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Neuronal metabotropic glutamate receptor 8 protects against neurodegeneration in CNS inflammation

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Neuronal metabotropic glutamate receptor 8 protects against neurodegeneration in CNS inflammation

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Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system with continuous neuronal loss. Treatment of clinical progression remains challenging due to lack of insights into inflammation-induced neurodegenerative pathways. Here, we show that an imbalance in the neuronal receptor interactome is driving glutamate excitotoxicity in neurons of MS patients and identify the MS risk–associated metabotropic glutamate receptor 8 (*GRM8*) as a decisive modulator. Mechanistically, GRM8 activation counteracted neuronal cAMP accumulation, thereby directly desensitizing the inositol 1,4,5-trisphosphate receptor (IP3R). This profoundly limited glutamate-induced calcium release from the endoplasmic reticulum and subsequent cell death. Notably, we found *Grm8*-deficient neurons to be more prone to glutamate excitotoxicity, whereas pharmacological activation of GRM8 augmented neuroprotection in mouse and human neurons as well as in a preclinical mouse model of MS. Thus, we demonstrate that GRM8 conveys neuronal resilience to CNS inflammation and is a promising neuroprotective target with broad therapeutic implications.

Introduction

Multiple sclerosis (MS) is the predominant nontraumatic cause of neurological disability in young adults and thereby constitutes a substantial healthcare and socioeconomic burden (Reich et al., 2018). Its pathogenesis has been mostly attributed to an infiltration of autoreactive immune cells into the central nervous system (CNS) with concurrent demyelination and neuroaxonal degeneration (Dendrou et al., 2015). Although immunomodulatory treatments effectively suppress inflammatory relapses of the disease, neurodegeneration is not halted. Therefore, increasing neuronal resilience to inflammatory stress in MS constitutes a major unmet clinical need (Friese et al., 2014).

Neuronal loss in MS and its animal model, experimental autoimmune encephalomyelitis (EAE), is initiated by continuous inflammatory insults. Infiltrating immune cells, together with CNS-resident microglia, releases multiple inflammatory mediators that induce synaptic loss (Di Filippo et al., 2018) and disturb neuroaxonal integrity (Nikić et al., 2011). It has been proposed that production of reactive oxygen and nitrogen species, together with iron deposition, damages neuronal mitochondria with subsequent metabolic failure (Campbell et al., 2011; Stephenson et al., 2014). Disruption of neuronal ion homeostasis (Friese et al., 2007) and aggregation of neuronal proteins might further drive neuroaxonal demise (Schattling et al., 2019). However, identifying druggable targets that specifically induce neuronal resilience has been notoriously difficult due to lack of insights into key modulators of injurious neuronal stress responses or severe adverse effects of their modulation. For example, dysregulated neuronal calcium influx has been proposed to drive neuronal loss in primary and secondary neurodegenerative diseases (Hardingham et al., 2001), but broad inhibition of calcium influx results in significant reduction of neuronal functionality (Yasuda et al., 2017; Rowland et al., 2005). Moreover, only few molecular targets have been

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identified with neuroprotective properties that are separable from their impact on inflammatory responses, such as the acid-sensing ion channel 1 (Friese et al., 2007), transient receptor potential melastatin 4 (Schattling et al., 2012), the integrated stress response (Stone et al., 2019), nucleocytoplasmic shuttling (Haines et al., 2015), or the mitochondrial matrix protein cyclophilin D (Forte et al., 2007). Therefore, further dissection of neuron-intrinsic mechanisms that are dysregulated in response to inflammation is critical to identify treatment strategies that counteract neurodegeneration.

A pathological feature shared between primary neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (Dong et al., 2009), and MS is neuroinflammation (Ransohoff, 2016) together with elevated glutamate levels in the brain (Srinivasan et al., 2005) and the cerebrospinal fluid (Sarchielli et al., 2003) that likely contributes to neuronal injury. This excessive amount of extracellular glutamate, the main excitatory amino acid, results from intracellular release of dying cells, active secretion by immune cells (Birkner et al., 2020), and impaired glutamate reuptake (Macrez et al., 2016) that collectively induce cell death in neurons by unregulated calcium accumulation. Thus, tight control of glutamate is critical to preserve homeostasis, ensuring neuronal functionality. Central players in this delicate balance are excitatory ionotropic glutamate receptors (iGluRs) and $G\alpha_{q/11}$-coupled metabotropic glutamate receptors (mGluRs) that are opposed by inhibitory $G\alpha_i$-coupled mGluRs (Reiner and Levitz, 2018). Although blocking iGluRs is protective in EAE (Smith et al., 2000), their clinical use remains challenging due to lack of specificity and severe neuropsychiatric adverse effects (Kalia et al., 2008). Moreover, different approaches to block $G\alpha_{q/11}$-coupled mGluRs, such as metabotropic glutamate receptor 1 (GRM1) or GRM5, failed to show neuroprotective efficacy in EAE (Sulkowski et al., 2013). While genetic variants of iGluR and mGluR have been associated with MS risk and severity (Baranzini et al., 2009; Briggs et al., 2011), which glutamate receptor signaling network modulates inflammation-induced neurodegeneration remains elusive.

In this study, we set out to investigate neuron-specific stress responses in an inflammatory environment and compared transcriptional signatures and receptor interactome networks of neurons that were exposed to defined stressors with transcriptional responses of neurons in the CNS of MS patients and EAE mice. We demonstrate that glutamate stress signature genes have the strongest enrichment across all MS and EAE datasets and identify the regulatory network of the MS risk–associated inhibitory GRM8 to be robustly enriched in neurons of MS patients. Reasoning that increasing GRM8 activity might be limiting neurodegeneration, we found that pharmacological activation of GRM8 was neuroprotective in mouse neurons in vitro and reduced inflammation-induced neurodegeneration in vivo. Accordingly, *Grm8*-deficient mice showed more severe neurodegeneration during CNS inflammation. Mechanistically, we can show that GRM8 negatively regulates cAMP-dependent sensitization of inositol 1,4,5-trisphosphate (IP3) receptors (IP3Rs), thereby limiting glutamate-induced calcium release from the ER. Importantly, we were able to successfully translate these findings to human MS brains and human induced pluripotent stem cell (hiPSC)–derived neurons. These results support the activation of GRM8 as a broad therapeutic strategy to enhance neuronal resilience by counteracting glutamate excitotoxicity in neurodegeneration.

Results

Neuronal glutamate receptor signaling during CNS inflammation

To identify pathways that drive inflammation-induced neurodegeneration, we first compiled transcriptional signatures from primary neurons that were exposed to defined challenges, such as virally triggered inflammation (Daniels et al., 2017), glutamate excitotoxicity (Zhang et al., 2007), proteasomal inhibition (Choy et al., 2011), oxidative stress (Peng et al., 2012), protein aggregation (Kramer et al., 2018), or energy deprivation (Yap et al., 2013; signature genes are provided in Table S1). We then overlapped these signatures with bulk mRNA sequencing of MS gray (Durrenberger et al., 2015) and white matter lesions (Hendrickx et al., 2017), as well as neuronal transcriptomes derived from single-nucleus mRNA sequencing of MS cortices (Schirmer et al., 2019) and white matter (Jäkel et al., 2019) by gene set enrichment analysis (GSEA; Fig. 1 A). Notably, signature genes of glutamate excitotoxicity showed the highest enrichment across all MS datasets (Fig. 1, A and B; and Fig. S1 A), supporting that neuronal glutamate signaling is a major driver in MS neurodegeneration.

Due to the heterogeneity of neuronal populations, we next investigated stress responses in neuronal subtypes (Schirmer et al., 2019). We detected that the transcriptional signature of glutamate excitotoxicity (Fig. 1 C) as well as protein aggregation (Fig. S1 B) were significantly enriched in all subtypes, whereas inflammatory gene signatures were restricted to layer 5/6 excitatory neurons and parvalbumin-positive interneurons (Fig. S1 C). Hence, our results indicate that dysregulated glutamate signaling, together with protein aggregation, displays general pathological features of neurons that are chronically exposed to inflammation in MS, independent of subtype.

Previously, some glutamate receptor genes (*N*-methyl-D-aspartate [NMDA] receptor [NMDAR] subunits *GRIN2A*, *GRIN2B*; the kainate receptor subunits *GRIK1*, *GRIK2*, *GRIK4*, *GRIK5*; the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA] receptor subunit *GRIA1*; and the metabotropic glutamate receptor *GRM8*) have been associated with MS disease severity (Baranzini et al., 2010, 2009; Strijbis et al., 2013; Wang et al., 2011). To investigate whether they contribute to our observed dysregulated glutamate signaling, we first compared their neuron-specific mRNA expression in control and MS patients (Schirmer et al., 2019; Jäkel et al., 2019), but we did not find any differences that could explain the disturbed glutamate signaling (Fig. 1 D and Fig. S1 D). Since the activity of transmembrane receptors heavily depends on mechanisms other than changes in mRNA expression, such as spatial organization, coincidental ligand binding, or desensitization (Strasser et al., 2017; Packiriswamy and Parameswaran, 2015), we next assessed the receptor activity by analyzing their downstream gene regulatory networks. Therefore, we employed the reconstruction of gene regulatory networks (ARACNe) reverse

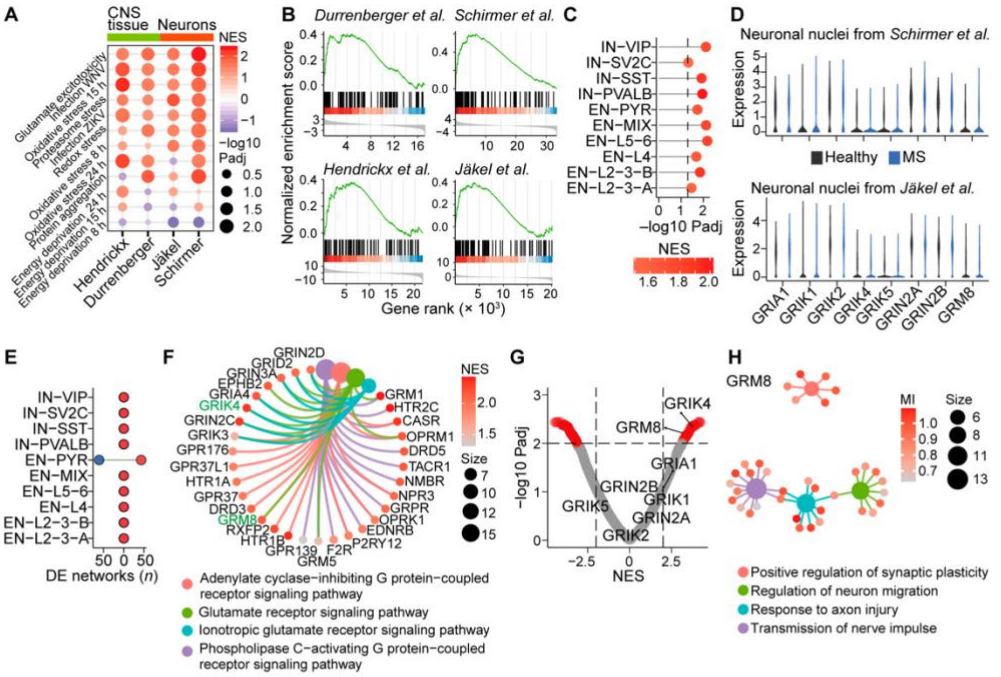

Figure 1. **Neuronal receptor interactomes in MS. (A)** GSEA of transcriptional signatures of defined neuronal stressors in MS CNS tissue or neurons. Rows are arranged in descending fashion by summed NESs across all MS datasets. **(B)** GSEA of glutamate stress signature in the respective MS transcriptomes. Transcriptomes in A and B were taken from Durrenberger et al. (2015), Hendrickx et al. (2017), Jäkel et al. (2019), and Schirmer et al. (2019). **(C)** Enrichment of glutamate stress signature in neuronal subsets from the cortices of MS patients. Dashed line represents significance threshold of FDR-adjusted P < 0.05. We classified subtypes provided by Schirmer et al. (2019) as inhibitory neurons (INs) that were defined by expression of vasoactive intestinal peptide (IN-VIP), synaptic vesicle glycoprotein 2C (IN-SV2C), somatostatin (IN-SST), parvalbumin (IN-PVALB), and excitatory neurons (ENs) from distinct layers (EN-L2-3A, -B, L4, L5-6), EN-PYRs, and a population without known identifier (EN-Mix). **(D)** Relative gene expression of MS-associated glutamate receptors in neuronal nuclei from MS patients. Transcriptomes were taken from Jäkel et al. (2019) and Schirmer et al. (2019). **(E)** The number of differentially regulated receptor interactomes with an FDR-adjusted P < 0.01 in neuronal subtypes from MS patients. **(F)** The top four significantly up-regulated biological themes and defining receptor interactomes in EN-PYRs. MS-associated glutamate receptors are labeled in green. **(G)** Volcano plot of receptor interactomes in EN-PYRs. Significantly enriched networks (FDR-adjusted P < 0.05) are labeled in red. MS-associated glutamate receptors are indicated. **(H)** Significantly enriched biological themes in the GRM8 regulatory network. Color represents mutual inference (MI), and size shows the number of genes in each respective biological theme. WNV, West Nile virus; ZIKV, Zika virus.

engineering algorithm (Margolin et al., 2006) and created neuron-specific receptor interactomes out of 502 available mRNA sequencing datasets (receptors are provided in Table S2; datasets are listed in Table S3) of healthy and stressed in vitro and in vivo mouse neuronal transcriptomes. Subsequently, we compared the obtained receptor networks (receptor interactomes are provided in Table S4) between distinct neuronal subtypes of MS patients and healthy controls. We found that excitatory pyramidal neurons (EN-PYRs) showed robust (P_{adj} < 0.01) down-regulation (n = 61) and up-regulation (n = 43) of transmembrane receptor networks (Fig. 1 E), which is consistent with their severe affliction in MS (Magliozzi et al., 2010). Notably, the regulatory networks of glutamate receptor signaling (Fig. 1 F and Fig. S1 E), and here in particular the MS-associated *GRIK4* and *GRM8* (Fig. 1 G and Fig. S1 F), were strongly enriched in EN-PYRs of MS patients. Intriguingly, the regulatory network of *GRM8* was enriched for genes that modulate neuroaxonal repair (Fig. 1 H). Therefore, we hypothesized that GRM8 activation could contribute to neuronal resilience during CNS inflammation and decided to mechanistically explore GRM8-dependent pathways in inflammation-induced glutamate excitotoxicity.

