Sonderen et al. 2016; Bien et al. 2017). Circulating CASPR2-Abs were also detected during pregnancy in mothers of children with autism spectrum disorder (Brimberg et al. 2016) or intellectual disability (Coutinho, Jacobson et al. 2017), hinting that gestational transfer of maternal CASPR2-Abs to the foetus contributes to the development of neuropsychiatric disorders in the progeny, and recent studies reveal that offspring mice exposed *in utero* to CASPR2-Abs develop neuropsychiatric phenotypes (Brimberg et al. 2016; Coutinho, Menassa et al. 2017).

CASPR2 is a neurexin-related cell-adhesion molecule expressed in axons, where it acts as a membrane scaffold for the clustering of Shaker-like K^+ channels (VGKC) at the juxtaparanodal region of myelinated axons (Poliak et al. 1999; Scott et al. 2017). However, roles for CASPR2 in regulating the excitation/inhibition balance and neuronal migration have been suggested (Penagarikano et al. 2011). More recently, a synaptic function for Caspr2 has been proposed, since abnormal spine development and a reduced number of excitatory and inhibitory synapses (Anderson et al. 2012; Gdalyahu et al. 2015), as well as accumulation of cytoplasmic aggregates of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors (AMPARs) (Varea et al. 2015) were observed upon loss of Caspr2.

Despite the relevant roles of the *CNTNAP2* gene and its encoded protein CASPR2 for human brain function and their implication in disease mechanisms, the cellular and molecular mechanisms mediated by CASPR2 remain unclear. In the present study, we address the hypothesis that Caspr2 regulates glutamatergic function by testing whether Caspr2 loss of function affects activity-dependent changes in the trafficking of AMPARs, crucial for the expression of synaptic plasticity mechanisms, and by evaluating a potential pathogenic effect of patients CASPR2-Abs in glutamatergic transmission.

Materials and Methods

Animals

For electrophysiological studies, C57BL/6J mice (Jackson Laboratories) were raised under a normal 12-h light/dark cycle until the beginning of experiments. For primary cultures of cortical neurons, pregnant Wistar rats were used. For primary cultures of hippocampal neurons, either Wistar pups at postnatal Day 0 (P0) or pregnant *Cntnap2* KO mice [B6.129(Cg)*Cntnap2*^{tm1Pele}/J; Jackson Laboratory] were used. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Johns Hopkins University, Baltimore, USA, or by the Portuguese National Authority for Animal Health (DGAV).

Human Samples and IgG Purification

Plasma exchange samples of a 72-year-old male patient with anti-CASPR2 encephalitis were used in this study. The patient presented with Morvan's syndrome secondary to high levels of CASPR2 autoantibodies, with progressive neuropathic pain, lack of perception with goosebumps, and memory complaints, with no indication of paraneoplastic causes. Serum from a healthy age- and sex-matched subject was kindly donated. Written informed consent was obtained from both patient and healthy control. Plasma antibody titre was determined through a live cell-based assay as previously reported (Coutinho, Menassa et al. 2017). End-point titration at which a positive signal was still observed for patient antibodies was 1:6400. Human immunoglobulins (IgGs) were purified from healthy or patient plasma samples as previously described (Coutinho, Menassa et al. 2017), and their concentration determined: healthy pIgG—21.2 mg/mL; patient pIgG—15.1 mg/mL. Details can be found in Supplementary Material.

Primary Neuronal Cultures, Neuronal Transfection and *In vitro* Paradigms

Primary cultures of cortical and hippocampal neurons were prepared as previously described in Santos et al. (2012); Coutinho, Menassa et al. (2017), and kept in culture until DIV 13–18. DNA plasmids (Anderson et al. 2012) for neuronal transfection to deplete Caspr2 expression were a kind gift from Dr Thomas Südhof (Stanford University, USA), and were recombinantly expressed in DIV 7 cortical cultures using a previously described protocol (Santos et al. 2012). To induce synaptic scaling, neurons were treated with 1 μM Tetrodotoxin (TTX) or TTX and 100 μM D-5-aminophosphonovalerate (APV) for 48 h at DIV 11. To study CASPR2-Abs, hippocampal or cortical neurons were incubated either with diluted patient serum at 1:100 or with serum-purified human IgGs for 1–7 h. All biochemical and immunocytochemical assays were performed as previously described in Santos et al. (2012); Mele et al. (2014); Coutinho, Menassa et al. (2017). Details can be found in Supplementary Material.

Stereotactic Surgeries

Stereotactic surgeries were performed as previously described (Petrus et al. 2015). Briefly, C57BL/6J mice were injected onto the primary visual cortex (V1) with either recombinant lentivirus or 200 ng of purified human IgGs in a stereotactic apparatus. To specifically target V1-layer 2/3, calculated stereotactic coordinates were: bregma −3.6 mm, lateral 2.5 mm, depth 0.36 mm. Mice injected with human IgGs were recovered for 7 h before sacrifice. Lentiviral-infected mice were returned to the animal colony for 4–5 weeks post-infection before experimental paradigms were initiated. Details can be found in Supplementary Material.

Visual Deprivation Paradigms

Visual deprivation was induced in the form of dark exposure (DE), as described previously (Goel and Lee 2007). Briefly, mice were kept in a dark room for 2 days, and cared for using infra-red vision goggles under dim infra-red light. Age-matched control (normally reared [NR]) animals were continuously raised in normal light conditions for the same duration. Details can be found in Supplementary Material.

Electrophysiology

Acute primary visual cortical slices and electrophysiological methods have been previously described (Goel et al. 2006). AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) were pharmacologically isolated with 1 μM TTX, 20 μM bicuculline and 100 μM APV and measured in whole-cell patch-clamp configuration. Cells were held at −80 mV, digitised at 10 kHz with a data acquisition board and acquired through custom software. At least, 200 consecutive mEPSCs for each cell were analysed with a detection threshold set at 3 times the root mean square noise level. Details can be found in Supplementary Material.

Data Analysis and Statistics

All results are presented as normalised mean ± SEM. Biochemical and immunocytochemistry data are presented from at least 3 different experiments, performed in independent preparations. Mann–Whitney test was used for 2-group comparisons. Comparisons across multiple groups were performed with the Kruskal–Wallis analysis of variance followed by Dunn's Multiple Comparison test, or with a 2-way ANOVA analysis followed by Tukey's multiple comparison test. Significance of cumulative distributions was calculated with the Kolmogorov–Smirnov test. For all tests, $P < 0.05$ was considered statistically significant.

Results

Caspr2 Regulates the Trafficking and Synaptic Content of AMPARs

We immunolabelled cultured rat cortical neurons for Caspr2, and found the Caspr2 signal to be significantly distributed along both axons and dendrites (Fig. 1A). Given the high expression of Caspr2 in axons, to evaluate the dendritic localisation of Caspr2 without interference from axonal staining, we sparsely transfected cortical neurons with a construct encoding HA-tagged human CASPR2 and observed expression of HA-CASPR2 in axons and MAP2-labelled dendrites (Supplementary Fig. S1A). We then co-stained cultured neurons for Caspr2, for the postsynaptic scaffolding protein PSD95 and for the presynaptic vesicular glutamate transporter vGluT1 (Fig. 1A); the signal overlap of Caspr2 with synaptic clusters positive for both