Pre- and post-synaptic localization of GRM8 in neurons

GRM8 is an inhibitory mGluR that could potentially counteract glutamate excitotoxicity and confer neuroprotection in CNS inflammation. Since the function of GRM8 is poorly understood, we first characterized its CNS distribution and cellular localization in the mouse to get an indication of its contribution to neuronal responses during CNS inflammation. We observed strong *Grm8* mRNA expression in mouse cortex and spinal cord

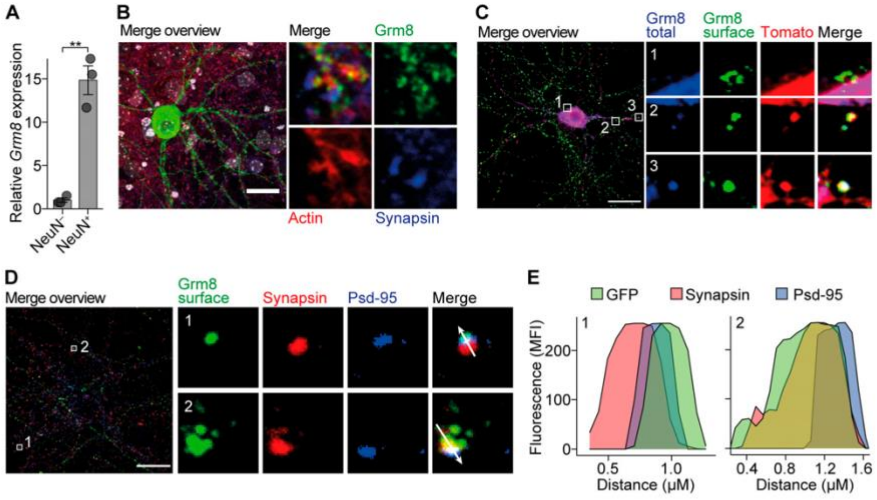

Figure 2. **Grm8 is located pre- and post-synaptically. (A)** *Grm8* expression in sorted NeuN-positive and NeuN-negative nuclei of the spinal cord. All groups, $n = 3$. Data are shown as mean ± SEM. FDR-adjusted unpaired two-tailed *t* test was used with **, $P < 0.01$. **(B–D)** EGFP was inserted at an extracellular domain of *Grm8* adjacent to its signal peptide (EGFP-*Grm8*). Neurons were transfected with EGFP-*Grm8* alone (B and D) or with EGFP-*Grm8* and a tdTomato-containing expression vector to visualize the entire neuronal morphology (C). Subsequently, living neurons were incubated with antibodies against EGFP at 4°C to visualize membrane-bound EGFP-*Grm8* (Grm8 surface), or antibodies against EGFP were applied to fixed and permeabilized neurons to visualize total EGFP-*Grm8* (Grm8 total). This was combined with immunostaining against the indicated markers of neuronal and synaptic morphology. Scale bars, 20 µm. **(E)** Histogram plots showing fluorescence intensity along the arrows of representative synapses from D of surface Grm8 (GFP), synapsin, and Psd-95.

(Fig. S2, A–C). Moreover, we found expression of *Grm8* to be neuron specific, which was reflected by a 15-fold enrichment of *Grm8* in sorted mouse spinal cord NeuN-positive nuclei as compared with NeuN-negative nuclei (Fig. 2 A and Fig. S2 D). As existing antibodies raised against GRM8 showed unspecific staining (data not shown), we transfected primary mouse neuronal cultures with fluorescently tagged *Grm8*—enhanced GFP (EGFP) was inserted at the N-terminal extracellular domain adjacent to the signal peptide—to clarify the subcellular localization of GRM8. By applying antibodies directed against EGFP on living transfected neurons at 4°C to prevent receptor recycling, we were able to visualize surface-bound Grm8. Although previous antibody stainings reported presynaptic localization (Ferraguti and Shigemoto, 2006), in our transfected neurons, we observed a perisynaptic localization at neuronal somata and dendritic spines (Fig. 2, B–E; and Fig. S2, E and F). This close proximity to neighboring excitatory glutamate receptors might allow GRM8 to efficiently modulate glutamate-induced excitotoxicity in neurons.

GRM8 activation is neuroprotective by suppressing ER calcium release

Next, we investigated the potential of GRM8 to modulate glutamate-mediated neuronal loss. We compared glutamate-challenged *Grm8*-deficient (Duvoisin et al., 2005) with WT primary mouse neurons that were pretreated with a positive allosteric modulator of GRM8 AZ12216052 (AZ; Jantas et al., 2014; Rossi et al., 2014) or vehicle control. Following glutamate exposure, there was increased injury of *Grm8*$^{-/-}$ neurons compared with WT neurons (Fig. 3 A and Fig. S2 G) that was accompanied by transcript induction of proapoptotic caspase-8 (*Casp8*) and repression of the prosurvival genes FBJ osteosarcoma oncogene (*Fos*) and brain-derived neurotrophic factor (*Bdnf*; Fig. S2, H and I). Moreover, pharmacological activation of Grm8 by AZ rescued WT but not *Grm8*$^{-/-}$ neurons from glutamate excitotoxicity (Fig. 3 A). We observed no differences in baseline viability, apoptotic potential, and glutamate receptor expression between WT and *Grm8*$^{-/-}$ neurons (Fig. S2, J–L). Since cytosolic and nuclear calcium accumulation has been proposed to drive glutamate excitotoxicity (Lau and Tymianski, 2010), we next analyzed whether GRM8-mediated modulation of neuronal calcium levels could explain its protection against neuronal hyperexcitation. Application of glutamate to spontaneously active neurons or electrically silenced neurons resulted in an NMDAR-dependent nuclear calcium accumulation over time (Fig. 3 B). However, neuronal activation of Grm8 with AZ resulted in reduced nuclear and cytosolic calcium accumulation compared with vehicle treatment (Fig. 3, B and C). Accordingly, *Grm8*$^{-/-}$ neurons showed an exaggerated nuclear and cytosolic calcium accumulation (Fig. 3, D and E). Similarly, blocking synaptic glutamate reuptake (Fig. S3, A and B) or specifically triggering NMDAR and mGluR activity (Fig. S3, C and D) resulted in enhanced calcium accumulations and cell death (Fig. S3, E and F) in *Grm8*-deficient neurons compared with WT

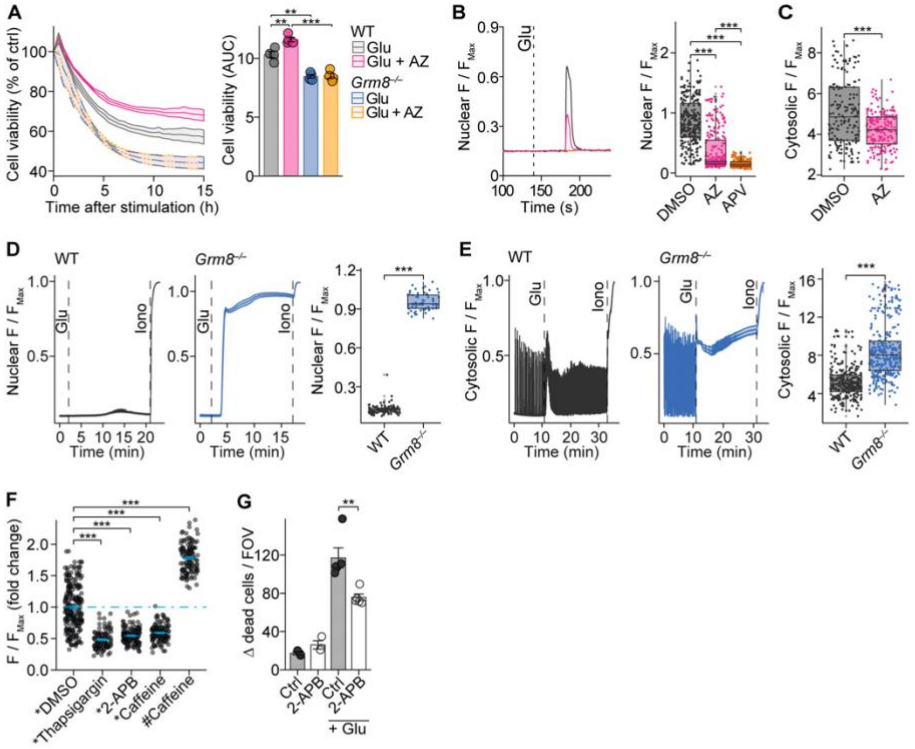

Figure 3. **Grm8 protects against glutamate-induced calcium accumulation. (A)** RealTime-Glo Cell Viability Assay of WT and *Grm8*$^{-/-}$ mouse neurons ± AZ pretreatment that were exposed to glutamate. All groups, *n* = 4. **(B and C)** Nuclear (B; DMSO, *n* = 247; AZ, *n* = 269; APV, *n* = 185) and cytosolic (C; DMSO, *n* = 227; AZ, *n* = 213) calcium recordings in glutamate-exposed mouse neurons that were pretreated with AZ. Data are shown as median ± interquartile range. **(D and E)** Nuclear (D; WT, *n* = 91; *Grm8*$^{-/-}$, *n* = 64) and cytosolic (E; WT, *n* = 298; *Grm8*$^{-/-}$, *n* = 324) calcium recordings in glutamate-exposed WT and *Grm8*$^{-/-}$ mouse neurons. Data are shown as median ± interquartile range. **(F)** Mouse neuronal calcium levels after emptying the ER (pretreatment with thapsigargin or 2-APB or caffeine) or enhancing ER release probability (caffeine) with subsequent (*) or concurrent (#) glutamate exposure. Data are normalized to glutamate-induced calcium increase after DMSO pretreatment. DMSO, *n* = 231; thapsigargin, *n* = 105; 2-APB, *n* = 145; caffeine pretreatment, *n* = 134; caffeine concurrent treatment, *n* = 123. **(G)** Mouse neuronal cultures were exposed to glutamate ± pretreatment with 20 µM 2-APB, and dead cells were counted. Ctrl, *n* = 3; 2-APB, *n* = 3; Glu + Ctrl, *n* = 5; Glu + 2-APB, *n* = 5. If not stated otherwise, data are shown as mean ± SEM. FDR-adjusted unpaired two-tailed *t* test was used with **, P < 0.01; ***, P < 0.001. FOV, field of view.

neurons. *Grm8* deficiency or activation by AZ did not change neuronal baseline calcium level (Fig. S3, G and H). Thus, GRM8 activation is neuroprotective by reducing glutamate-induced calcium accumulation.

The ER constitutes the major intracellular calcium store, which can be released upon stimulation. To explore its contribution to glutamate toxicity, we emptied the ER calcium store by pretreatment with thapsigargin or caffeine, both of which resulted in reduced glutamate-induced calcium accumulation (Fig. 3 F). Similarly, inhibition of ER calcium release by blocking IP3R with 2-APB led to reduced calcium accumulation and ameliorated glutamate-induced neuronal injury (Fig. 3, F and G; and Fig. S3, I and J). Of note, simultaneous application of glutamate and caffeine that increases the ER calcium release probability further increased the glutamate-induced calcium response (Fig. 3 F). Together, this supports the notion that calcium release from the ER and iGluR-mediated external calcium entry synergistically drive glutamate excitotoxicity.

GRM8 inhibits ER-mediated calcium release via cAMP and IP3R signaling

As GRM8 has been reported to reduce excitatory synaptic transmission (Rossi et al., 2014; Gosnell et al., 2011), we hypothesized that its effect to counteract glutamate excitotoxicity is mediated by inhibiting calcium release from the ER (Chen-Engerer et al., 2019). To test this hypothesis, we pharmacologically isolated mGluR-specific calcium responses (Fig. 4 A) that were dependent on sarco/ER calcium-ATPase, IP3R, and

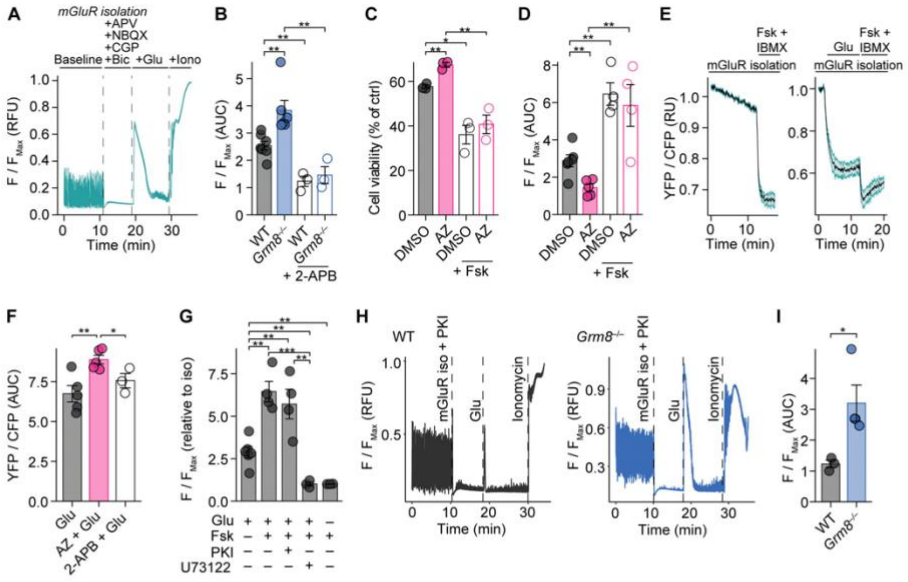

Figure 4. **Grm8 inhibits IP3R-dependent calcium release. (A and B)** Isolated mGluR calcium response (A) in WT and *Grm8*$^{-/-}$ mouse neurons with or without pretreatment with 2-APB (B; WT, $n = 7$; *Grm8*$^{-/-}$, $n = 6$; WT + 2-APB, $n = 3$; *Grm8*$^{-/-}$ + 2-APB, $n = 3$). **(C and D)** Cell viability (C; all groups, $n = 3$) and isolated mGluR calcium response (D; DMSO, $n = 6$; AZ, $n = 5$; DMSO + Fsk, $n = 3$; AZ + Fsk, $n = 3$) after AZ with or without forskolin (Fsk) pretreatment and subsequent glutamate application. **(E and F)** Mouse neuronal cAMP response during isolated mGluR activation (E) and AZ or 2-APB pretreatment (F; Glu, $n = 5$; Glu + AZ, $n = 5$; 2-APB + Glu, $n = 3$). **(G)** Isolated mGluR calcium response in WT neurons that were additionally treated with forskolin, PKI, or PLC inhibitor (U73122) in the indicated combinations (Glu, $n = 6$; Glu + Fsk, Glu + Fsk + PKI, $n = 4$; Glu + Fsk + U73122, Fsk, $n = 3$). **(H and I)** Representative calcium traces (H) and quantification (I) of isolated mGluR calcium response from WT ($n = 3$) and *Grm8*$^{-/-}$ ($n = 4$) primary neurons that were additionally pretreated with PKI. For quantification of calcium and cAMP, AUC was used; if not stated otherwise, data are shown as mean ± SEM. FDR-adjusted unpaired two-tailed *t* test was used with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. RFU, relative fluorescence units.

phospholipase C (PLC) activation (Fig. S3 K). *Grm8*-deficient primary neurons showed enhanced mGluR-mediated calcium release from the ER that was abolished by inhibiting IP3R activity (Fig. 4 B). Of note, *Grm8* deficiency did not affect neuronal IP3R expression (*Itpr1–3*; Fig. S3 L). Congruently, AZ pretreatment reduced the mGluR-dependent cytosolic calcium increase in WT but not *Grm8*$^{-/-}$ primary neurons (Fig. S3, M–P). We concluded that Grm8 activation desensitizes IP3R-mediated calcium release from the ER.