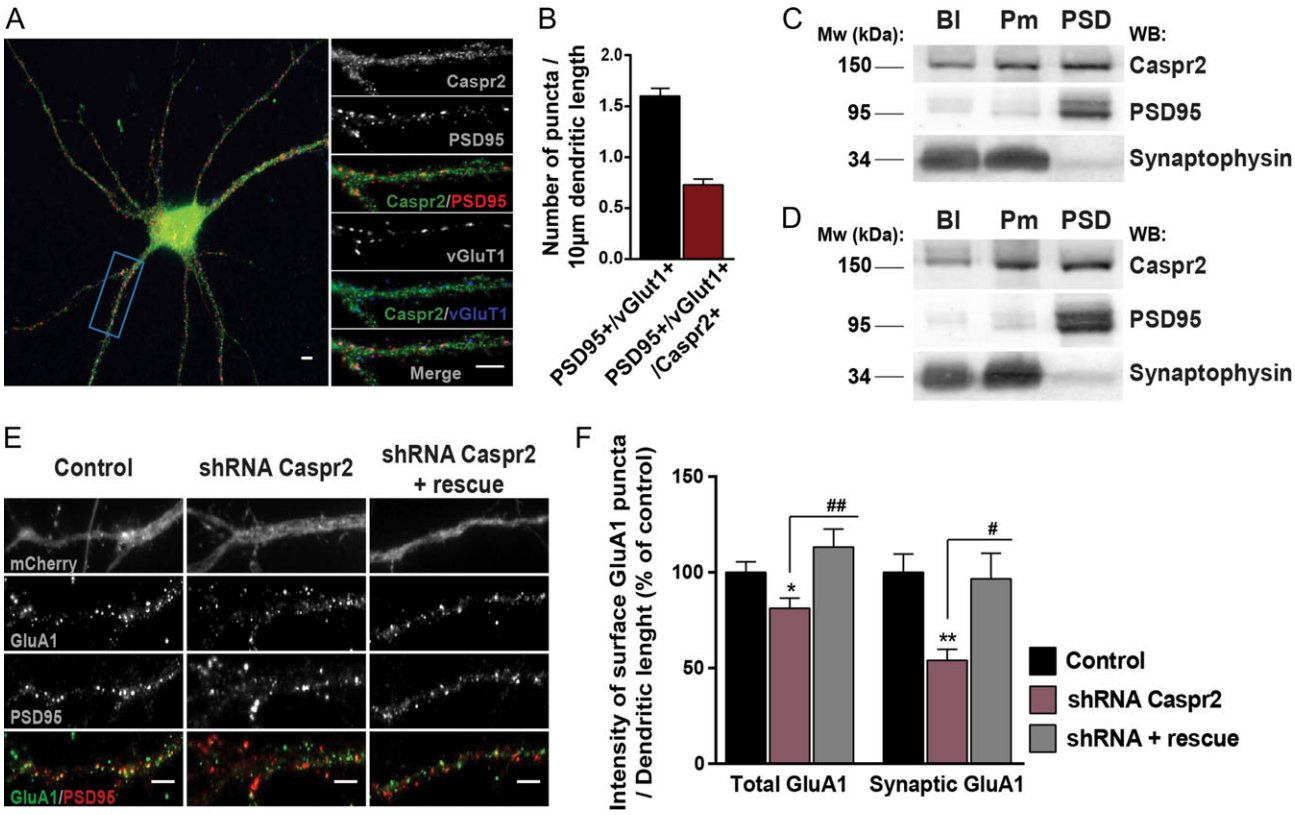

Figure 1. Caspr2 is required to regulate synaptic clusters of surface AMPARs. (*A*) Immunolabelling of Caspr2, PSD95, and vGlut1 in cortical neurons. *Scale bars* = 5 μm. (*B*) Number of glutamatergic synapses (PSD95+/vGlut1+) per 10 μm dendritic length that contain Caspr2 (PSD95+/vGlut1+/Caspr2+). [Independent experiments (N) ≥ 3, number of cells per condition (*n*) ≥ 30 cells]. (*C*, *D*) Representative blots of subcellular fractionations of mouse whole brain **(C)** and cortical **(D)** lysates (Bl), plasma membrane (Pm) fractions, and purified PSD95-enriched postsynaptic densities (PSD). (*E*) Immunolabelling of surface GluA1 and PSD95 in mCherry-expressing cortical neurons transfected with a control empty-vector or the Caspr2-shRNA, or co-transfected with the Caspr2-shRNA and a shRNA-resistant Caspr2 mutant. *Scale bars* = 5 μm. (*F*) Fluorescence intensity of total and PSD95-co-localised synaptic clusters of surface GluA1 was analysed. ($N \geq 3$, $n \geq 30$ cells); in **(F)** Kruskal–Wallis test, Dunn's post hoc test, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control, and $\#P < 0.05$, $\#\#P < 0.01$ relative to Caspr2-shRNA. In **(B)** and **(F)**, results are presented as mean ± SEM.

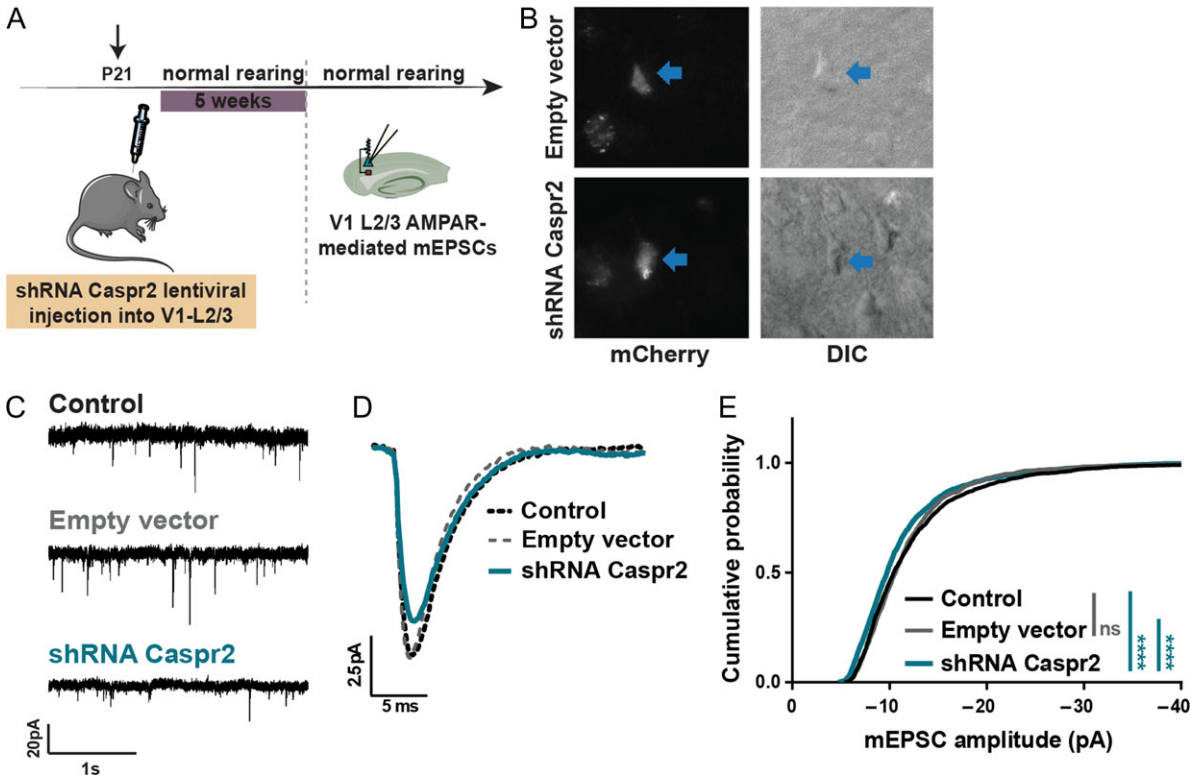

Figure 2. Caspr2 regulates AMPAR function *in vivo* in the mouse primary visual cortex. (*A*) Caspr2-shRNA or empty vector-encoding lentivirus were injected into the mouse V1-L2/3, and AMPAR-mediated mEPSCs were recorded from V1-L2/3 pyramidal neurons. (*B*) Representative image of mCherry-expressing V1-L2/3 pyramidal neurons from lentiviral-infected mice. (*C*, *D*) Comparison of representative mEPSC traces **(C)** and average mEPSC traces **(D)** recorded from either control mice (black, $n = 13$ cells), empty vector-infected (grey, $n = 9$ cells) or shRNA Caspr2-infected mice (blue, $n = 12$ cells). (*E*) Cumulative distribution of mEPSC amplitudes of empty vector-(grey) and shRNA Caspr2-infected mice (blue) plotted against control littermates (Kolmogorov–Smirnov test, ****$P < 0.0001$; ns—non-significant). No changes in the mEPSC frequency or kinetics were observed. See also Supplementary Table S1.

PSD95 and vGluT1 was determined (Fig. 1A, B). We found that 45.5 ± 3.6% of all excitatory synapse clusters contained Caspr2 (Fig. 1B). Furthermore, Caspr2 is expressed in PSD95-enriched postsynaptic densities (PSD) isolated from the total mouse brain (Fig. 1C), or from mouse cortex (Fig. 1D). In agreement with previous studies showing altered inhibitory function in the cortex of *Cntnap2*$^{-/-}$ mice (Bridi et al. 2017), we found that Caspr2 is also expressed in 61.2 ± 3.2% of inhibitory synapses, detected by the overlap of clusters positive for both gephyrin and vGAT (Supplementary Fig. S1B, C).

Based on the localisation of Caspr2 at excitatory synapses, and on the role of Caspr1, a homologous family member of Caspr2, in regulating AMPAR traffic in hippocampal neurons (Santos et al. 2012), we hypothesised that Caspr2 may regulate excitatory synaptic function. To investigate if Caspr2 affects AMPAR trafficking, we transfected cortical neurons with a specific shRNA sequence previously validated to knockdown (KD) Caspr2 expression (Anderson et al. 2012), and evaluated the cell surface and synaptic distribution of GluA1-containing AMPARs (Fig. 1E). Quantitative immunofluorescence analysis of mCherry-expressing transfected neurons revealed that shRNA-mediated loss of Caspr2 resulted in decreased intensity of total and synaptic cell surface GluA1 clusters (Fig. 1F). To exclude the contribution of off-target effects of the Caspr2-shRNA, neurons were co-transfected with the Caspr2-shRNA and a shRNA-resistant mutant construct of Caspr2 (Anderson et al. 2012). Expression of this mutant rescued the KD-mediated decrease in total and synaptic GluA1 cluster intensity, indicating that the observed defects were specifically caused by the loss of Caspr2 (Fig. 1E, F). Caspr2 depletion did not affect PSD95 dendritic clustering (Supplementary Fig. S2A, C) nor the intensity of GluA2 clusters (Supplementary Fig. S2A, B). These observations indicate that Caspr2 regulates the trafficking of GluA1-AMPARs to the cell surface and into synapses.

Caspr2 is Required for *In vivo* Regulation of AMPAR Function

To understand if Caspr2 is relevant for AMPAR function *in vivo*, we injected lentivirus encoding the Caspr2-shRNA and co-expressing mCherry into layer 2/3 of the mouse primary visual cortex (V1) (Fig. 2A). Infected pyramidal neurons in V1-layer 2/3 of injected animals at P60 were identified by the pyramid-shaped soma with the apical dendrite pointing to the pia, and expression of mCherry fluorescence (Fig. 2B). We measured AMPAR-mediated mEPSCs (Fig. 2C). There was a marked decrease in the amplitude of average trace of mEPSCs recorded from Caspr2 shRNA-infected animals (Fig. 2D—blue trace), and the cumulative distribution of Caspr2 shRNA-mEPSC amplitudes was significantly shifted towards smaller values (Fig. 2E—blue trace), with no changes in the current frequency (Supplementary Table S1). These observations indicate that *in vivo* loss of Caspr2 impairs basal AMPAR-mediated synaptic transmission in the mouse visual cortex.

CASPR2 Autoantibodies Impair AMPAR Function

Autoantibodies targeting CASPR2 have been recently described in encephalitis patients with severe cognitive defects and

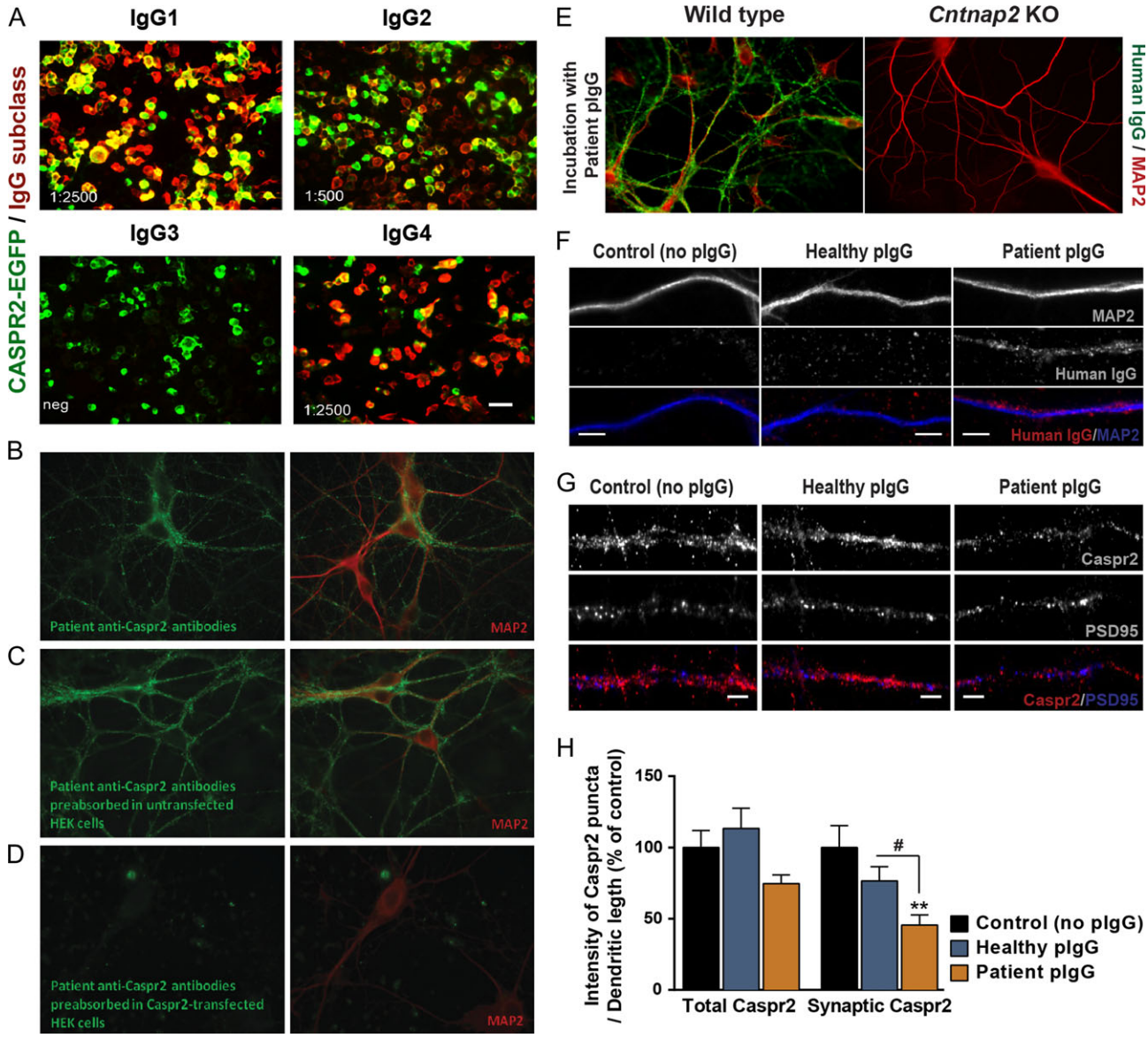

Figure 3. CASPR2 autoantibodies, predominantly of the IgG1 and IgG4 subclasses, fail to bind to *Cntnap2* KO hippocampal neurons and significantly alter the expression of Caspr2 in cortical dendrites. (*A*) Immunolabeling of human CASPR2-EGFP-transfected HEK293 cells (green) incubated for 1 h with the plasma of a patient with anti-CASPR2 encephalitis. Human immunoglobulins (IgGs; red) are immunolabelled with anti-human IgG antibodies specific for each IgG subclass. End-point titration for each subclass is given in the lower left corner of each panel. (*B*) Immunolabelling of human IgGs (green) and the neuronal marker MAP2 (red) in hippocampal neurons incubated for 1 h with the patient plasma. (*C, D*) To confirm the specificity of patient IgGs for CASPR2, and test for the presence of autoantibodies against other neuronal protein targets, patient plasma was pre-absorbed in (*C*) untransfected HEK293 cells, or (*D*) HEK293 cells expressing human CASPR2, and incubated with hippocampal neurons. Plasma pre-absorbed in CASPR2-expressing cells fails to stain hippocampal neurons. (*E*) Immunolabelling of human IgGs (green) and the neuronal marker MAP2 (red) in either wild-type or *Cntnap2* KO hippocampal neurons incubated for 1 h with IgGs purified from the patient plasma (patient pIgGs). Patient pIgGs bind strongly to the surface of WT, but not of *Cntnap2* KO hippocampal neurons. (*F*) Immunolabelling of human IgGs (red) and the dendritic neuronal marker MAP2 (blue) in cortical neurons incubated for 7 h with IgGs purified from either the patient plasma (patient pIgGs) or from healthy control (healthy pIgGs). *Scale bars* = 5 μm. (*G*) Immunolabelling of Caspr2 clusters in cortical neurons incubated for 7 h with healthy or patient pIgGs. *Scale bars* = 5 μm. (*H*) Fluorescence intensity of total and PSD95-co-localised synaptic clusters of Caspr2 was quantified. $N = 3$, $n \geq 30$ cells; Kruskal–Wallis test, Dunn's post hoc test, $^{**}P < 0.01$ compared with control, $\#P < 0.05$ relative to healthy pIgG. Results are presented as mean ± SEM.