Since GRM8 activation has been shown to increase $G\alpha_i$ activity (Duvoisin et al., 2010), we reasoned that decreasing cytosolic levels of cAMP is responsible for restricting IP3R-evoked calcium release (Taylor, 2017). Therefore, we investigated whether pharmacological increase of cAMP affects glutamate excitotoxicity. We observed that forskolin-mediated acute increase of cAMP synergistically enhanced glutamate-mediated calcium accumulation (Fig. S3 Q) and cell death (Fig. 4 C). More specifically, cytosolic cAMP accumulation enhanced mGluR-dependent calcium release from the ER, overriding the protective effect of AZ (Fig. 4 D). To directly verify that stimulatory mGluR activation increases cAMP that is counteracted by GRM8 activity, we used primary neurons derived from a transgenic fluorescence resonance energy transfer (FRET)–based cAMP biosensor mouse (Börner et al., 2011). Isolated mGluR activation resulted in an increase of intracellular cAMP (Fig. 4 E and Fig. S3 R). Moreover, pretreatment with AZ, but not blocking IP3R, reduced the glutamate-induced increase of cAMP, indicating that GRM8 counteracts glutamate-induced cAMP production upstream of the IP3R (Fig. 4 F). Thus, GRM8 protects from glutamate-induced neurotoxicity by limiting cAMP-mediated IP3R sensitization that reduces calcium release from the ER.

Next, we thought that the cAMP-induced IP3R sensitization could be mediated by activation of protein kinase A (PKA). Notably, we found that pretreatment of primary neurons with the PKA inhibitor (PKI; 5-24) did not limit the enhancing effect of forskolin on the mGluR-specific calcium response. By contrast, isolated mGluR and forskolin-enhanced mGluR calcium response could be completely abolished by treating cells with the PLC inhibitor U73122 (Fig. 4 G). Thus, cAMP accumulation sensitizes IP3Rs and thereby increases calcium release from the ER independent of PKA activity. Accordingly, treatment of *Grm8*$^{-/-}$ primary neurons with PKI did not rebalance the isolated mGluR calcium response (Fig. 4, H and I). Together, glutamate

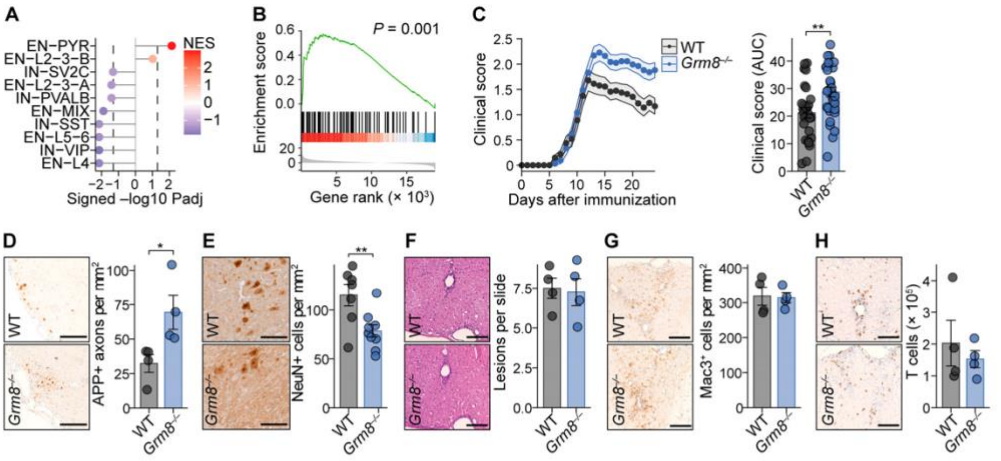

Figure 5. ***Grm8* deficiency aggravates neurodegeneration and clinical disability in EAE. (A)** Transcriptional enrichment of human cortical neuron subtype–defining genes from Schirmer et al. (2019) and mouse spinal cord ChAT-positive motor neurons from Schattling et al. (2019). Dashed lines represent the significance threshold of FDR-adjusted P < 0.01. **(B)** GSEA of transcriptional glutamate stress signature in ranked gene list from Schattling et al. (2019; NES, 0.573). **(C)** WT (n = 27) and *Grm8*$^{-/-}$ (n = 31) mice were subjected to EAE. Pooled data from three independent experiments are shown. AUC was quantified. WT, n = 27; *Grm8*$^{-/-}$, n = 31. **(D and E)** Histopathological quantification of damaged APP-positive axons during acute inflammation 15 d after immunization (D; all groups, n = 4) and neuronal loss in the chronic phase 30 d after immunization (E; WT, n = 9; *Grm8*$^{-/-}$, n = 7) of WT and *Grm8*$^{-/-}$ EAE mice. **(F–H)** Histopathological quantification of inflammatory lesions (F) and Mac3-positive cells (G) and FACS quantification of T cell infiltration (H) during acute phase of EAE 15 d after immunization. All groups, n = 4. Scale bars, 100 µm. Data are shown as mean values ± SEM. FDR-adjusted Mann–Whitney U test was used with *, P < 0.05; **, P < 0.01.

engagement of neuronal activatory mGluRs results in cAMP accumulation that directly sensitizes IP3Rs and hence controls cytosolic calcium levels and cell death, which is limited by GRM8 activity.

GRM8 activation as a neuroprotective strategy in CNS inflammation in vivo

To examine whether our in vitro findings could be translated into in vivo models of CNS inflammation, we investigated the neuroprotective potential of GRM8 activation in the MS mouse model of EAE. Since inflammation in C57BL/6 EAE mice strongly affects motor neurons in the mouse spinal cords, we first probed whether mouse motor neurons show a transcriptional similarity to layer 5 pyramidal neurons from human cortices, which was indeed the case (Fig. 5 A). Moreover, similar to MS pathology, motor neurons from EAE animals (Schattling et al., 2019) showed enrichment of gene transcripts that are indicative of glutamate excitotoxicity (Fig. 5 B). As these overlapping key characteristics support translatability of neuronal responses in mice to humans during CNS inflammation, we compared *Grm8*$^{-/-}$ and WT animals that were subjected to EAE. In accordance with our in vitro findings, *Grm8*$^{-/-}$ mice showed an exacerbated EAE disease course compared with WT animals, especially in the chronic phase of EAE (pooled data from three independent experiments are shown in Fig. 5 C; results from individual EAE experiments are provided in Fig. S4, A–C, and Table S5), while disease onset was unaltered (Fig. S4 D). There was an increased number of amyloid precursor protein (APP)–positive axons, a marker for axonal injury, in the acute phase of EAE (Fig. 5 D) and an extensive loss of neurons in the ventral horn of the spinal cord (Fig. 5 E) and demyelination in the dorsal columns (Fig. S4 E) of the spinal cord in the chronic phase of EAE. Importantly, healthy WT and *Grm8*$^{-/-}$ mice did not differ in axonal and neuronal counts in vivo and synaptic density in vitro (Fig. S4, F–I). As we detected *Grm8* expression in plasmacytoid dendritic cells but not in other immune cell subsets (Fig. S4 J), we examined whether the ameliorated neuronal loss in *Grm8*$^{-/-}$ mice could be partly explained by altered immune cell activation or infiltration. However, genetic deletion of *Grm8* neither affected the proliferation of $MOG_{35–55}$-specific T cells by recall stimulation ex vivo (Fig. S4, K) nor impacted on frequencies, absolute numbers, or activation of dendritic cell subsets or T cells during disease onset (Fig. S4, L and M). Moreover, the numbers of inflammatory lesions and infiltrating immune cells were not altered in *Grm8*$^{-/-}$ mice compared with WT mice during the acute phase of EAE (Fig. 5, F–H; and Fig. S4 N). Together, *Grm8* deficiency results exclusively in higher neuronal vulnerability to inflammation-induced neurodegeneration.

To then test whether specific activation of GRM8 is neuroprotective in the preclinical mouse model of MS, we subjected WT and *Grm8*$^{-/-}$ EAE to daily injections of 1 mg/kg body weight AZ i.p. starting on the day of disease onset. AZ treatment ameliorated the disease course in WT (Fig. S5, A and B) but not in *Grm8*$^{-/-}$ mice (pooled data from two independent experiments are shown in Fig. 6 A; results from individual EAE experiments

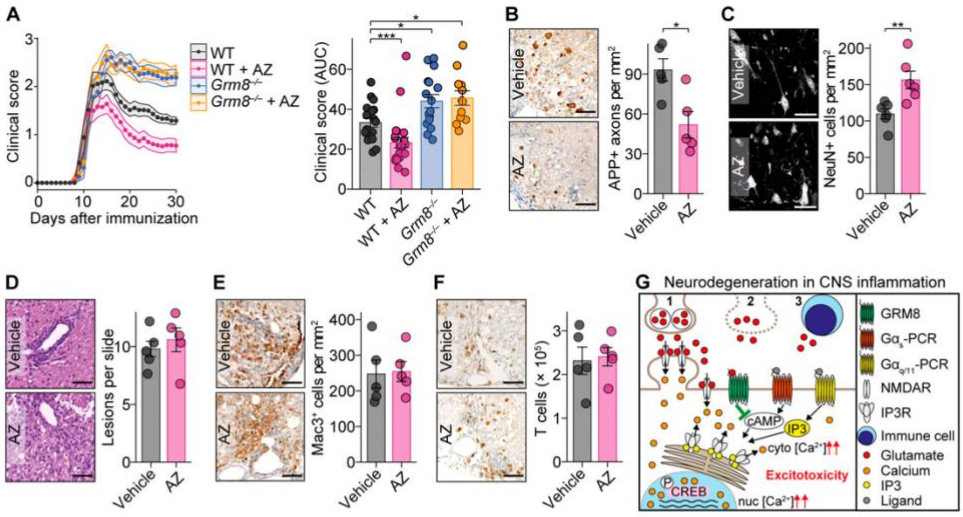

Figure 6. **Activation of Grm8 is neuroprotective in EAE. (A)** Disease course of WT and *Grm8*$^{-/-}$ mice that were subjected to EAE and were treated from disease onset with either vehicle or AZ. Pooled data from two independent experiments are shown. WT, $n = 18$; *Grm8*$^{-/-}$, $n = 16$; WT + AZ, $n = 23$; *Grm8*$^{-/-}$ + AZ, $n = 12$. **(B and C)** Histopathological quantification of damaged APP-positive axons during acute inflammation 15 d after immunization (B; all groups, $n = 5$) and neuronal loss in chronic phase 30 d after immunization (C; all groups, $n = 6$) of EAE mice that were either vehicle or AZ treated. **(D–F)** Histopathological quantification of inflammatory lesions (D) and Mac3-positive cells (E) and FACS quantification of T cell infiltration (F) during acute phase of EAE 15 d after immunization. All groups, $n = 5$. **(G)** Graphical summary showing detrimental effects of glutamate excess derived by spillover (1), necrotic cell death (2), and secretion from immune cells (3) in CNS inflammation and the counteracting neuroprotective signaling by GRM8 activation. Scale bars, 100 µm. Data are shown as mean values ± SEM. FDR-adjusted Mann–Whitney *U* test was used with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

are provided in Fig. S5, C and D, and Table S5), confirming the specificity of the compound in this in vivo model. AZ treatment in WT-EAE was accompanied by fewer APP$^+$ damaged axons (Fig. 6 B) and less neuronal loss (Fig. 6 C). Moreover, day of disease onset (Fig. S5 E), proliferation of MOG_{35-55}-specific T cells (Fig. S5 F), activation and composition of dendritic cell subsets and T cells (Fig. S5, G and H), the number of lesions, and CNS immune cell infiltration during EAE were not affected by treatment with AZ (Fig. 6, D–F; and Fig. S5 I). Taken together, we conclude that GRM8 activity determines neuronal resilience to inflammation-induced glutamate excitation in this mouse model of MS (Fig. 6 G).

Glutamate excitotoxicity in MS

Finally, we investigated whether our mouse findings could be translated back to humans. Therefore, we first assessed *GRM8* expression by RNAscope in situ hybridization and found it similarly expressed in control brain tissue as compared with normal-appearing gray matter (NAGM) and cortical lesions of MS patients (Fig. S5 J). To find molecular evidence of sustained glutamate exposure for neurons in MS, we analyzed the neuronal hallmark of glutamate excitotoxicity: the phosphorylation of serine 133 of cAMP response element-binding protein (pCREB; Hardingham and Bading, 2002). We observed a two-fold increase of pCREB-positive neurons in NAGM and a four-fold increase in cortical MS lesions compared with brain sections of non-neurological disease control individuals (Table S6). The strongest pCREB up-regulation was evident in neurons of epilepsy patients (Fig. 7, A and B), representing a pathology that can be attributed to glutamate hyperexcitation (Park et al., 2003; Zhu et al., 2012; Beaumont et al., 2012). Reassuringly, we found that hiPSC-derived excitatory neurons (Fig. S5, K–M; Harberts et al., 2020) strongly induced pCREB after glutamate challenge that was blocked by inhibiting NMDAR or IP3R-dependent calcium release from the ER (Fig. 7, C and D; and Fig. S5 N). This corroborated the importance of calcium release from internal stores also for human glutamate excitotoxicity. Since we found robust *GRM8* expression in hiPSC neurons (Fig. S5 O), we investigated whether GRM8 activation could counteract the pCREB up-regulation that we observed in neurons of MS brains and under excitotoxic treatment. We found that pretreatment with AZ significantly reduced pCREB up-regulation after glutamate application (Fig. 7 E), while AZ alone did not change pCREB baseline levels (Fig. S5 P). Notably, AZ did not affect inward currents of iGluRs (Fig. 7 F), thereby supporting our notion that GRM8 activity induces neuronal resilience by decreasing IP3R sensitivity independently of ion flux through the cell membrane. Last, to more closely mimic MS pathophysiology, we challenged hiPSC neurons with IFN-γ and TNF-α, two abundant cytokines in neuroinflammation (Becher et al., 2017), in combination with glutamate. Also, in response to this challenge, AZ-treated hiPSC neurons showed