psychiatric symptoms, but their pathogenesis is not fully characterised. To explore the mechanisms underlying the pathogenesis of CASPR2 autoantibodies, we used human IgGs preparations from a patient with CASPR2-Ab autoimmune encephalitis, and tested their specificity of binding to CASPR2 (Coutinho, Menassa et al. 2017). Human IgGs in the plasma of the patient have high titre of both CASPR2-IgG1 and -IgG4 subtypes (Fig. 3A), and bind strongly to hippocampal neurons, which did not occur when plasma was previously pre-absorbed in human CASPR2-transfected HEK293 cells (Fig. 3B–D). Moreover, purified IgGs (pIgGs) from the patient plasma failed to bind to hippocampal neurons isolated from *Cntnap2* KO mice (Fig. 3E), indicating specific detection of Caspr2. We incubated cortical neurons for 7 h with pIgGs, which specifically bind to dendritic compartments (Fig. 3F), and evaluated their effect on the dendritic and synaptic distribution of Caspr2 (Fig. 3G). Quantitative analysis revealed that incubation with IgGs purified from the patient plasma (patient pIgG; 200 ng/mL) decreased the intensity of total and

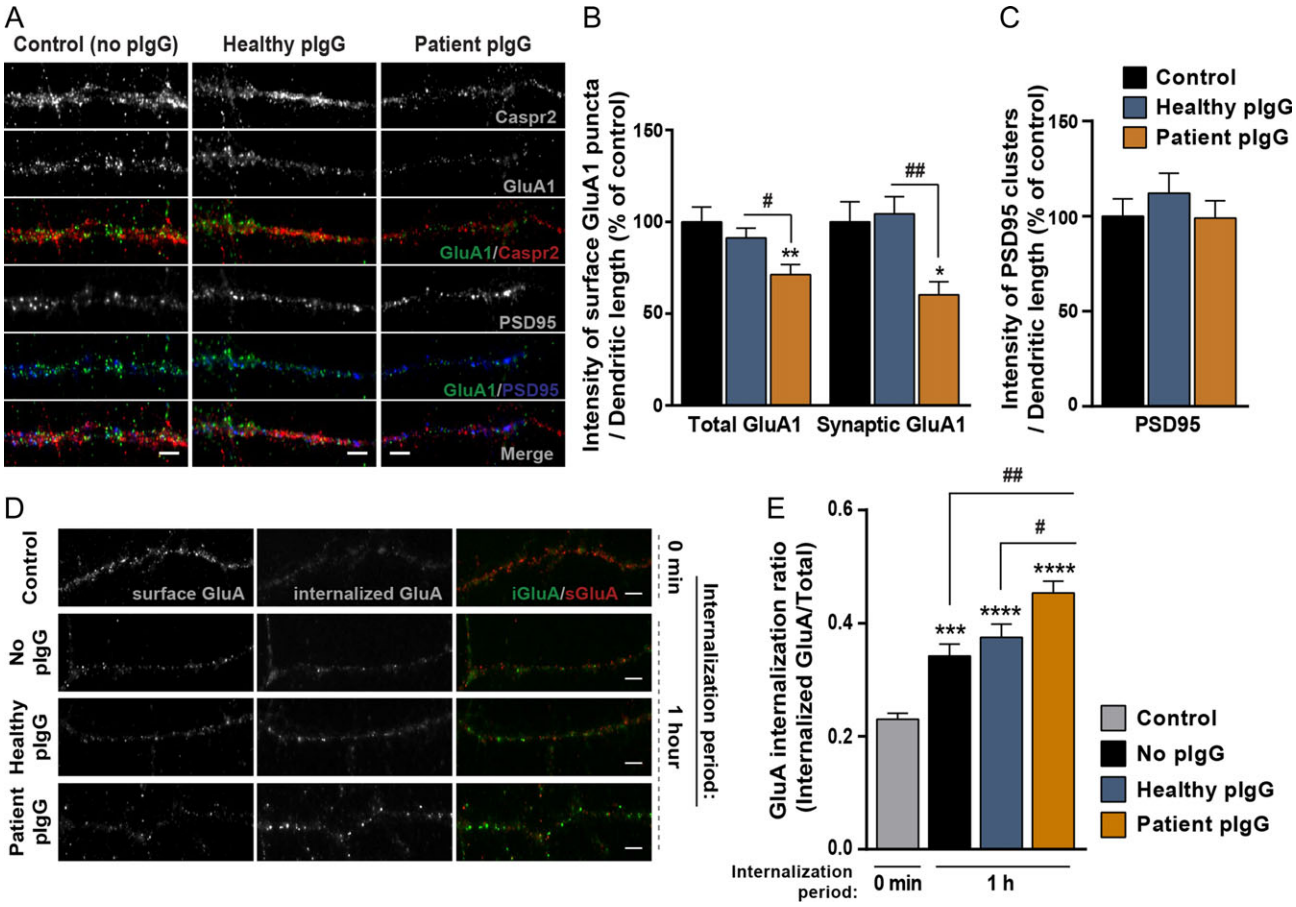

Figure 4. CASPR2 autoantibodies decrease the synaptic expression of surface AMPAR clusters by increasing their internalisation from the cell surface. (*A*) Immunolabelling of surface GluA1, Caspr2, and PSD95 clusters in cortical neurons incubated with healthy or patient pIgGs. *Scale bars* = 5 μm. (*Caspr2 and PSD95 representative image panels are identical to* Fig. 3G). (*B, C*) Fluorescence intensity of (B) total and PSD95-co-localised synaptic clusters of surface GluA1, and (C) PSD95 clusters was quantified. ($N = 3$, $n \geq 30$ cells; in (B), Kruskal–Wallis test, Dunn's post hoc test, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control, $\#P < 0.05$, $\#\#P < 0.01$ relative to healthy pIgGs). (*D*) Immunolabelling of surface and internalised AMPAR clusters following a 10-min incubation of live neurons with an antibody against an extracellular epitope of GluA subunits ("feeding"). Neurons were then incubated with 200 ng/mL of either healthy or patient pIgGs for up to 1 h at 37 °C to promote internalisation of antibody-bound AMPARs. *Scale bars* = 5 μm. (*E*) Fluorescence intensity of surface and internalised clusters of AMPARs was analysed and the receptor internalisation ratio (internalised/total GluA) was calculated. $N = 2$, $n \geq 40$ cells; Kruskal–Wallis test, Dunn's post hoc test: $^{***}P < 0.001$, $^{****}P < 0.0001$ when comparing incubated cells after 1 h of internalisation to the control (0 min) condition; $\#P < 0.05$, $\#\#P < 0.01$ when multiple comparisons were done between conditions after 1 h of internalisation. In (B), (C), and (E), results are presented as mean ± SEM.

synaptic dendritic clusters of Caspr2, as determined using an antibody against the intracellular C-terminus of the protein, when compared with control cells or neurons incubated with IgGs purified from a healthy control (healthy pIgGs; Fig. 3H). We further tested whether CASPR2-Abs can be internalised over time. Cultured cortical neurons were incubated during 1 or 7 h with either healthy or patient pIgGs, fixed and then immunolabelled with an excess concentration of an anti-human fluorophore-tagged secondary antibody to stain human IgGs bound to the cell surface. After permeabilization, cells were immunolabelled with a second anti-human secondary antibody, tagged with a different fluorophore, to stain potentially internalised human IgGs, and further stained against MAP2 to visualize dendritic processes. This analysis showed significant intracellular presence of anti-human antibodies in neurons incubated with the patient CASPR2-Abs, supporting the possibility that CASPR2-Abs and associated Caspr2 are internalised (Supplementary Fig. S3).