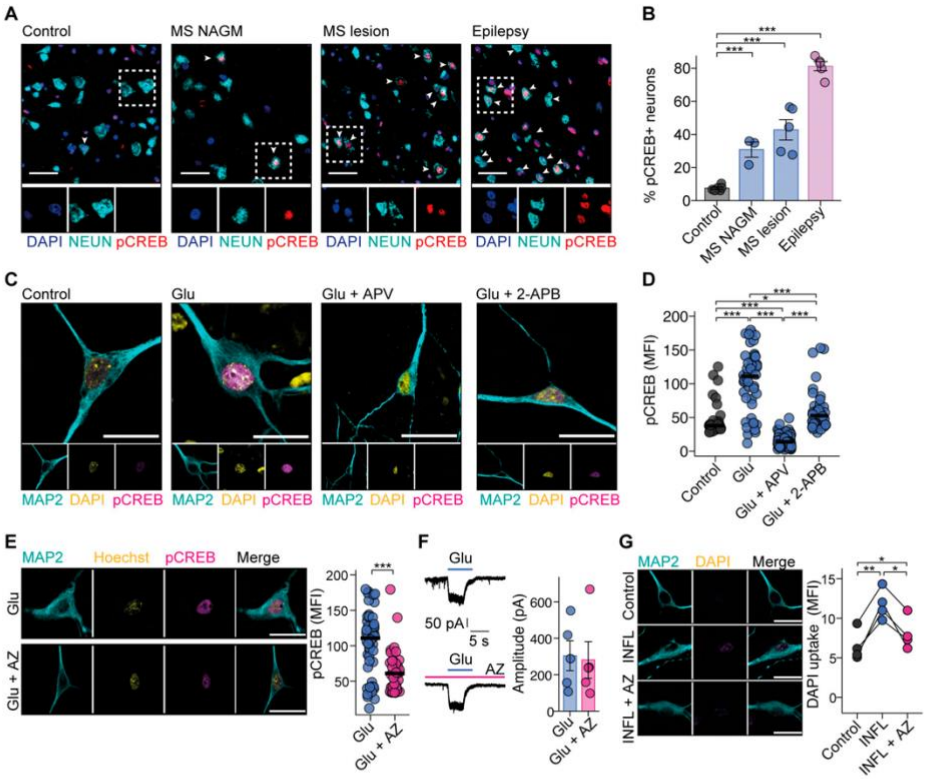

Figure 7. **Activation of GRM8 protects human neurons from glutamate excitotoxicity. (A and B)** Representative images (A) and quantification (B) of percentage of pCREB-positive neurons in brain sections of MS NAGM or cortical MS lesions and epilepsy patients compared with control individuals without neurological diseases. Controls, $n = 6$; MS NAGM, $n = 3$; MS lesions, $n = 5$; epilepsy, $n = 5$. Scale bars, 25 µm. Data are shown as mean ± SEM. FDR-adjusted unpaired two-tailed *t* test was used. **(C and D)** Representative images (C) and quantification (D) of pCREB immunofluorescence in hiPSC neurons that were untreated (control) or that were treated with sham solution, 50 µM APV, or 50 µM 2-APB and subsequently stimulated with 20 µM glutamate (Glu) for 20 min. Control, $n = 38$; glutamate, $n = 50$; Glu + APV, $n = 78$; Glu + 2-APB, $n = 49$. Scale bars, 20 µm. **(E)** pCREB immunofluorescence of hiPSC neurons that were treated with AZ and subsequently exposed to 20 µM glutamate for 20 min. Glu, $n = 50$; Glu + AZ, $n = 37$. Data are shown as median values. Scale bars, 20 µm. **(F)** Patch-clamp recording of inward currents in hiPSC neurons that were incubated in ACSF containing 0.5 µM TTX and 20 µM bicuculline and sham or 1 µM AZ and subsequently were exposed to 10 µM glutamate (Glu) for 4 s. Peak amplitude was used for quantification. Glu, $n = 5$; Glu + AZ, $n = 5$. **(G)** DAPI uptake by hiPSC neurons that were treated with AZ or vehicle and subsequently exposed to IFN-γ, TNF-α, and glutamate. INFL, inflammation. All groups, $n = 4$. Scale bars, 20 µm. FDR-adjusted paired two-tailed *t* test was used. If not stated otherwise, data are shown as mean ± SEM. FDR-adjusted unpaired two-tailed *t* test was used with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

reduced cell death compared with vehicle-treated hiPSC neurons (Fig. 7 G and Fig. S5 Q). This supports that human GRM8 activation exhibits a neuroprotective effect in an excitatory and inflammatory environment.

Discussion

Here, we investigated the pathophysiology of neurodegeneration in CNS inflammation and identified glutamate excitotoxicity as a critical component. Excessive activation of NMDAR by elevated glutamate has been proposed to drive several primary neurodegenerative diseases, such as Alzheimer's disease (Zott et al., 2019) and Parkinson's disease (Kalia and Lang, 2015), but also MS (Baranzini et al., 2010). In CNS inflammation, glutamate can derive from multiple sources, such as activated Th17 cells that secrete higher levels of glutamate in MS patients' CSF compared with healthy individuals (Birkner et al., 2020). Furthermore, glutamate is actively released from presynaptic vesicles by neurons in a hypoxic environment and passively set free from dying neurons (Wroge et al., 2012) that can further promote neuronal loss. Increased levels of glutamate in the CNS result in excessive activation of NMDAR and subsequent

sustained calcium influx from the extracellular space (Hardingham and Bading, 2010). Thus, ionic disbalance in neurons could drive mitochondrial injury, accumulation of oxidized free radicals, and activation of neuronal regulated cell death (Friese et al., 2014). Although elevated glutamate levels have been described in brains of MS patients by magnetic resonance spectroscopy (Baranzini et al., 2010), it has been unclear whether the sustained increase of glutamate contributes to inflammation-induced neurodegeneration or is a by-product of inflammatory activity (Macrez et al., 2016). Here we used pCREB as a durable molecular marker for continuous glutamate exposure in human neurons and found it strongly up-regulated in glutamate-exposed hiPSC neurons and in MS lesions. This indicates that glutamate excitotoxicity directly contributes to neuronal loss in CNS inflammation. Notably, pCREB levels were also elevated in NAGM, suggesting glutamate-driven neurodegeneration independently of inflammatory lesions in MS. This might contribute to cognitive deficits and cerebral atrophy in MS patients that cannot solely be explained by the spatial distribution of lesions (Kaufmann et al., 2019).

To identify receptors that modulate neuronal resilience during glutamate excitotoxicity in CNS inflammation, we analyzed neuronal receptor networks in single-cell RNA-sequencing data of MS patients. Network construction and subsequent transcriptional network deconvolution (Lachmann et al., 2016) have been successfully used to identify master regulators of numerous cancer types (Alvarez et al., 2016) and neuronal loss in a mouse model of Parkinson's disease (Brichta et al., 2015). In contrast to available neuronal regulatory networks that used expression of different brain regions from healthy mice (Brichta et al., 2015), we used expression data of healthy and challenged neurons from in vitro primary cultures and in vivo mouse models of neurodegenerative and neuroinflammatory diseases. Our receptor interactome includes stress and steady-state responses as recent advances in transcriptomic analyses revealed an induction of pathways that were traditionally assigned to immune cells, such as IFN signaling, also to be active in neurons during inflammation (Di Liberto et al., 2018; Schattling et al., 2019) and aging (Dulken et al., 2019). We found that, in MS patients, mainly EN-PYRs were affected by a dysregulated receptor interactome that was driven by glutamate activity. This may indicate a neuronal subtype–specific vulnerability to glutamate exposure, as suggested by previous neuropathological studies (Magliozzi et al., 2010; Jürgens et al., 2016).

To counteract the disbalanced receptor interactome, we focused on GRM8 as one of the inhibitory mGluRs. We chose GRM8 as it was associated with MS disease severity (Baranzini et al., 2009; Briggs et al., 2011), and we detected its regulatory network that was associated with neuroaxonal repair to be significantly elevated in pyramidal neurons of MS patients. Thus far, pharmacological inhibition of stimulatory GRM1 and GRM5 signaling did not affect the disease course in EAE (Sulkowski et al., 2013) or the group 2 mGluRs GRM2 and GRM3 (Sun et al., 2013). Moreover, group 3 mGluRs GRM4 and GRM7 are highly expressed in nonneuronal cells, while GRM6 is only expressed in retinal ON-bipolar cells (Peachey et al., 2017), therefore constituting them as unsuitable neuroprotective targets. Similarly, *Grm4* deficiency primarily affects dendritic cells that skew T cell differentiation toward Th17 cells and increases inflammatory activity in EAE (Fallarino et al., 2010). By contrast, GRM8 is an appealing drug target, as it is predominantly expressed in neurons and its activation has been reported to protect undifferentiated neuroblastoma cells against doxorubicin (Jantas et al., 2016) and the mitochondrial toxin $MPP4^+$ (Jantas et al., 2014). This suggests that GRM8 activation might exert neuroprotective properties. Other than MS, gene variants of GRM8 have been mostly associated with psychiatric disorders, such as major depressive disorder (Howard et al., 2019) and schizophrenia (Bolonna et al., 2001). In accordance, behavioral studies of mice that are deficient in *Grm8* showed higher levels of anxiety (Duvoisin et al., 2005).

Our data demonstrate that CNS inflammation continuously activates GRM8, as its dependent regulatory network is particularly active in pyramidal neurons of MS patients. This could be interpreted as a neuroprotective countermeasure during chronic glutamate exposure. Accordingly, *Grm8*-deficient neurons were more prone to glutamate excitotoxicity, while pharmacological activation of GRM8 by using AZ was able to further augment neuroprotection in mouse and human neurons. Moreover, daily AZ treatment of mice undergoing EAE profoundly counteracted neurodegeneration. We chose allosteric modulation of GRM8 by AZ, as it provides mechanistic advantages compared with orthosteric agonists. Instead of directly activating GRM8, it increases the physiological signaling initiated from binding of glutamate, with potentially minimized unphysiological receptor activity and the risk for adverse effects (Wootten et al., 2013). Moreover, its structural similarity to other allosteric modulators against Grm1 (Yohn et al., 2020), Grm5 (Haas et al., 2017), and Grm7 (Klar et al., 2015) supports its direct action on the CNS after i.p. treatment. Nevertheless, when considering GRM8 as a therapeutic target, it is important to determine potential unwanted adverse effects. *Grm8* deficiency in mice resulted in mild insulin resistance and weight gain (Duvoisin et al., 2005). Moreover, GRM8 is expressed in glutamatergic neurons of the enteric nervous system and enhances intestinal motility (Tong and Kirchgessner, 2003). Additionally, in the immune system, we found *Grm8* expression exclusively in plasmacytoid dendritic cells in mice. However, we did not observe any differences in immune cell infiltration and the extent of inflammatory lesions during EAE. Further, daily AZ treatment did not affect $Grm8^{-/-}$ mice, indicating that AZ treatment counteracted inflammation-induced neurodegeneration by specifically promoting *Grm8* activity in neurons.

Mechanistically, GRM8 has been associated with supporting a negative feedback of presynaptic neurotransmitter release. Electrophysiologic recordings of prepulse inhibition (Gosnell et al., 2011) and immunolabeling in the olfactory bulb (Kinoshita et al., 1996) and lateral perforant pathway (Shigemoto et al., 1997) supported this notion of a presynaptic localization. However, its precise subcellular localization was unknown. By expressing fluorescently tagged *Grm8* in cortical neurons, we could now observe pre- and post-synaptic as well as surface localization at neuronal somata. Although overexpression experiments have to be interpreted with caution, the close proximity of GRM8 to excitatory

synapses might allow it to monitor and counteract glutamate spillover (Arnth-Jensen et al., 2002) and subsequent hyperexcitation. Despite electrophysiological recordings showing that activation of GRM8 reduced synaptic transmission in the stria terminalis (Gosnell et al., 2011), its mode of action and potential neuroprotective properties have not been investigated.

While glutamate toxicity has been mostly attributed to the influx of neuronal calcium from external sources (Hardingham and Bading, 2002), the contribution of internal calcium stores to excitotoxic cytosolic and nuclear calcium accumulation remains unclear. The ER is the main internal calcium source, and it extends throughout the entire neuron and releases calcium into the cytosol or quenches it to buffer high cytosolic levels (Wu et al., 2017). Calcium release from the ER is mediated by activation of IP3R and ryanodine receptors. Missense mutations of *Itpr1* have been found in patients suffering from spinocerebellar ataxia (Barresi et al., 2017; Hara et al., 2008), and cerebellum-specific deletion of *Itpr1* in mice induces severe ataxia and synaptic loss (Egorova et al., 2016; Kasumu et al., 2012), indicating the importance of ER calcium release for neuronal health. Moreover, β-amyloid aggregate-induced neurotoxicity could also be rescued by blocking IP3R activity (Demuro and Parker, 2013), indicating that unregulated ER calcium release plays an important role in neurodegeneration. Here, we show that IP3R-mediated calcium release from the ER heavily contributes to glutamate-induced excitotoxic calcium accumulation, endorsing its inhibition as an attractive neuroprotective strategy.

We found that activation of GRM8 counteracted this glutamate-induced excitotoxic calcium accumulation by limiting IP3R-dependent calcium release from the ER. Intriguingly, we observed that acute cAMP increase by forskolin strongly enhanced IP3R sensitivity, reinforcing glutamate excitotoxicity. Activation of GRM8 limited cAMP production and thereby decreased IP3R-dependent calcium release from the ER. There are at least two ways that cAMP can regulate IP3R sensitivity: (1) cAMP binding enables PKA to sensitize IP3R1 and IP3R2 or to desensitize IP3R3 by phosphorylation (Vanderheyden et al., 2009) or (2) phosphorylation-independent modulation by direct binding to low-affinity cAMP binding sites of IP3R (Tovey et al., 2008). As we observed an immediate calcium release from the ER by simultaneously applying forskolin and glutamate, and as inhibition of PKA did not reduce cAMP-enhanced calcium release, we assume that this supports a direct effect that is independent of phosphorylation (Gelens and Saurin, 2018). Thus, pathological cAMP accumulation by dysregulated metabotropic signaling likely contributes to neurodegeneration by promoting excessive calcium release from the ER through direct sensitization of IP3R. Although HEK cells (Konieczny et al., 2017) and osteoblasts (Buckley et al., 2001) react differently to IP3 than to IP3 together with cAMP, the direct interaction site of cAMP with different IP3R isotypes is currently unknown. However, this suggests that cAMP-mediated IP3R sensitization may be a widespread mechanism in different cell types that could be modulated by G protein–coupled receptor–targeted drugs (Hauser et al., 2017). Our data infer that IP3R sensitivity is a crucial determinant of neuronal calcium homeostasis and integrity, which are directly modulated by the druggable GRM8. Thus, the interplay between metabotropic signaling and internal calcium stores emerges as a central pathophysiological mechanism warranting further characterization in other neurodegenerative processes.

In summary, we demonstrate that GRM8 is a decisive player in an endogenous feedback mechanism to limit glutamate-induced excitotoxic calcium accumulation in neurons. Our findings are a rare example of a neuroprotective pathway sensu stricto that increases neuronal resilience without impacting the immune response during CNS inflammation (Friese et al., 2014). This commends GRM8 activation as a valuable therapeutic approach to counteract inflammation-driven neurodegeneration in MS and other neurological diseases that involve glutamate excitotoxicity.

Materials and methods

Mice

All mice (C57BL/6J WT [The Jackson Laboratory]; C57BL/6J *mGluR8*$^{-/-}$ [Duvoisin et al., 2005]; and FVB/NRJ Epac1-PLN [Sprenger et al., 2015]) were kept under specific pathogen-free conditions in the central animal facility of the University Medical Center Hamburg-Eppendorf (UKE). We used adult mice (6–20 wk old) of both sexes; mice were sex and age matched in all experiments. We did not observe sex-specific differences in any of the experiments; therefore, the sexes were reported together.

EAE

We immunized mice subcutaneously with 200 µg MOG_{35-55} peptide (Schafer-N) in CFA (Difco; catalog no. DF0639-60-6) containing 4 mg ml^{-1} *Mycobacterium tuberculosis* (Difco; catalog no. DF3114-33-8). In addition, we injected 200 ng pertussis toxin (Calbiochem; catalog no. CAS70323-44-3) i.p. on the day of immunization and 48 h later. We scored animals daily for clinical signs by the following system: 0, no clinical deficits; 1, tail weakness; 2, hindlimb paresis; 3, partial hindlimb paralysis; 3.5, full hindlimb paralysis; 4, full hindlimb paralysis and forelimb paresis; 5, premorbid or dead. Animals reaching a clinical score ≥4 were euthanized according to the regulations of the local Animal Welfare Act. Where indicated, animals were injected i.p. with 1 mg kg^{-1} body weight AZ 12216052 (Tocris; catalog no. 4832) starting from the day of disease onset. We used littermate controls in all EAE experiments. AZ was prediluted in DMSO, and the final injection consisted of 10% DMSO ± AZ, 40% polyethylene glycol (Thermo Fisher Scientific; catalog no. P/3676/08), and 50% Dulbecco's PBS (Pan Biotech). The results and number of animals from independent EAE experiments are provided in Table S5. For recall assays (described below), mice were treated with DMSO vehicle control or AZ from day 3 after immunization for 6 d and were used for experiments 9 d after immunization. The investigators were blind to the genotype and treatment in the EAE experiments.

hiPSC-derived neurons

We maintained hiPSCs (ZIPi013-B; Tandon et al., 2018) under feeder-free conditions on Matrigel (Corning)-coated plates in

mTeSR1 medium (STEMCELL Technologies; catalog no. 85850). For neuronal induction, we dissociated hiPSCs with Accutase and seeded them at a density of 3 × 10^6 cells per well on AggreWell800 plates (10,000 cells per embryoid body; STEMCELL Technologies) in SMADi neural induction medium (STEMCELL Technologies; catalog no. 08582) supplemented with 10 µM Y-27632 (STEMCELL Technologies; catalog no. 72302). On day 6, embryoid bodies were harvested and cultivated on Matrigel-coated plates in SMADi neural induction medium for 12 d. Newly formed neural rosettes were manually picked and cultured for another 4 d. To release neural precursor cells (NPCs), neural rosettes were dissociated with Accutase and were maintained for several passages at high density in Neural Progenitor Medium (STEMCELL Technologies; catalog no. 05833) on Matrigel-coated plates. We differentiated hiPSC-derived NPCs into neurons as previously described (Brennand et al., 2011; Djuric et al., 2015; Zhang et al., 2016) with some modifications. Briefly, NPCs were seeded at a density of 5 × 10^4 cells/cm^2 in Neural Progenitor Medium onto poly-L-ornithine/laminin–coated plates. After 24 h, we replaced the medium by neural differentiation medium (day 0 of differentiation) composed of Neurobasal Plus Medium (Gibco BRL; catalog no. A3582901) containing 1× B27 Plus Supplement (Gibco BRL; catalog no. A3582801), 1× N2 Supplement-A (STEMCELL Technologies; catalog no. 07152), 1× MEM nonessential amino acids (Gibco BRL; catalog no. 11140050), 1 µg ml^{-1} laminin (Sigma-Aldrich; catalog no. 11243217001), 1 µM dibutyryl-cAMP (STEMCELL Technologies; catalog no. 73882), 10 ng ml^{-1} L-ascorbic acid (STEMCELL Technologies; catalog no. 72132), 10 ng ml^{-1} brain-derived neurotrophic factor (STEMCELL Technologies; catalog no. 78005), and 10 ng ml^{-1} glia-derived neurotrophic factor (STEMCELL Technologies; catalog no. 78058). To promote a glutamatergic neuronal cell type, 5 µM cyclopamine (STEMCELL Technologies; catalog no. 72072) was additionally added to the medium during the first week of differentiation. In the second week, we supplemented neuronal differentiation medium with 2 µM cytarabine (Sigma-Aldrich; catalog no. BP383) in order to reduce proliferation of non-neuronal cells. On day 14, the cells were detached using Accutase and reseeded onto 12-mm-diameter coverslips. Thereafter, cells were maintained for up to 18–20 wk to increase maturity.

Primary mouse neuronal cultures

For primary cortical cultures, we euthanized pregnant C57BL/6J, FVB/NRJ Epac1-PLN, or *mGluR8*$^{+/-}$mice. To ensure comparability between genotypes, we used only embryos from heterozygous breeding. We reserved tissue of each embryo for genotyping and isolated the cortex, dissociated, and plated cells at a density of 10^5 cells per 1 cm^2 on poly-D-lysine–coated wells (5 µM; catalog no. A-003-M; Sigma-Aldrich). If not stated otherwise, cells were maintained in Neurobasal Plus Medium (supplemented with B27 Plus, penicillin, streptomycin, and L-glutamine; Gibco BRL; catalog no. A3582901) at 37°C, 5% CO_2, and a relative humidity of 98% and treated with 1 µM cytarabine (Sigma-Aldrich; catalog no. BP383) at 1 d in vitro (1 div) to inhibit glial cell proliferation. If no cytarabine was applied, cells were maintained in neurobasal medium (supplemented with B27, penicillin, streptomycin, and L-glutamine; Gibco BRL). Throughout, we used cultures after 14–23 div for experiments.

GSEA

We downloaded published expression data from the Gene Expression Omnibus (GEO) and derived murine neuronal stress signatures from GSE10470, GSE22087, GSE22465, GSE22997, GSE109177, and GSE122121. We selected glutamate-regulated genes from Zhang et al. (2007); a murine dataset of non-inflamed and inflamed neurons from EAE derived from GSE104897; and human datasets of healthy individuals and MS patients from GSE10800, GSE26927, GSE118257, and PRJNA544731. We analyzed microarray datasets by the standard *limma* pipeline (Ritchie et al., 2015). We contrasted stressed neurons against control neurons or MS brain tissue against nondiseased control brain tissue, respectively. We analyzed RNA-sequencing datasets by a standard *DESeq2* pipeline (Love et al., 2014). We identified neuronal transcript counts from single-nucleus sequencing datasets by the annotation provided by GEO and summed up counts for each gene and for each individual. The resulting expression matrix consisted of the neuronal transcription profile of every individual. We analyzed differential gene expression (DE) between MS patients and nondiseased individuals by *DESeq2*. To create ranked gene lists, we arranged DE results from healthy MS comparisons by *limma*-derived moderated *t*-statistics for GSE10800 and GSE26927 or by *DESeq2*-derived Wald statistics for GSE104897, GSE118257, and PRJNA544731. For neuronal stress signatures, we only considered genes with a positive fold change and false discovery rate (FDR)–adjusted $P < 0.05$. When >100 genes fulfilled the criteria, only the top 100 most significant genes were used to get comparable gene set sizes. When genes were represented by multiple probes, the one with the highest absolute deviation around the median was considered. To avoid batch effects and interspecies differences of gene expression, we did not directly compare differentially regulated genes but rather assessed differentially regulated biological themes that consist of gene groups that represent biological functions across species. Therefore, we performed GSEA using clusterProfiler (Yu et al., 2012).

Regulatory network analysis

Raw read counts of 502 neuron-specific mRNA sequencing datasets of in vitro healthy and challenged neuronal cultures and in vivo mouse models of psychiatric, neurodegenerative, neuroinflammatory, and metabolic diseases were retrieved from the Sequence Read Archive and were aligned to the mouse reference genome (mm10) using STAR version 2.4 (Dobin et al., 2013) with default parameters; overlap with annotated gene loci was counted with featureCounts version 1.5.1 (Liao et al., 2014). The regulatory network was reverse engineered using ARACNe (Lachmann et al., 2016). ARACNe was run with 100 bootstrap iterations using all probes that mapped to a set of 1,101 mouse transmembrane receptors, which were defined as genes as members of Gene Ontology identifier GO:0003700, "transmembrane signaling receptor activity," and its respective offspring. Olfactory receptor genes were excluded from the

analysis. As recommended for bootstrap ARACNe analysis, we used 0 data processing inequality tolerance and a threshold for mutual inference $P < 10^{-7}$. Genome-wide expression signatures of neuron subtype–specific changes in MS patients compared with nondiseased controls were computed as described above from Schirmer et al. (2019). We used the annotation provided by GEO for filtering different neuron subtypes. DE between MS patients and nondiseased controls was tested by *DESeq2* (Love et al., 2014). The receptor interactomes of different neuron subtypes from MS patients compared with nondiseased controls were computed by Virtual Inference of Protein-activity by Enriched Regulon (Viper; Alvarez et al., 2016) using ranked gene lists of each neuronal subtype from MS patients compared with nondiseased controls and the regulatory transmembrane receptor network we created as input. The FDR-adjusted P value and normalized enrichment score (NES) were computed by comparison with a null model that was generated by permuting the samples uniformly at random 1,000 times. Subsequent enrichment analysis was performed using clusterProfiler (Yu et al., 2012).

Chemicals

The used chemicals and the respective function, supplier, catalog number, and concentration that we used in vitro are depicted in Fig. S3 I and Table S7.

RNAscope in situ hybridization

We performed RNAscope fluorescent in situ hybridization using the RNAscope Fluorescent Multiplex Kit V2 (Advanced Cell Diagnostics; catalog no. 323100) according to the manufacturer's protocol. Probes against human Hs-*Snap25*-C3 (catalog no. 518851-C3) and Hs-*Grm8* (catalog no. 563351) were commercially available from Advanced Cell Diagnostics, Inc. RNAscope human samples were scanned using the Pannoramic 250 FLASH II (3DHISTECH) Digital Slide Scanner at 20× magnification. *GRM8*$^+$*SNAP25*$^+$ neurons were quantified by a blinded experimenter using Pannoramic Viewer software (3DHISTECH) and Fiji (National Institutes of Health [NIH] image analysis software) or with a custom-made script, which was based on Cognition Network Language (Definiens Cognition Network Technology; Definiens Developer XD software).

Immunohistochemistry, immunohistopathology, and immunocytochemistry

The used primary and secondary antibodies and the respective antigen, host species, supplier, catalog number, and dilution are listed in Table S8. Human CNS tissue was fixed with 4% paraformaldehyde and embedded in paraffin as described previously (Kreutzfeldt et al., 2013). To prevent unspecific binding, we performed antigen retrieval. Human sections were scanned using the Pannoramic 250 FLASH II (3DHISTECH) Digital Slide Scanner at 20× magnification. Positive signals in a field of view of 1.2 mm^2 were quantified using CaseViewer software (3DHISTECH). Mouse spinal cord tissue was obtained and processed as described previously (Schattling et al., 2012). Mouse sections were analyzed with a Zeiss LSM 700 confocal microscope. For histopathology, we used hematoxylin (blue color) and immunolabeling that we visualized using the avidin–biotin complex technique with 3,3′-diaminobenzidine (brown stain). We analyzed slides with a NanoZoomer 2.0-RS digital slide scanner and NDP.view2 software (Hamamatsu). We quantified CD3- and Mac3-positive cells as well as APP deposits in the white matter tract of the spinal cord using a customized counting mask with Fiji (ImageJ). NeuN-positive cells (neurons) were manually counted in the ventral horn outflow tract of the spinal cord. For Luxol fast blue staining, we quantified the positive area in the white matter of the spinal cord using a customized counting mask with Fiji (ImageJ). Analysis conditions were standardized across all conditions. At least three images were analyzed per animal, and the mean per animal was used for subsequent statistical comparisons. For immunocytochemistry of hiPSC neurons and mouse neurons, we cultivated cultures on 12-mm-diameter coverslips, fixed them with 4% paraformaldehyde, incubated them in 10% normal donkey serum containing 0.1% Triton X-100, and subsequently performed immunolabeling. For surface staining, we incubated transfected cultures in ice-cold medium for 30 min with the primary anti-GFP antibody (1:200), subsequently fixed them, and applied the secondary antibody before permeabilization (1:500) in 10% NDS. Afterward, the staining protocol was continued as described above. To visualize neuronal morphology, we used actin-stain 555 phalloidin (1:100; Cytoskeleton; catalog no. PHDH1-A) and actin-stain 670 phalloidin (1:100; Cytoskeleton; catalog no. PHDN1-A). To measure the influence of glutamate on human neuronal pCREB regulation, we pretreated hiPSC neuron cultures with 1 µM AZ, 50 µM 2-amino-5-phosphonovaleric acid (APV), 50 µM 2-APB, or 0.1% DMSO (vehicle) and stimulated them for 20 min with 20 µM glutamate or 0.1% PBS. We visualized stained cells by confocal microscopy (see above).

RealTime-Glo cell viability assay

We mixed RealTime-Glo (Promega; catalog no. G9711) MT cell viability substrate and NanoLuc Enzyme together, added it to neuronal cultures, and incubated them for 5 h for equilibration of luminescence signal before the respective treatments were applied. We recorded luminescence with a Spark 10M multimode microplate reader (Tecan) at 37°C and 5% CO_2 every 30 min over a total time period of 20–24 h. We used at least five technical replicates per condition. For analysis, every well's data point was normalized to its last value before the stressor was added and then normalized to the mean of the control wells for every time point. Thereby we controlled for well-to-well seeding variability. For statistical analysis, we compared either area under the curve (AUC) or endpoint.

CellTiter-Glo cell viability assay

24 h after stimulation of neuronal cultures, the CellTiter-Glo Luminescent Cell Viability Assay (Promega; catalog no. G7570) was performed according to the manufacturer's protocol. We recorded luminescence with a Spark 10M multimode microplate reader (Tecan).

Real-time PCR

We reverse transcribed RNA to cDNA with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher

Scientific) according to the manufacturer's instructions. We analyzed gene expression by real-time PCR performed in an ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Gene Expression Assays (Thermo Fisher Scientific) for *Grm8* (Mm00433840_m1), *Grm4* (Mm01306128_m1), *Grm6* (Mm00841148_m1), *Grm7* (Mm01189424_m1), *Fos* (Mm00487425_m1), *Bdnf* (Mm00432069_m1), *Grin1* (Mm00433790_m1), *Grin2a* (Mm00433802_m1), *Grin2b* (Mm00433820_m1), *Gria1* (Mm00433753_m1), *Grik1* (Mm00446882_m1), *Slc1a2* (Mm00441457_m1), *Itpr1* (Mm00444937_m1), *Itpr2* (Mm00439907_m1), *Itpr3* (Mm01306070_m1), *Tbp* (Mm00446971_m1), *GRM8* (Hs00945353_m1), and *TBP* (Hs00427620_m1). We calculated gene expression as $2^{-\Delta Ct}$ relative to *Tbp* (mouse) or *TBP* (human) as the endogenous control.

Isolation of CNS-infiltrating immune cells and flow cytometry

CNS-infiltrating immune cells from EAE animals during the inflammatory phase 12–17 d after immunization were isolated and quantified as we described previously (Ufer et al., 2016). We stained single-cell suspensions in the presence of TruStain Fc receptor block (BioLegend) and used Alexa Fluor 750 NHS Ester (Invitrogen) for live/dead discrimination. The antibodies and the respective antigen, host species, supplier, catalog number, clone, and dilution are listed in Table S8. Data were acquired on an LSR II FACS analyzer (BD Biosciences).

Recall assay

For antigen-specific recall assays, 9 d after immunization of the mice, 2.5×10^5 draining inguinal lymph node cells were prepared and cultured in 96-well round-bottom plates for 72 h with the indicated concentrations of MOG_{35-55} peptide, a vehicle control, or plate-coated anti-CD3ε (1 µg/ml; BioLegend; catalog no. 100340) together with soluble anti-CD28 (1 µg/ml; BioLegend; catalog no. 102116) as a positive control. During the last 16 h of culture, cells were pulsed with 1 µg/ml BrdU (catalog no. 423401). Single-cell suspensions were stained for surface antigens in the presence of TruStain Fc receptor block (BioLegend), and Fixable Viability Stain 780 (BD Biosciences; catalog no. 565388) was used to discriminate dead cells. Cells were fixed (fixation buffer; BioLegend; catalog no. 420801) and permeabilized using 0.5% Triton X-100, followed by incubation with 40 KU/ml DNase I (Merck; catalog no. 260913-10MU) in PBS with Ca^{2+} and Mg^{2+} for 1 h at 37°C. After DNA digestion, incorporated BrdU was detected by incubation with an anti-BrdU AF647-coupled antibody. The antibodies and the respective antigen, host species, supplier, catalog number, clone, and dilution are listed in Table S8. Data were acquired on an LSR II FACS analyzer (BD Biosciences). Representative gating strategies will be provided upon request.