We then evaluated whether CASPR2-Abs might impact the distribution of cell surface GluA1-AMPARs (Fig. 4A). In fact, incubation of cortical neurons with patient pIgGs significantly decreased the intensity of surface GluA1 total and synaptic clusters (Fig. 4B), without affecting the intensity of PSD95 dendritic clusters (Fig. 4C). This result suggests that CASPR2 autoantibodies may perturb the role of Caspr2 in the regulation of AMPAR synaptic traffic, possibly through changes in the molecular mechanisms underlying receptor endocytosis and/or recycling. To further explore this, we started by evaluating the effect of CASPR2-Abs on AMPAR internalisation. Since internalisation is expected to be fast following antibody exposure, we first tested whether 1 h incubation with patient CASPR2-Abs is enough to lead to decreased GluA1 surface levels. We found that in fact after 1 h incubation with patient pIgGs surface GluA1 levels are decreased (Supplementary Fig. S4A, B) to a similar extent as with the 7 h incubation timepoint (Fig. 4A, B). We also tested total GluA1 levels, and found them to be unchanged in neurons incubated for 1 h with patient pIgGs when compared with both control or healthy pIgG-incubated cells (Supplementary Fig. S4C, D), further arguing in favour of increased AMPAR endocytic rates sustaining the decrease in surface AMPAR levels upon CASPR2-Ab incubation. To test this, we performed antibody-feeding

experiments, in which neurons were first incubated for 10 min at RT with an antibody against an extracellular epitope in AMPAR subunits, followed by 1 h incubation with either healthy or patient pIgGs at 37 °C to induce internalisation of antibody-bound AMPARs. We analysed the surface and internalised signal of AMPAR clusters (Fig. 4D), and calculated the receptor internalisation ratio (internalised/total GluA, Fig. 4E). We observed that AMPARs are significantly internalised following 1 h incubation at 37 °C, and found that, in neurons incubated for 1 h with patient pIgGs, the internalisation ratio of AMPARs is significantly higher than that of either control (no pIgG) or healthy pIgG-incubated neurons (Fig. 4E). These findings indicate that CASPR2-Abs decrease the synaptic content of surface AMPAR clusters by promoting AMPAR internalisation from the cell surface.

To investigate *in vivo* if the disruptive effect of CASPR2-Abs in the regulation of AMPAR trafficking is relevant for AMPAR function, we injected patient or healthy IgGs (200 ng) into mouse V1-layer 2/3 (Fig. 5A) and measured AMPAR-mediated mEPSCs in V1-layer 2/3 pyramidal neurons (Fig. 5B). The average mEPSC trace of mice injected with patient pIgGs was strikingly smaller than control or healthy pIgG traces (Fig. 5C—yellow trace), and the cumulative distribution of patient pIgG-mEPSC amplitudes was significantly shifted towards smaller values (Fig. 5D; patient pIgG: yellow). No changes were detected in mEPSC frequency or current kinetics (Supplementary Table S2). These observations reveal that CASPR2-Abs impair the localisation of Caspr2 to excitatory synapses and perturb AMPAR-mediated synaptic transmission *in vivo*.

Caspr2 is Essential for Homoeostatic Synaptic Scaling

Activity-dependent regulation of AMPAR traffic is fundamental for several modalities of synaptic plasticity (Shepherd and Huganir 2007). Synaptic scaling is a form of homoeostatic plasticity that adjusts the overall synaptic strength of a neuron through compensatory changes in the synaptic accumulation of AMPARs, to maintain neuronal networks in-balance when perturbations in activity occur (Turrigiano 2008; Fernandes and Carvalho 2016). Failure in neuronal synaptic homoeostasis is considered an underlying pathology in several cognitive disorders (Ramocki and Zoghbi 2008; Wondolowski and Dickman 2013; Mullins et al. 2016). We asked whether the role of Caspr2 in the regulation of AMPARs impinges on mechanisms of neuronal homoeostasis. To evaluate if Caspr2 expression is itself regulated by neuronal activity, we treated cortical neurons for 48 h with either the voltage-gated Na^+-channel blocker TTX (1 µM) to inhibit action potential firing, or TTX together with APV (100 µM) for additional blockade of NMDAR function. As previously described (Ju et al. 2004; Wierenga et al. 2005), we observed that TTX or TTX + APV treatment resulted in a homoeostatic upregulation of the GluA1 subunit of AMPARs, which was paralleled by an increase in total Caspr2 (Fig. 6A, B). A significant increase in the intensity of total and synaptic GluA1 clusters following blockade of neuronal activity with TTX + APV was detected (Fig. 6C–E). Importantly, when transformed by a scaling factor, the scaled TTX + APV distribution completely superimposed with the control cumulative distribution, indicating a multiplicative effect (a defining characteristic of synaptic

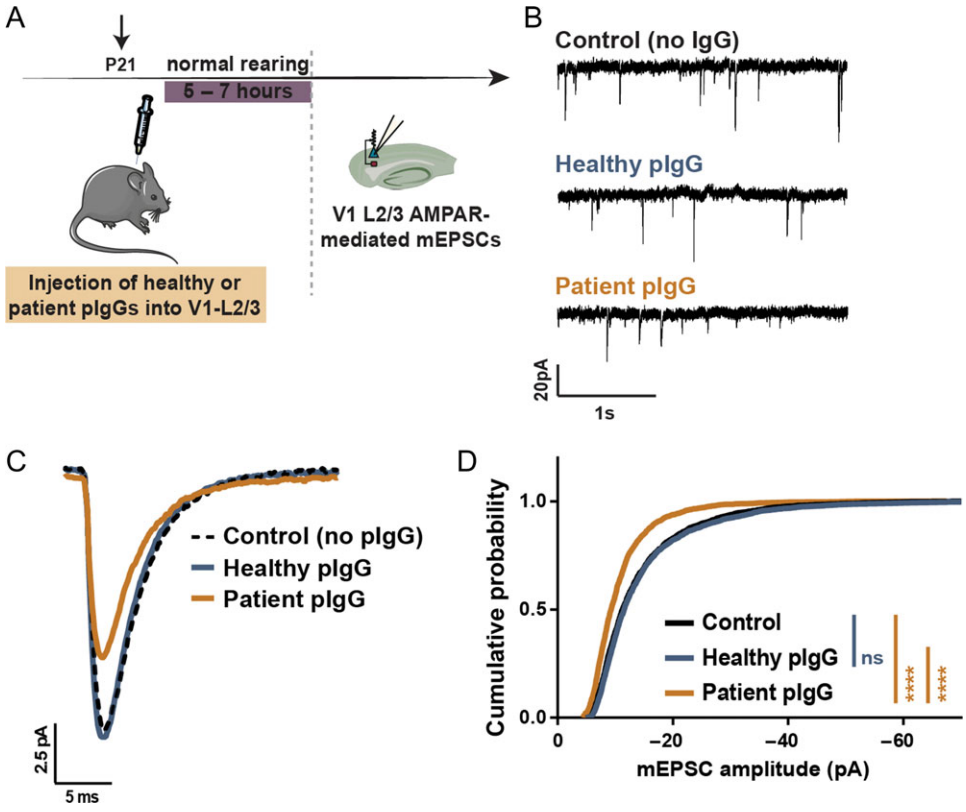

Figure 5. CASPR2 autoantibodies perturb AMPAR function *in vivo* in the mouse primary visual cortex. (A) Healthy or patient pIgGs were injected into L2/3 of the mouse V1, and AMPAR-mediated mEPSCs recorded from V1-L2/3 pyramidal neurons. **(B, C)** Representative mEPSC traces **(B)**, and average mEPSC traces **(C)** recorded from control animals (n = 7 cells—black), or from mice injected with either healthy (n = 10 cells—blue), or patient pIgGs (n = 9 cells—yellow). **(D)** Cumulative distribution of mEPSC amplitudes of mice injected with healthy (blue) or patient pIgGs (yellow) plotted against control mice (black). (Kolmogorov–Smirnov test, ****$P < 0.0001$, ns—non-significant). No changes in mEPSC frequency or kinetics were observed. See also Supplementary Table S2.

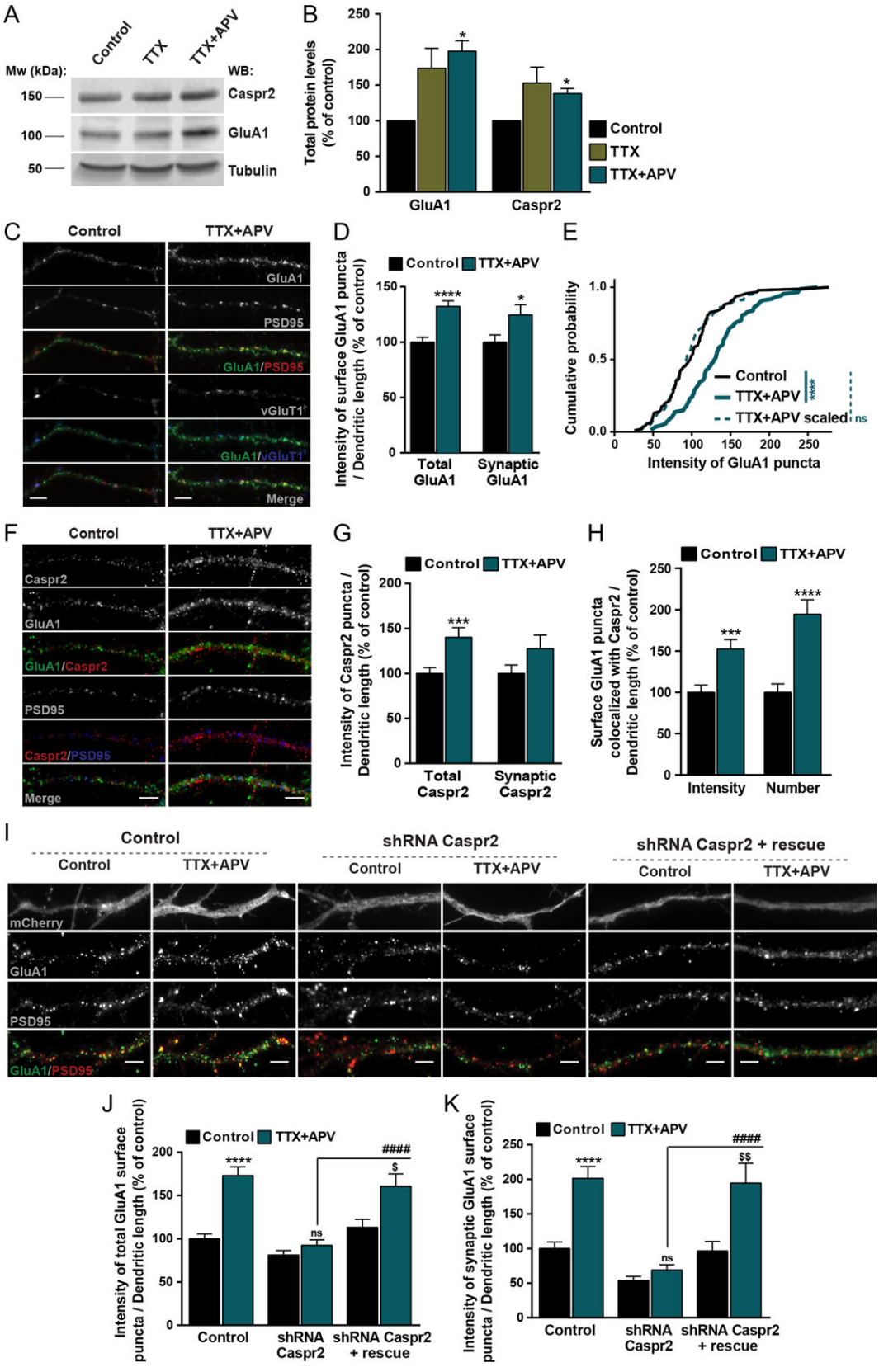

Figure 6. Caspr2 is regulated by neuronal activity, and its loss prevents synaptic upscaling of GluA1-containing AMPARs. **(A)** Representative blot of GluA1 and Caspr2 levels from cortical neurons treated for 48 h with TTX or TTX and APV. **(B)** Total protein levels of GluA1 and Caspr2 were determined. ($N = 4$; Kruskal–Wallis test, Dunn's post hoc test, $^*P < 0.05$ compared with control). **(C)** Immunolabelling of surface GluA1, PSD95, and vGlut1 clusters in cortical neurons treated for 48 h with TTX + APV. *Scale bars* = 5 μm. **(D)** Fluorescence intensity of total and synaptic (co-localised with PSD95 and vGlut1) clusters of surface GluA1 was quantified. ($N \geq 3$, $n \geq 30$

scaling). Furthermore, we observed that TTX + APV treatment significantly increased the fluorescence intensity of Caspr2 clusters (Fig. 6F, G), and the intensity and number of cell surface GluA1 clusters co-localised with Caspr2 (Fig. 6H).

We then investigated if Caspr2 is required for the expression of synaptic scaling. In fact, neurons expressing the Caspr2-shRNA failed to trigger the homoeostatic scaling of GluA1 (Fig. 6I–K), which was fully restored by the expression of the shRNA-resistant form of Caspr2 (Fig. 6I–K). Overall, these findings indicate that Caspr2 is regulated by neuronal activity and necessary for the synaptic scaling of GluA1-AMPARs.

Caspr2 is Required for Experience-Dependent Homoeostatic Plasticity

We asked whether Caspr2 function *in vivo* impinges on mechanisms of experience-dependent homoeostatic plasticity that are fundamental for sensory processing (Whitt et al. 2014). Such mechanisms have been characterised in the mouse visual cortex through manipulations of visual experience: 2 days of visual deprivation (in the form of dark exposure – DE, Fig. 7A) causes a homoeostatic increase of AMPAR-mediated mEPSCs in V1-layer 2/3 pyramidal neurons (Goel et al. 2011) that is accompanied by a specific upregulation in protein levels of the GluA1 subunit of AMPARs (Goel et al. 2011) (Fig. 7B, C), suggesting that a prolonged absence of visually-driven activity scales up excitatory synapses in V1-layer 2/3. We evaluated if Caspr2 expression can be regulated by visual experience *in vivo*, and observed that dark exposing (DE) P60 mice for 2 days significantly increased total protein levels of Caspr2 in the V1 when compared with normal reared (NR) mice (Fig. 7B, D), whereas it did not change Caspr2 expression in P21-30 mice (Supplementary Fig. S5), indicating that this effect is developmentally regulated.

To investigate whether Caspr2 is required for visually-driven experience-dependent homoeostatic plasticity in P60 animals, mice infected for expression of the Caspr2-shRNA were dark exposed for 2 days (Fig. 7E) and AMPAR-mediated mEPSCs of V1-layer 2/3 pyramidal neurons were recorded (Fig. 7F). Both in non-infected control or empty vector-infected mice, DE increased the amplitude of average mEPSC traces and significantly shifted the cumulative distribution of mEPSC amplitudes to larger values when compared with NR (Fig. 7G, H—control; Fig. 7I, J—empty vector). Average frequency and kinetics of mEPSCs remained unaltered following DE of both control and empty vector-infected mice (Supplementary Table S1). Importantly, dark exposure of Caspr2 shRNA-mice failed to upscale the average mEPSC trace (Fig. 7K), and the cumulative distribution of DE amplitudes instead showed a small but statistically significant shift towards smaller values than that of NR Caspr2-shRNA (Fig. 7L), suggesting a downscaling effect. Overall, these observations indicate that levels of Caspr2 in the V1 are regulated by visual experience, and that loss of Caspr2 hinders the scaling up of AMPAR function induced by visual deprivation, suggesting that Caspr2 is essential for visually-driven experience-dependent homoeostatic plasticity.