DAPI cell toxicity assay

hiPSC neurons were incubated with either 1 µM AZ or 0.1% DMSO for 1 h and then stimulated with 200 µM glutamate, 100 ng ml^{-1} IFN-γ (PeproTech; catalog no. 315-05), and 50 ng ml^{-1} TNF-α (PeproTech; catalog no. 315-01A). After 24 h, we added 5 µM DAPI (Invitrogen) to the culture for 10 min and performed immunostaining as described above for a neuronal marker (Map2; see above) and propidium iodide (PI; 1:1,000; BioLegend; catalog no. 421301) to identify all nuclei. We used neuronal nuclei as the region of interest to quantify DAPI uptake by mean fluorescence intensity (MFI) as a measure of neuronal cell damage (Fig. S5 Q).

Calcium imaging

We seeded primary neuronal cultures on either the Ibidi 60 µ-Dish Quad (catalog no. 80411) or High (catalog no. 81158) with a glass bottom. To measure cytosolic calcium changes, we infected neuronal cultures with an AAV7 containing pAAV-Syn-GCamp6f-WPRE-SV40 (Chen et al., 2013; Addgene; 100837) at 8–12 div with a 10,000–20,000-fold multiplicity of infection. AAV particles were produced according to the standard procedures of the UKE vector facility. We acquired images with a confocal LSM 700 laser scanning confocal microscope (Zeiss) every 0.48 s with 20× magnification in an imaging chamber maintaining 37°C and 5% CO_2. Infected cultures were imaged in the respective culture medium. We isolated mGluR signaling by applying 25 µM bicuculline, 2 µM CGP 55845, 50 µM APV, 20 µM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX) and 20 µM DL-threo-b-benzyloxyaspartate (DL-TBOA) and subsequently applied 20 µM glutamate. Response-modifying chemicals were applied simultaneously to the isolation mix. Since Gcamp6f is not expressed in the nucleus (Dana et al., 2019), we used Fluo-4 acetoxymethyl ester (Thermo Fisher Scientific; catalog no. 14201) to measure nuclear calcium. For that, we incubated neuronal cultures in medium with 5 µM Fluo-4 acetoxymethyl ester for 30 min at 37°C and 5% CO_2. Then, cells were rinsed three times and left to equilibrate in imaging buffer (10 mM glucose, 140 mM $NaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 20 mM Hepes, and 2 mM $CaCl_2$, pH 7.4) for at least 30 min before imaging. If indicated, 1 µM tetrodotoxin (TTX) was added to electrically silence the cultures. In general, we recorded the first 5–10 min of baseline activity before applying the indicated chemicals. At the end of recording, we applied 10 µM ionomycin to induce maximum cellular calcium response that was used for normalization. Specific assay details and concentrations can be found in the respective figure legends. For data analysis, we measured mean fluorescence values of every cell using Fiji software (NIH) and normalized it to either the maximal calcium response after ionomycin challenge (indicated as F/F_{Max}) or to the mean fluorescence of the baseline (indicated as $F/F_{Baseline}$). For each cell, we calculated maximal, minimal, mean, and AUC of the calcium response using a custom R script. If not stated otherwise, AUC was used for statistical comparisons.

cAMP imaging

We seeded primary neuronal cultures from pregnant FVB/NRJ Epac1-PLN mice on 25-mm-diameter coverslips and imaged them at div 21. The imaging setup has been described in detail elsewhere (Sprenger et al., 2012). Briefly, we washed coverslips twice with imaging buffer (see above) and subjected them to mGluR isolation (see above) with additional treatment of either 0.1% DMSO (vehicle), 50 µM 2-APB, or 1 µM AZ. After a stable FRET ratio was reached, we recorded for 1 min as a baseline and subsequently stimulated cultures with 10 µM glutamate for at

least 10 min. As a viability control, 50 µM forskolin and 50 µM 3-isobutyl-1-methylxanthine were finally added. We recorded FRET measurements using an inverted fluorescent microscope (Nikon Ti) and Fiji software. The FRET donor CFP was excited at 440 nm using a CoolLED light source. The exposure time was 10 ms, and images in CFP and YFP emission were acquired every 5 s. For data analysis, we normalized YFP/CFP ratios to mean fluorescence of the baseline measurement. For each cell, we calculated maximal, minimal, mean, and AUC using a custom R script.

Neuronal nuclei isolation and flow cytometry

Nuclei of mouse spinal cords were isolated with the Nuclei Isolation Kit (Sigma-Aldrich; catalog no. NUC101) according to the manufacturer's protocol. To obtain neuronal nuclei, we stained nuclei with PI (1:2,000; see above) and a primary labeled antibody directed against NeuN (1:500). Then we sorted PI^+NeuN^+ nuclei by using a BD Aria III cell sorter (BD Biosciences). We processed RNA for real-time PCR as described above.

Electrophysiological recordings of hiPSC neurons

For patch-clamp experiments, artificial cerebrospinal fluid (ACSF) with low magnesium was used as an extracellular solution. ACSF was oxygenated during experiments with 95% (vol/vol) O_2 and 5% CO_2 (pH 7.3–7.4) containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25.0 $NaHCO_3$, 0.2 $MgCl_2$, 2.0 $CaCl_2$, and 25 glucose. The internal recording pipette solution contained (in mM): 120 $KMeSO_4$, 20 KCl, 10 Hepes, 0.2 EGTA, 2 $MgCl_2$, 4 Na_2ATP, and 0.3 Na_2GTP; pH was adjusted to 7.3 with KOH. If indicated in the respective figures, 0.5 µM TTX, 50 µM APV, 20 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 20 µM bicuculline, 10 µM glutamate, or 1 µM AZ was added to ACSF or was applied for 4–8 s. All experiments were done at room temperature (22–25°C). Somatic whole-cell voltage-clamp and current-clamp recordings were obtained from visually identified hiPSC neurons with a 40× objective of a Zeiss Axioskop 2 FS Plus microscope. Borosilicate glass capillaries (GC150F-10; Harvard Apparatus) were pulled (Flaming/Brown micropipette puller, model P-97; Sutter Instrument) and had a resistance of 3–5 MΩ when filled with internal solutions. Membrane currents and action potentials were recorded with an EPC9 amplifier (HEKA Elektronik) using Patchmaster software. Only recordings with an access resistance <25 MΩ were evaluated. Series resistance was compensated to 70–80%. Neurons were perfused continuously (1–1.5 ml min^{-1}) with carbonated ACSF.

Vector construction and transfection

To visualize Grm8 localization, we inserted EGFP at the N-terminal extracellular domain next to the 33-amino acid–long signal peptide. Sequentially, EGFP (Primer_f_1, Primer_r_2 from pcDNA3-EGFP), *Grm8* signal peptide (Oligo_f_1, Oligo_f_2), and mm*Grm8* without signal peptide (Primer_f_3, Primer_r_4 from mouse brain cDNA) were inserted into a temporary backbone. Primers, oligonucleotides, and the respective restriction sites are listed in Table S9. For the final construct, we used a modified pAAV-hSyn-EGFP as a backbone. pAAV-hSyn-EGFP was a gift from Bryan Roth (Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC; Addgene 50465; http://n2t.net/addgene:50465; Research Resource Identifier Addgene_50465). First, EGFP was replaced with a multiple cloning site (Oligo_f_3, Oligo_f_4), and then SP-EGFP-*Grm8* (Primer_f_5, Primer_r_4) was inserted, resulting in the pAAV-hSyn-SP-EGFP-mm*Grm8* construct, which we used to transfect primary neuronal cultures alone or together with a tdTomato expression construct at div 1 with 500 ng DNA and Lipofectamine 3000 (Invitrogen; catalog no. L3000001) according to the manufacturer's protocol.

Statistical analysis

The statistical analyses applied during the bioinformatics analysis are detailed in the respective sections of the article. Flow cytometric data were analyzed by using FlowJo software (FlowJo LLC). Images were analyzed by using Fiji software (NIH). Patch-clamp data were analyzed by using Fitmaster (HEKA Elektronik) and Igor Pro 6.03 (Wavemetrics). Experimental data were analyzed within the R environment (version 1.2.5001) on a Mac OS X. Unless stated otherwise, the data are presented as mean ± SEM, and differences between two experimental groups were determined by using unpaired, two-tailed Student's *t* tests and were FDR corrected for multiple comparisons. Statistical analysis of the clinical scores in the EAE experiments was performed by applying a Mann-Whitney *U* test to the AUCs for each animal. The exact number of experiments is provided in the figure legends. Significant results are indicated by $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Data and materials availability

The datasets analyzed during the study are available in the GEO database, and the corresponding accession numbers are listed in the Material and methods section. Signature gene lists for neuronal stressors are provided in Table S1; Sequence Read Archive identifier and fastq download links of datasets used for ARACNe are listed in Table S2; input receptors for ARACNe are shown in Table S3; and the neuronal receptor network is provided in Table S4. The R code used for live-cell imaging analysis, GSEA, and the transmembrane receptor regulatory network is available from the corresponding author on reasonable request.

Study approval

All animal care and experimental procedures were performed according to institutional guidelines and conformed to the requirements of the German Animal Welfare Act. Ethical approvals were obtained from the State Authority of Hamburg, Germany (approval no. 15/81, ORG713). As human tissue could no longer be assigned to a human being, the analyses did not constitute a "research project on humans" in the sense of section 9, paragraph 2, of the Hamburg Chamber of Commerce Act for the Health Professions and therefore did not require consultation in accordance with section 15, paragraph 1, of the Professional Code of Conduct for Physicians in Hamburg. The use of hiPSCs was approved by the ethics committee of the Kiel University, Germany (A145/11), and is further described at https://

www.sciencellonline.com/technical-support/ethical-statement.html.

Online supplemental material

Fig. S1 shows the expression of MS-associated glutamate receptors and neuronal receptor interactomes in different neuronal subsets of MS patients and healthy controls. Fig. S2 characterizes *Grm8* mRNA expression in different tissues and cell types and that *Grm8*$^{-/-}$ and WT neurons do not differ in baseline viability and glutamate receptor expression. Fig. S3 shows that *Grm8*$^{-/-}$ neurons have enhanced calcium accumulation in different glutamate-dependent stress assays and further supports that metabotropic glutamate signaling depends on IP3R signaling and is modulated by cAMP. Fig. S4 shows that *Grm8*$^{-/-}$ and WT animals do not differ in baseline axonal and synaptic density and the immune cell infiltration during the acute phase of EAE but have more demyelination. Fig. S5 shows that Grm8 activation by chronic application of AZ does not alter the immune response in the acute phase of EAE and electrophysiological recordings that support the excitatory differentiation of hiPSC neurons and neuron-specific *Grm8* expression in hiPSC neuronal cultures. Table S1 lists neuronal stress signature genes that were used for GSEA in Fig. 1. Table S2 lists datasets, identifiers, and fastq download links for datasets that were used for ARACNe. Table S3 includes Ensembl gene names of receptors that were used as input for ARACNe. Table S4 shows the neuronal receptor network output from ARACNe. Table S5 lists the results and the number of animals used in individual EAE experiments. Table S6 summarizes clinical data for brain specimens. Table S7 lists chemicals, Table S8 lists antibodies, and Table S9 lists primers and oligonucleotides that we used for creating overexpression constructs.

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Author contributions: M.S. Woo conducted most of the experiments and bioinformatic analyses. N. Rothammer and J.B. Engler optimized the neuronal nuclei isolation method. L. Binkle helped with neuronal cell culture and cloning strategies. G. Di Liberto, I. Wagner, K. Egervari, and D. Merkler conducted human histopathology and RNAscope experiments. U. Haferkamp and O. Pless established and characterized hiPSC neurons. S. Hornig performed patch-clamp experiments. J. Raber and R.M. Duvoisin generated transgenic Grm8 mice and provided reagents and expertise. M.S. Woo, N. Rothammer, F. Ufer, and S. Bauer performed EAE in Grm8-KO animals. J.K. Sonner helped with immunophenotyping. M.S. Woo, F. Ufer, and M.A. Friese designed the experiments for the study and analyzed the data. M.S. Woo, F. Ufer, J.B. Engler, and M.A. Friese wrote the initial version of the manuscript. M.A. Friese conceived, supervised, and funded the study. All coauthors contributed to the editing and discussion of the manuscript and approved the final version.

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Supplemental material

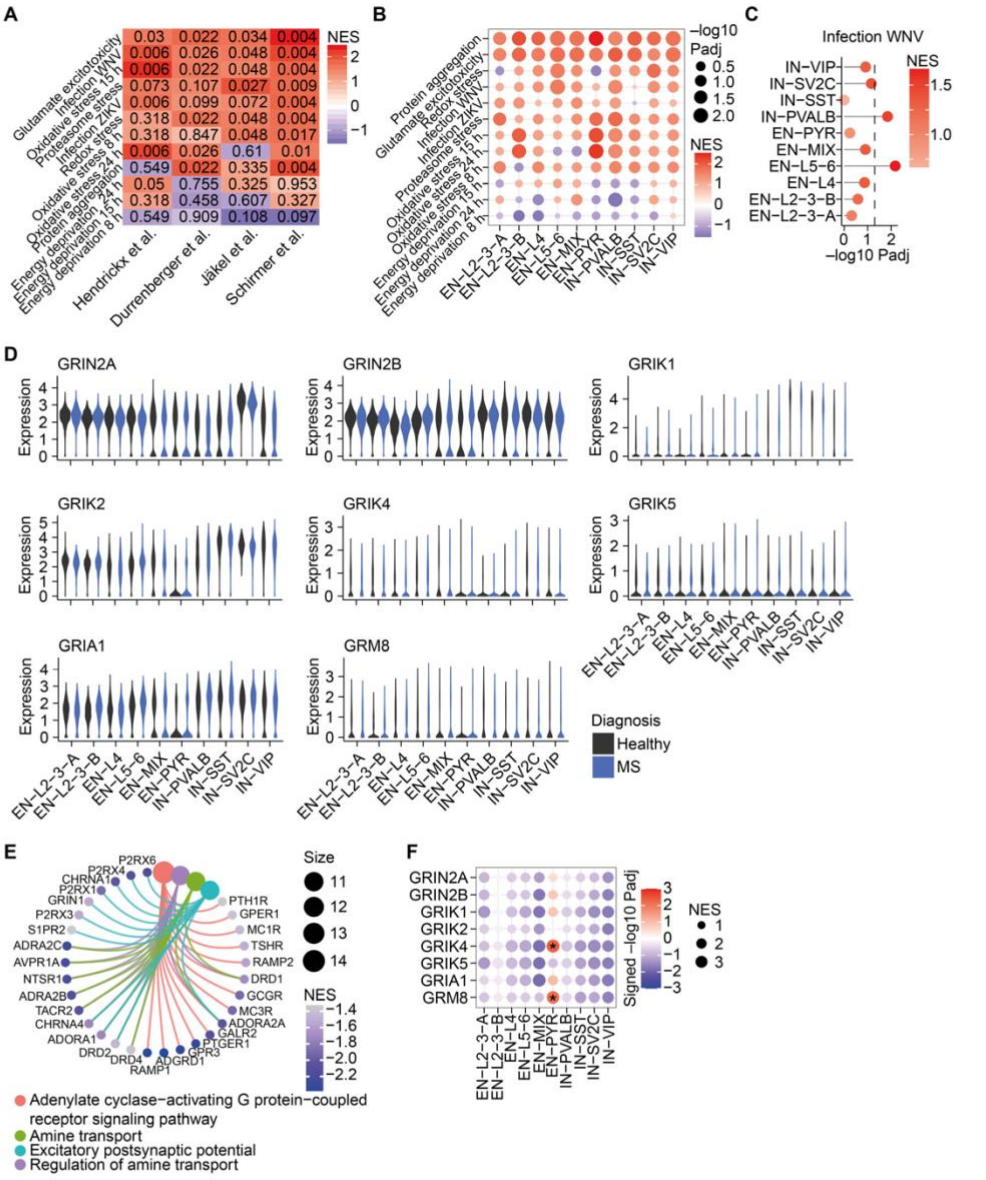

Figure S1. **Neuron-specific transcriptional stress signatures in MS. (A)** FDR-adjusted P values of the NESs of neuronal stress signature gene transcriptomes in respective MS brain specimens. Rows are sorted by cumulative NESs across MS transcriptomes. **(B)** Transcriptional enrichment of neuronal stress signature genes in respective neuron subtypes of MS brains from Schirmer et al. (2019). Size shows negative $\log_{10}$ FDR-adjusted P value; color represents NES. **(C)** Enrichment of gene signature genes from primary neurons that were transduced with West Nile virus (WNV) in depicted neuron subtypes of MS brains from Schirmer et al. (2019). **(D)** Relative gene expression of MS-associated *GRIN2A*, *GRIN2B*, *GRIK1*, *GRIK2*, *GRIK4*, *GRIK5*, *GRIA1*, and *GRM8* in different neuronal subtypes in brains of control and MS patients from Schirmer et al. (2019). **(E)** Top overrepresented biological themes in down-regulated receptor networks in pyramidal neurons of MS patients. **(F)** Heatmap of enrichment of MS-associated glutamate receptors in depicted neuron subtypes in MS patients from Schirmer et al. (2019). Significant enrichment with FDR-adjusted P < 0.01 is labeled with asterisks. Size represents NES. ZIKV, Zika virus.

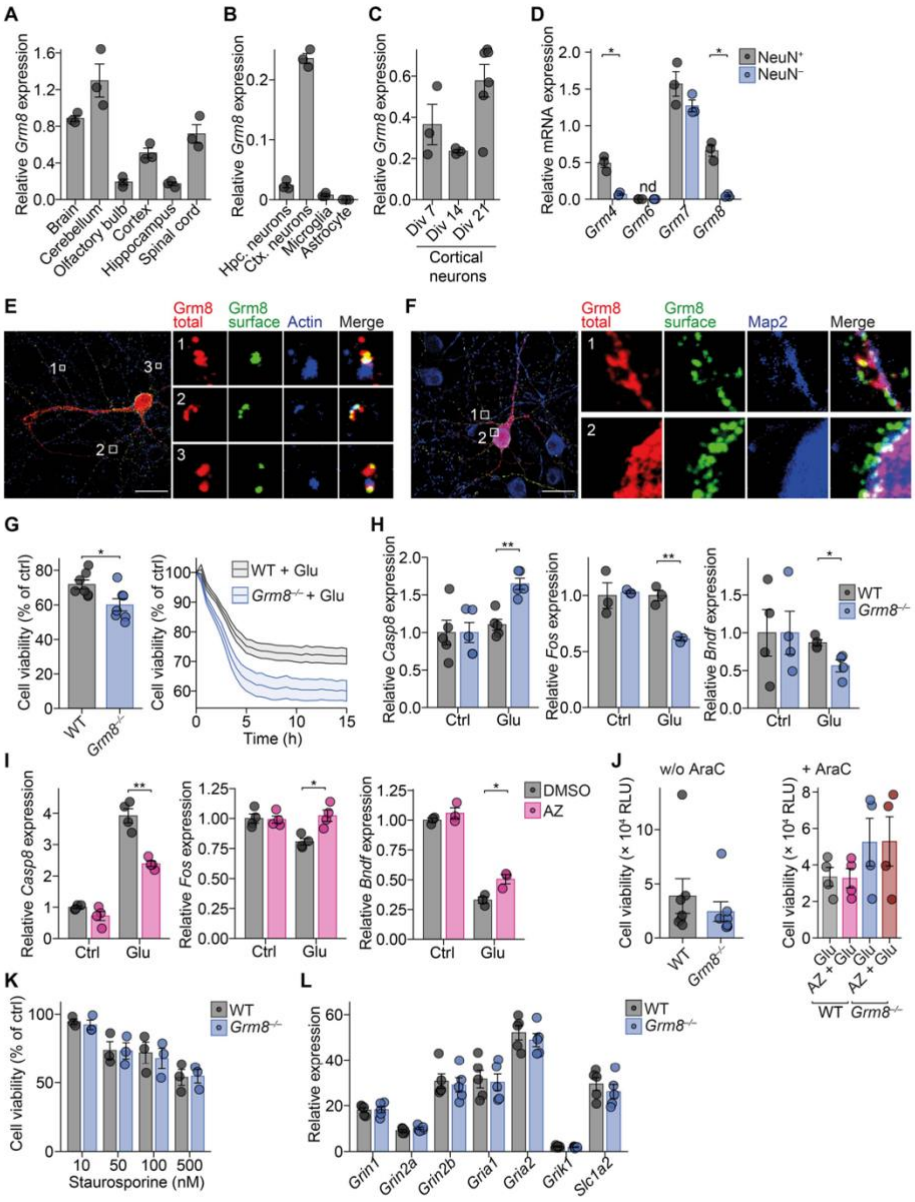

Figure S2. **Grm8 is located in close proximity to pre- and post-synapses and does not influence receptor expression. (A–C)** *Grm8* mRNA expression in indicated mouse tissue (A); hippocampal (hpc) neuronal, cortical (ctx) neuronal, and astrocyte cultures in vitro and sorted microglia (B); and in cortical neuronal cultures at 7, 14, and 21 div (C). All groups, $n = 3$. **(D)** mRNA expression of group 3 metabotropic glutamate receptors in sorted NeuN-positive and NeuN-negative nuclei from the spinal cords of healthy mice. All groups, $n = 3$. **(E and F)** Immunostaining of neuronal cultures that were transfected with EGFP-tagged *Grm8* cDNA and stained for surface and total EGFP and indicated proteins. Scale bars, 20 µm. **(G)** RealTime-Glo Cell Viability Assay of WT and *Grm8*$^{-/-}$ primary mouse neuronal cultures that were not depleted from glial cells and subjected to 20 µM glutamate for 15 h. All groups, $n = 7$. Data are normalized for each time point to the respective untreated neurons (Ctrl). **(H)** Relative mRNA expression of *Casp8* (left), *Fos* (middle), and *Bdnf* (right) in WT and *Grm8*$^{-/-}$ primary mouse neurons

16 div without glial cell depletion 4 h after application of 10 µM glutamate. Data were normalized to WT controls. *Casp8*, *n* = 5; *Fos*, *n* = 3; *Bdnf*, *n* = 4. **(I)** Relative mRNA expression of *Casp8* (left), *Fos* (middle), and *Bdnf* (right) in primary mouse neuronal cultures that were treated with 1 µM AZ for 24 h and were subsequently stimulated with 20 µM glutamate for 4 h. Data were normalized to DMSO-treated control. *Casp8*, *n* = 4; *Fos*, *n* = 4; *Bdnf*, *n* = 3. **(J)** RealTime-Glo Cell Viability Assay baseline relative luminescence units (RLU) without glial cell depletion of WT and *Grm8*$^{-/-}$ (without cytarabine [AraC]; left) and with glial cell depletion (+AraC; right); without AraC, *n* = 7; +AraC, *n* = 4. **(K)** RealTime-Glo Cell Viability Assay endpoint of WT and *Grm8*$^{-/-}$ primary mouse neurons 15 h after exposure to staurosporine in indicated concentrations. All groups, *n* = 3. **(L)** mRNA expression of indicated glutamate receptors in WT and *Grm8*$^{-/-}$ primary mouse neurons. All groups, *n* = 5. Data are shown as mean ± SEM. FDR-adjusted unpaired two-tailed *t* test was used with *, P < 0.05; **, P < 0.01.

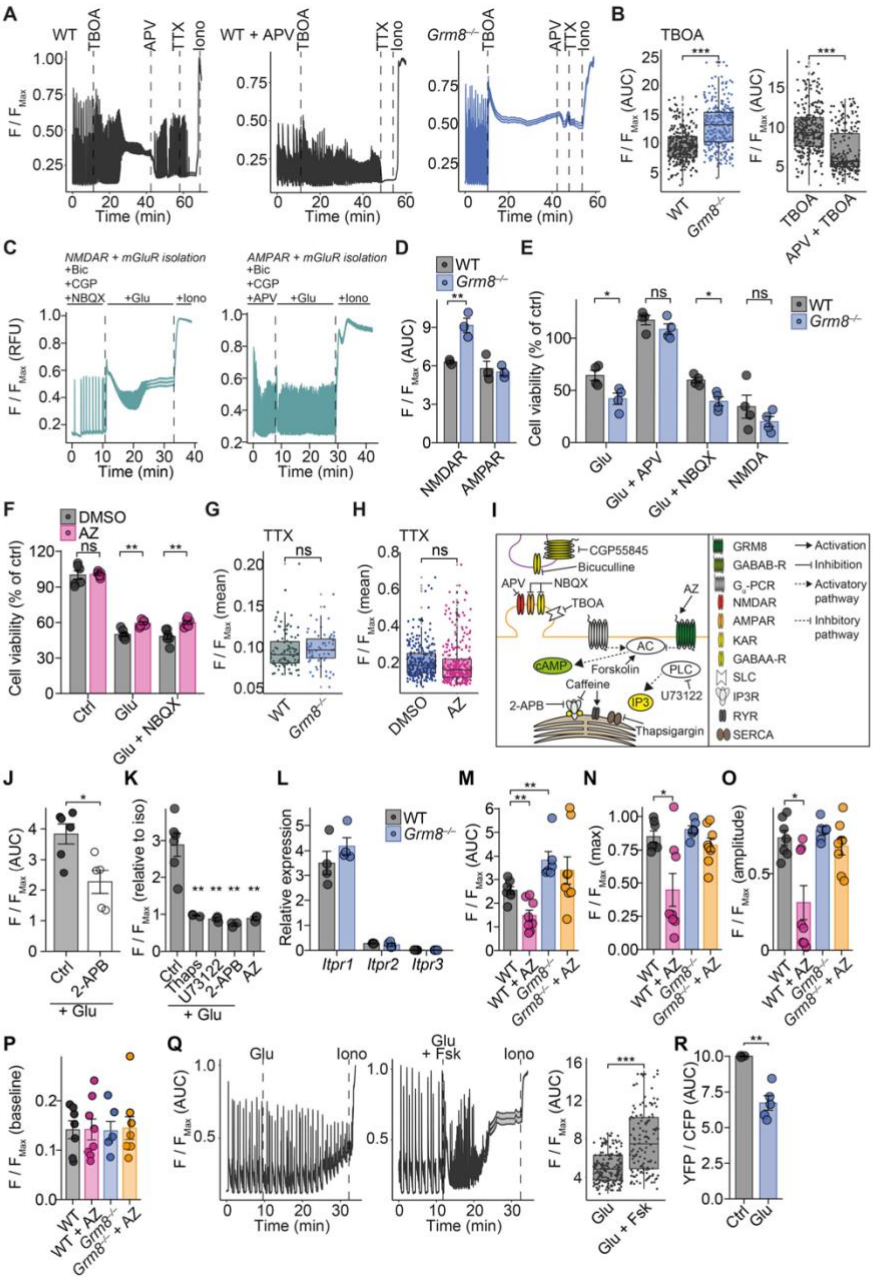

Figure S3. ***Grm8*-deficient neurons show stronger glutamate-induced calcium accumulation. (A)** Representative calcium traces of WT (left) and *Grm8*$^{-/-}$ (right) neuronal cultures without glial cell depletion that were sequentially challenged with 50 µM TBOA, 50 µM APV (only left and right panel), 1 µM TTX, 8 µM

ionomycin, and WT neuronal cultures that were similarly treated but additionally incubated with 50 µM APV during TBOA challenge (middle). **(B)** Left, quantification of AUC of cytosolic calcium in WT (n = 307) and *Grm8*$^{-/-}$ (n = 314) neurons that were challenged with 50 µM TBOA for 30 min. WT, n = 307; *Grm8*$^{-/-}$, n = 314. Right, quantification of neuronal cultures that were challenged with 50 µM TBOA ± 50 µM APV at the same time for 30 min. TBOA, n = 296; APV + TBOA, n = 233. **(C and D)** Isolated mGluR and NMDAR (left) or AMPA receptor (AMPAR; right) activation in mouse WT and *Grm8*$^{-/-}$ neurons. All groups, n = 3. **(E)** RealTime-Glo Cell Viability Assay endpoint of WT and *Grm8*$^{-/-}$ primary neurons that were exposed to 20 µM glutamate, 20 µM glutamate and 50 µM APV, 20 µM glutamate and 10 µM NBQX, and NMDA. All groups, n = 4. **(F)** CellTiter-Glo Viability Assay of primary mouse neurons that were treated with 0.1% DMSO or 1 µM AZ for 24 h and were subsequently exposed to control conditions, 20 µM glutamate, 20 µM glutamate, and 10 µM NBQX for 15 h. Data were normalized to DMSO-treated controls. All groups, n = 6. **(G and H)** Mean baseline calcium level of WT (n = 91) and *Grm8*$^{-/-}$ (n = 64) silenced neurons (G; WT, n = 91; *Grm8*$^{-/-}$, n = 64) and DMSO- and AZ-treated neurons (H; DMSO, n = 247; AZ, n = 269). **(I)** Graphical summary of chemicals and their respective targets and functions used for experiments. **(J)** Calcium response to glutamate of mGluR- and NMDAR-isolated mouse neuronal cultures that were treated with 0.1% DMSO or 50 µM 2-APB. Ctrl, n = 6; 2-APB, n = 5. **(K)** Mouse neuronal cultures were subjected to mGluR isolation protocol and were additionally incubated with 1 µM thapsigargin, 1.25 µM U73122, and 50 µM 2-APB for 10 min and subsequently with 20 µM glutamate or 1 µM AZ without glutamate. Ctrl, n = 7; other conditions, n = 3. Data were normalized to mean calcium level during isolation before application of glutamate. **(L)** Relative mRNA expression of IP3R paralogs *Itpr1*, *Itpr2*, and *Itpr3* in WT and *Grm8*$^{-/-}$ neuronal cultures. All groups, n = 4. **(M–P)** WT and *Grm8*$^{-/-}$ neuronal cultures were treated with 0.1% DMSO or 1 µM AZ, and subsequently isolated mGluRs were activated with glutamate. Quantification of AUC (M), maximal response (N), maximal amplitude (O), and mean baseline during mGluR isolation (P) is shown. WT, n = 7; *Grm8*$^{-/-}$, n = 6; WT + AZ, n = 7; *Grm8*$^{-/-}$ + AZ, n = 8. **(Q)** Spontaneously active cultures were exposed to 20 µM glutamate or 20 µM glutamate together with 10 µM forskolin (Fsk). Data are shown as median ± SEM. Glu, n = 184; Glu + Fsk, n = 132. **(R)** CFP/YFP ratios that negatively correlate with cAMP of mGluR-isolated neuronal cultures that were subsequently vehicle treated or 20 µM glutamate treated. Ctrl, n = 3; Glu, n = 5. Data are shown as mean ± SEM. FDR-adjusted unpaired two-tailed t test was used with *, P < 0.05; **, P < 0.01; ***, P < 0.001.