Discussion

We report that Caspr2 is present in approximately half of all excitatory synapses in rat cortical neurons, and enriched in PSD fractions from the mouse brain. Silencing of Caspr2 expression decreases the cell surface expression of GluA1-AMPARs *in vitro* at cortical synapses, and the amplitude of AMPAR-mediated mEPSCs *in vivo* in the mouse visual cortex. These findings are in agreement with reports indicating that Caspr2 is present in dendritic spines (Varea et al. 2015) and enriched in the synaptic plasma membrane (Bakkaloglu et al. 2008), and suggest an impairment in AMPAR trafficking and AMPAR-mediated synaptic transmission upon Caspr2 loss of function, accordant with the cytoplasmic AMPAR aggregates detected in *Cntnap2* KO cortical neurons (Varea et al. 2015).

AMPAR trafficking and localisation to synapses is dynamically modulated by several AMPAR-interacting intracellular (e.g., SAP97 and other MAGUKs, PICK1, GRIP1, protein 4.1 N) and transmembrane (e.g., stargazin and other TARPs, cornichons, SynDIG1) proteins, which regulate receptor membrane insertion, membrane lateral diffusion and anchoring to the PSD (Shepherd and Huganir 2007). We have previously identified Caspr1, the homologous family member of Caspr2, as an interactor of GluA1-AMPARs that regulates their trafficking and synaptic content (Santos et al. 2012). Although the mechanisms whereby Caspr2 regulates AMPAR trafficking are still unclear, one possibility is that Caspr2 might also be included in the AMPAR macrocomplex. Importantly, the effect of Caspr2 short-term depletion on AMPAR synaptic content that we report is independent of the role of Caspr2 on regulating synapse density/maturation (Anderson et al. 2012; Gdalyahu et al. 2015; Varea et al. 2015), since it does not affect synapse number as determined by the density of PSD95 clusters in cortical neurons (Supplementary Fig. S2A, C), or the frequency of mEPSCs in layer 2/3 V1 neurons (Supplementary Table S1). We detected a decrease in the surface and synaptic content of GluA1 (Fig. 1E, F), but not GluA2 (Supplementary Fig. S2A, B), upon Caspr2 silencing in cultured cortical neurons. Given that we found no changes in mEPSC current kinetics in layer 2/3 V1 neurons depleted of Caspr2 (Supplementary Table S1), it is possible that GluA1/2-AMPAR synaptic expression is impacted by Caspr2, whereas the GluA2/3-AMPAR population is spared. The decrease in GluA1/2-AMPA receptors at the surface and synapses is probably not sufficient to detect a decrease in the global population of cell surface GluA2-containing AMPA receptors.

Rapid changes in AMPAR trafficking and function at the synapse occur in response to different patterns of neuronal activity, which account for the expression of various forms of synaptic plasticity. Interestingly, the molecular players underlying such

cells; Mann–Whitney test, *P < 0.05, ****P < 0.0001 compared with control). **(E)** Cumulative distribution of GluA1 cluster intensities of TTX + APV-treated neurons (blue) plotted against control values (black). The original TTX + APV distribution was transformed by the best-fit equation and plotted (blue dashed line). (Kolmogorov–Smirnov test, ****P < 0.0001; ns—non-significant). **(F)** Immunolabelling of Caspr2, surface GluA1, and PSD95 clusters in cortical neurons treated for 48 h with TTX + APV. *Scale bars* = 5 μm. **(G, H)** Fluorescence intensity of total and synaptic clusters of Caspr2 **(G)**, and the intensity and number of surface GluA1 clusters that co-localise with Caspr2 **(H)** were quantified. ($N \geq 3$, $n \geq 30$ cells; Mann–Whitney test, ***P < 0.001, ****P < 0.0001 compared with control). **(I)** Immunolabelling of surface GluA1 and PSD95 in cortical neurons transfected with a control empty vector or the Caspr2-shRNA, or co-transfected with the Caspr2-shRNA and a shRNA-resistant Caspr2 mutant, and treated with TTX + APV for 48 h. *Scale bars* = 5 μm. (*Representative image panels of transfection genotypes in basal conditions of activity are identical to* Fig. 1E). **(J, K)** Fluorescence intensity of total **(J)** and synaptic **(K)** clusters of surface GluA1 was analysed. ($N \geq 3$, $n \geq 30$ cells; 2-way ANOVA test, Tukey's post hoc test, ****P < 0.0001 compared with control, ns relative to control Caspr2-shRNA, ####P < 0.0001 compared with TTX + APV Caspr2-shRNA, $P < 0.05, $$P < 0.01 relative to control Caspr2-shRNA+rescue). In ***(B)***, ***(D)***, ***(G)***, ***(H)***, ***(J)***, *and* ***(K)***, results are presented as mean ± SEM.

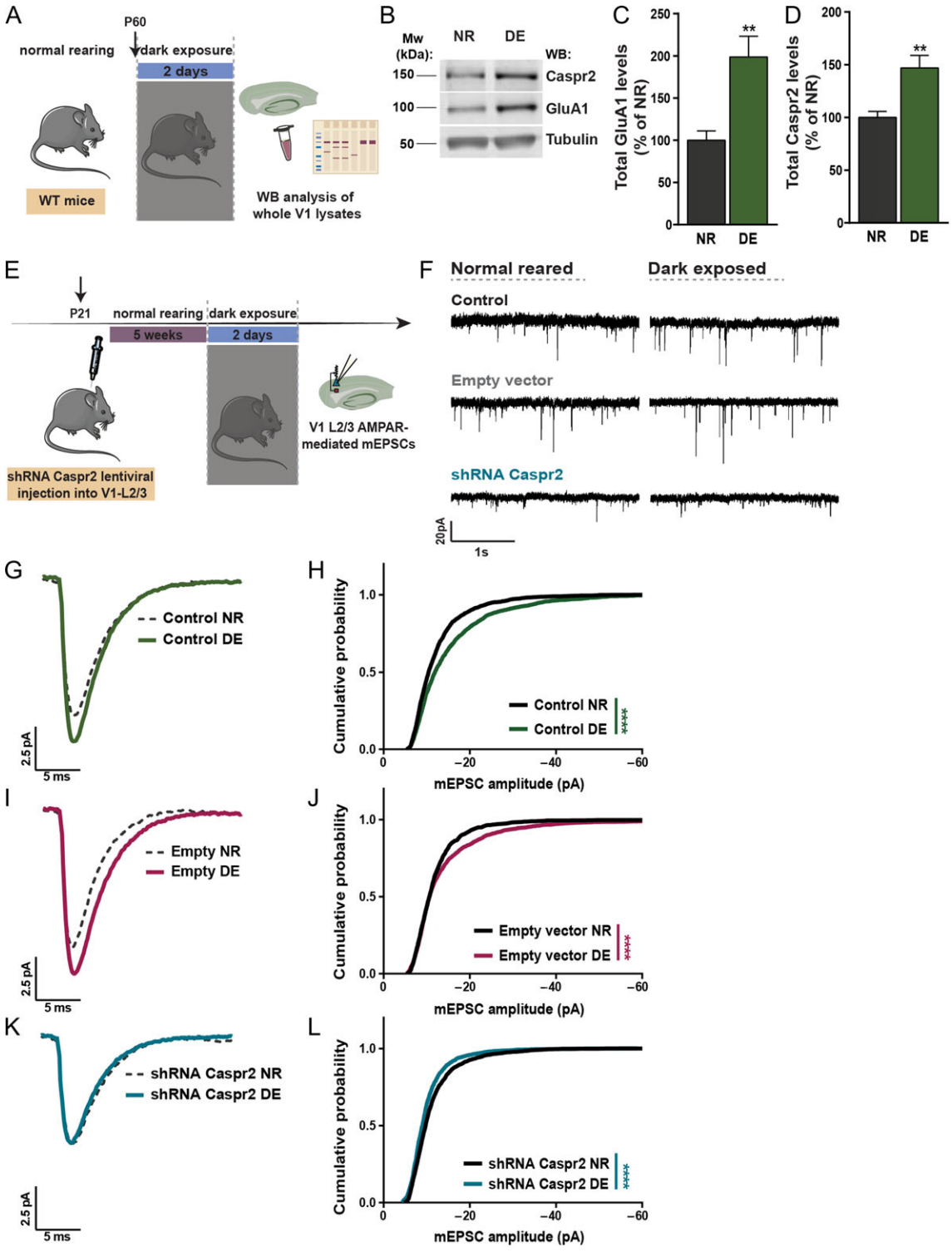

Figure 7. Paradigms of visual deprivation significantly increase Caspr2 levels in the mouse V1, and loss of Caspr2 in V1 prevents visual experience-dependent homeostatic upscaling of AMPAR-mediated mEPSCs. (A) Normal reared P60 mice were subjected to 2 days of visual deprivation. Whole V1 lysates were prepared for WB analysis. (B) Representative blots of GluA1 and Caspr2 levels in V1 lysates of NR or DE mice. (C, D) Total protein levels of GluA1 (C) and Caspr2 (D) were determined. (GluA1: $N \geq 5$; Caspr2: $N \geq 7$; Mann–Whitney test, $^{**}P < 0.01$ relative to NR). (E) shRNA Caspr2 or empty vector-encoding lentivirus were injected into V1-L2/3 of normal reared mice. Mice were then dark exposed for 2 days, and AMPAR-mediated mEPSCs from V1-L2/3 pyramidal neurons recorded. (F) Representative mEPSC traces of non-infected controls, or empty vector- or shRNA Caspr2-infected mice that were either NR (left) or subjected to visual deprivation (DE—right). (*Representative mEPSC traces of the different genotypes in normal reared conditions are identical to* Fig. 2C). (G, H) Average mEPSC traces (G), and cumulative distribution of mEPSC amplitudes (H) recorded from NR (black) or DE (green) control mice (NR, $n = 13$ cells; DE, $n = 8$ cells). (I, J) Average mEPSC traces (I), and cumulative distribution of mEPSC amplitudes (J) recorded from NR (black) or DE (pink) empty vector-infected mice (NR, $n = 9$ cells; DE, $n = 12$ cells). (K, L) Average mEPSC traces (K), and cumulative distribution of mEPSC amplitudes (L) recorded from NR (black) or DE (blue) shRNA Caspr2-infected mice (NR, $n = 12$ cells; DE, $n = 9$ cells). In (H), (J), and (L), Kolmogorov–Smirnov test, $^{****}P < 0.0001$.

trafficking mechanisms are often themselves activity-regulated (Shepherd and Huganir 2007; Fernandes and Carvalho 2016). Herein, we demonstrate that the expression of Caspr2 is regulated by neuronal activity *in vitro* and by visual experience in the mouse visual cortex. Furthermore, our findings reveal that loss of Caspr2 prevents homoeostatic synaptic scaling of AMPARs and hinders experience-dependent plasticity *in vivo* following prolonged visual deprivation. To our knowledge, we establish for the first time a relevant role for Caspr2 in the modulation of synaptic plasticity phenomena, in particular of neuronal synaptic homoeostasis. Homoeostatic plasticity mechanisms are thought to be of particular importance during periods of heightened plasticity, such as during development, sensory processing or adaptation to sensory environment changes, when massive remodelling of synaptic contacts and neuronal circuits occurs (Turrigiano 2008; Fernandes and Carvalho 2016). A relevant role for homoeostatic plasticity was just recently established in the regulation of sleep/wake physiology (Hengen et al. 2016; de Vivo et al. 2017; Diering et al. 2017), considered to be fundamental for memory consolidation and learning (Cirelli 2017). Most important, it has been proposed that a failure in neuronal synaptic homoeostasis may result in symptoms of several psychiatric disorders (Ramocki and Zoghbi 2008; Mullins et al. 2016). Moreover, some of the strongest susceptibility genes implicated in such disorders are important molecular players involved in the regulation of homoeostatic plasticity mechanisms (e.g., stargazin, MeCP2, FMRP) (Fernandes and Carvalho 2016). Indeed, emerging evidence from disease animal models shows alterations in homoeostatic signalling (Wondolowski and Dickman 2013), which is in accord to the findings uncovered in the present study. Herein, we propose that defects in homoeostatic plasticity are an underlying pathology in *CNTNAP2*/CASPR2-related neuropsychiatric disorders.

One phenotype reported in *Cntnap2* KO animal models is the loss of GABAergic interneurons and impaired GABAergic inhibition (Penagarikano et al. 2011; Anderson et al. 2012; Jurgensen and Castillo 2015; Hoffman et al. 2016; Bridi et al. 2017; Vogt et al. 2018). In particular, in layer 2/3 pyramidal neurons of the primary visual cortex, where we have found effects of Caspr2 depletion on excitatory currents (Figs 2, 5), *Cntnap2* KO mice showed reduced phasic inhibition and decreased $GABA_A$-receptor-mediated tonic inhibition (Bridi et al. 2017). In combination with the present study, and other reports on altered excitatory function upon Caspr2 manipulation (Anderson et al. 2012; Gdalyahu et al. 2015; Varea et al. 2015), these studies suggest that Caspr2 regulates the excitatory/inhibitory balance in different brain regions.

CASPR2 is a synaptic target of autoantibody-binding in autoimmune encephalitis, but the mechanisms underlying patients' symptoms remain elusive. A recent study found that autoantibodies against CASPR2 cause pain-related hypersensitivity in the periphery, and enhanced DRG cell excitability through regulation of Kv1 channel expression (Dawes et al. 2018). *In vitro*, it was found that IgG4-enriched CASPR2 antibodies inhibit the interaction of Caspr2 with contactin-2, but do not affect surface expression of Caspr2 in cultured hippocampal neurons (Patterson et al. 2018). We now find that a mixture of CASPR2-Abs of the IgG1 and IgG4 subtypes affects the neuronal distribution of Caspr2 in dendrites, significantly decreasing its synaptic expression (Fig. 3G, H). Additionally, we observed significant intracellular clusters of anti-human antibodies in neurons incubated with the patient CASPR2-Abs (Supplementary Fig. S3), supporting the possibility that CASPR2-Abs and associated Caspr2 are internalised. To our knowledge, this is the first report of a direct effect of CASPR2-Abs on endogenous Caspr2 levels, and, together with previous evidence in the literature, it is suggestive of different pathogenic mechanisms being triggered by CASPR2-Abs of different subclasses. Strikingly, we found a significant loss of surface AMPARs at the synapse in cortical neurons incubated with CASPR2-Abs (Fig. 4A, B), but no effects on synapse number (Fig. 4C), and *in vivo* injection of CASPR2-Abs in the mouse visual cortex significantly decreases AMPAR-mediated currents in layer 2/3 pyramidal neurons (Fig. 5A). These unsuspected data suggest that CASPR2-Abs may exert their pathogenicity by impeding the function of Caspr2 in regulating AMPAR synaptic traffic. Indeed, incubation with CASPR2-Abs increased the ratio of AMPA receptor internalisation in cultured cortical neurons (Fig. 4D, E). It is likely that CASPR2-Abs, by destabilising the synaptic localisation of Caspr2, affect AMPAR synaptic trafficking by increasing the rates of AMPAR endocytosis. One further possibility is that the interaction between Caspr2 and contactin-2 found to be perturbed by CASPR2-Abs (Patterson et al. 2018) is required for the role of Caspr2 in regulating AMPAR traffic. Importantly, our findings implicate dysfunction of glutamatergic transmission in the pathogenesis of anti-CASPR2 encephalitis, which may explain the severity of cognitive and psychiatric symptoms presented by patients, and may enable the development of more specific and efficient therapies for this type of encephalitis.

Altogether, the present study provides compelling evidence for a fundamental role of Caspr2 in the regulation of AMPAR synaptic traffic and function, as well as in the modulation of neuronal synaptic homoeostasis. Importantly, these mechanisms are disrupted in consequence of both *CNTNAP2* sequence variations and CASPR2 autoantibodies and most likely contribute to the pathogenesis of CASPR2-related neuropsychiatric disorders.

Supplementary Material

Supplementary material is available at *Cerebral Cortex* online.

Authors' Contributions

D.F., S.D.S., J.L.W., E.C, N.B., and T.R. performed experiments. E. C., M.I.L., and C.B. provided new reagents. D.F., S.D.S., J.L.W., and E.C. analysed data. D.F., H.-K.L., and A.L.C. designed experiments. D.F. and A.L.C. wrote the paper.

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Notes

Conflict of Interest: The authors declare no conflict of interest.

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