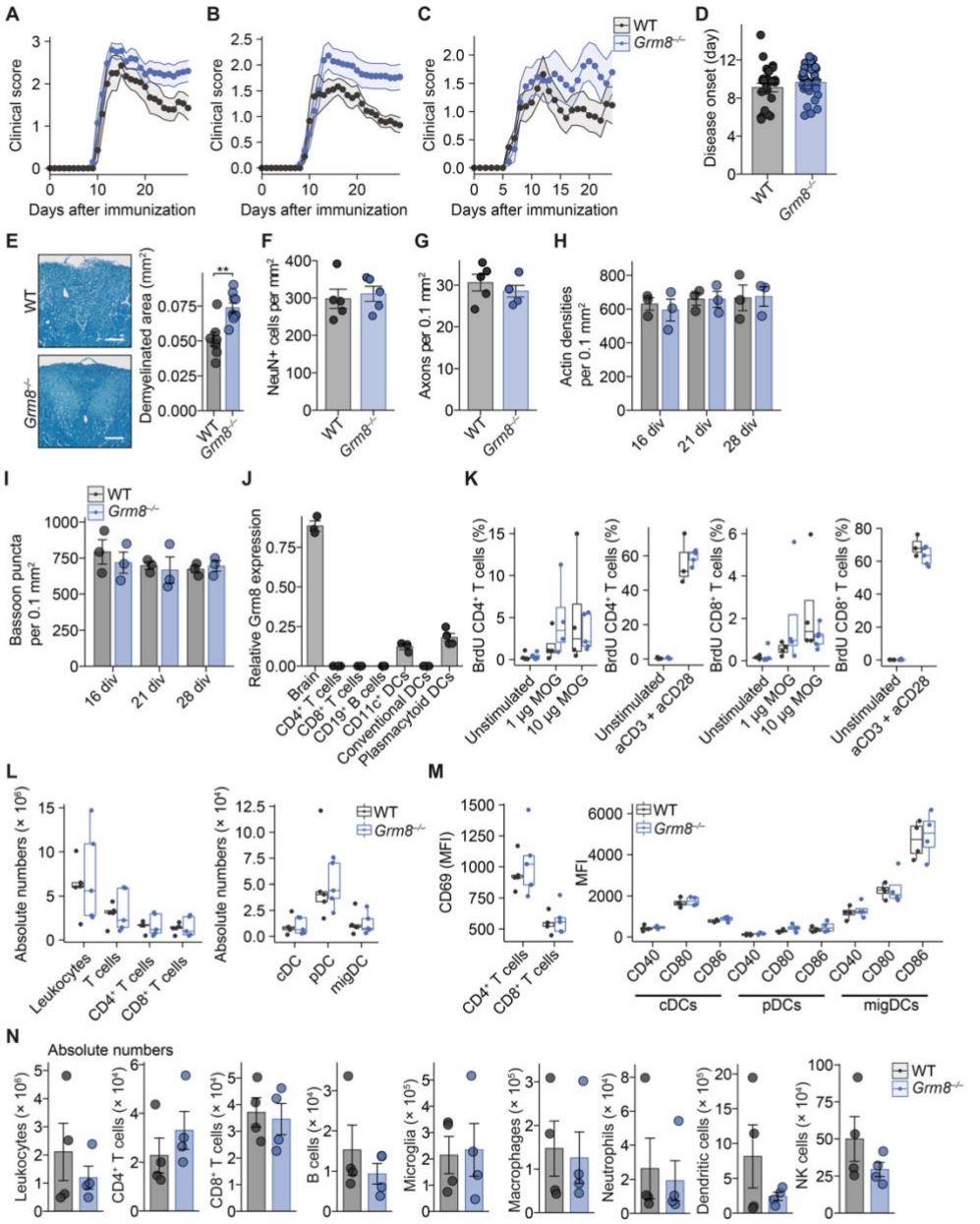

Figure S4. ***Grm8* deficiency does not alter baseline axonal and syaptic density or immune response in EAE. (A–C)** Disease course of individual EAEs that are shown as pooled data in Fig. 5 C. In A, WT, $n = 7$; *Grm8*$^{-/-}$, $n = 10$. In B, WT, $n = 9$; *Grm8*$^{-/-}$, $n = 13$. In C, WT, $n = 11$; *Grm8*$^{-/-}$, $n = 9$. Statistics are provided in Table S5. **(D)** Day of disease onset of WT and *Grm8*$^{-/-}$ animals that were subjected to EAE. WT, $n = 27$; *Grm8*$^{-/-}$, $n = 31$. **(E)** Quantification of demyelinated area

by Luxol blue staining in dorsal columns of spinal cords from WT (n = 7) and *Grm8*$^{-/-}$ (n = 9) mice in the chronic phase of EAE 30 d after immunization. **(F and G)** Number of neurons (F) and axons (G) in spinal cords of healthy WT and *Grm8*$^{-/-}$ mice. All groups, n = 5. **(H and I)** Actin densities (H) and bassoon puncta (I) of WT and *Grm8*$^{-/-}$ neuronal cultures at indicated div. **(J)** Relative *Grm8* mRNA expression in the mouse brain and in indicated immune cell subsets. All groups, n = 3. **(K)** Quantification of BrdU-positive T cells that were derived from draining lymph nodes 9 d after immunization and were restimulated with $MOG_{35–55}$ or CD3/CD28 antibodies as a positive control and pulsed with BrdU for 16 h. WT unstimulated, n = 5; 1 µg of MOG, n = 4; 10 µg of MOG n = 4; aCD3 + aCD28, n = 3; *Grm8*$^{-/-}$, unstimulated, n = 5; 1 µg of MOG n = 4; 10 µg of MOG, n = 4; aCD3 + aCD28, n = 5. **(L)** Quantification of immune cell populations that were derived from draining lymph nodes 9 d after immunization. WT, n = 5; *Grm8*$^{-/-}$, n = 5. **(M)** Quantification of MFI of the activation marker CD69 in T cells and activation markers CD40, CD80, and CD86 in depicted dendritic cell populations that were derived from draining lymph nodes 9 d after immunization of WT and *Grm8*$^{-/-}$ mice. For CD69, all groups, n = 4; for CD40, CD80, and CD86, all groups, n = 4. **(N)** Quantification of absolute numbers of CNS-infiltrating immune cell populations per spinal cord of WT and *Grm8*$^{-/-}$ mice during the acute phase of EAE 15 d after immunization. All groups, n = 4. Data are shown as mean ± SEM. FDR-adjusted unpaired two-tailed t test was used with **, P < 0.01. cDC, conventional dendritic cell; pDC, plasmacytoid dendritic cell; migDC, migratory dendritic cell.

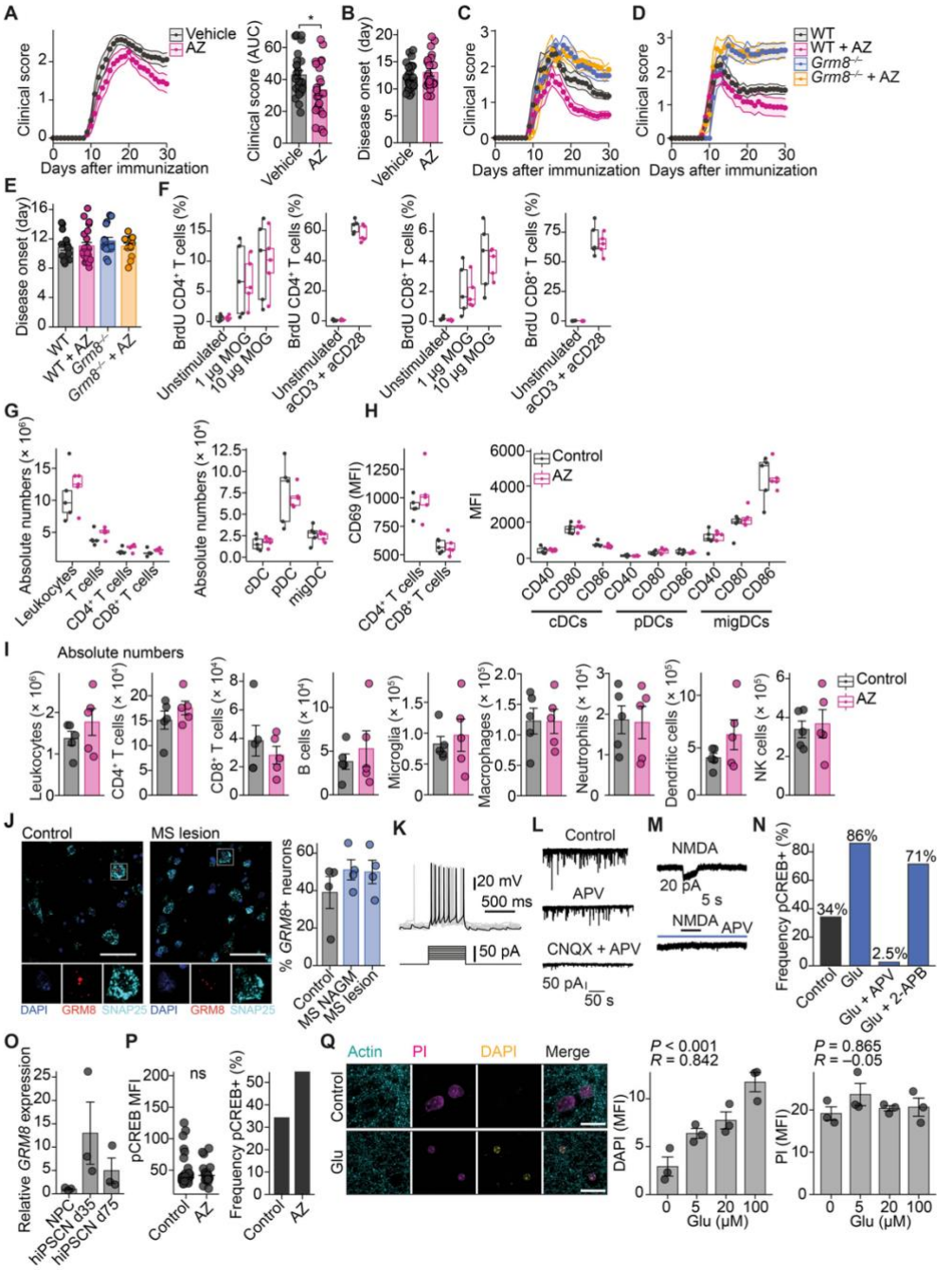

Figure S5. **Assessment of the immune response in EAE and hiPSC physiology after pharmacological Grm8 activation. (A and B)** Animals were subjected to EAE and were injected with either vehicle or AZ. AUC (A) and day of disease onset (B) were quantified. Vehicle, n = 26; AZ, n = 23. FDR-adjusted Mann–Whitney U test was used. **(C and D)** Disease course of individual EAEs that are shown as pooled data in Fig. 6 A. In C, WT, n = 10; *Grm8*$^{-/-}$, n = 8; WT + AZ, n = 12; *Grm8*$^{-/-}$ + AZ, n = 6. In D, WT, n = 8; *Grm8*$^{-/-}$, n = 8; WT + AZ, n = 11; *Grm8*$^{-/-}$ + AZ, n = 6. Statistics are provided in Table S5. **(E)** Day of disease onset of WT and *Grm8*$^{-/-}$ animals that were injected i.p. with a vehicle or AZ. WT, n = 18; *Grm8*$^{-/-}$, n = 17; WT + AZ, n = 23; *Grm8*$^{-/-}$ + AZ, n = 12. FDR-adjusted Mann–Whitney U test was used. **(F)** Quantification of BrdU-positive T cells that were derived from draining lymph nodes 9 d after immunization of mice that were treated for 6 d with DMSO vehicle (control) or AZ and were restimulated with MOG_{35-55} or CD3/CD28 as a positive control and pulsed with BrdU for 16 h. Control, n = 5; AZ, n = 5. **(G)** Quantification of immune cell populations that were derived from draining lymph nodes 9 d after immunization of mice that were treated for 6 d with DMSO vehicle (control) or AZ. Control, n = 5; AZ, n = 5. **(H)** Quantification of MFI of the activation marker CD69 in T cells and activation markers CD40, CD80, and CD86 in depicted dendritic cell populations that were derived from draining lymph nodes 9 d after immunization of mice that were treated for 6 d with DMSO vehicle (control) or AZ. Control, n = 5; AZ, n = 5. **(I)** Quantification of absolute numbers of CNS-infiltrating immune cell populations per spinal cord of mice that were treated with either vehicle or AZ during the acute phase of EAE 15 d after immunization. All groups, n = 5. **(J)** RNAscope fluorescence in situ hybridization of *GRM8* transcripts in brain sections of control individuals and MS NAGM and cortical lesions. All groups, n = 4. Scale bars, 50 µm. **(K)** In current clamp, stepwise increase of current injections in hiPSC neurons results in depolarization and neuronal firing. **(L)** In voltage clamp at −70 mV, subsequent application of APV and CNQX reduces spontaneous excitatory post-synaptic currents in hiPSC neurons. **(M)** Application of 50 µM NMDA for 4 s to hiPSC neurons in the presence of 0.5 µM TTX, 20 µM bicuculline, and 20 µM CNQX induces inward currents at a holding potential of −70 mV (17.4 ± 2.9 pA; n = 5) that can be completely blocked by 50 µM APV. **(N)** Frequency of pCREB-positive hiPSC neurons after stimulation with 20 µM glutamate, 20 µM glutamate with 50 µM APV, or 20 µM glutamate with 50 µM 2-APB for 20 min. Control, n = 38; Glu, n = 50; Glu + APV, n = 78; Glu + 2-APB, n = 49. **(O)** Relative mRNA expression of *GRM8* in undifferentiated human NPCs and 35 or 75 d after differentiation into hiPSC neurons. All groups, n = 3. **(P)** hiPSC neurons were treated for 20 min with either 0.1% DMSO or 1 µM AZ (pCREB-positive neurons; control, 54%; AZ, 34%). Control, n = 38; AZ, n = 22. **(Q)** Neuronal cultures were stimulated with indicated concentrations of glutamate for 2 h, and subsequently 5 µM DAPI was added for 15 min (yellow). After permeabilization, PI (magenta) was used to stain all nuclei and actin (cyan) to visualize neuronal morphology. Left, representative image of vehicle-treated (control) and 20 µM glutamate–stimulated cultures after 2 h. Middle, quantification of nuclear DAPI fluorescence after exposure to indicated glutamate concentration (R = 0.842). Right, quantification of nuclear PI fluorescence after exposure to indicated glutamate concentrations (R = −0.05). All groups, n = 3. Pearson correlation was used. Data are shown as mean ± SEM. Scale bars, 20 µm. FDR-adjusted unpaired two-tailed t test was used with *, P < 0.05. cDC, conventional dendritic cell; pDC, plasmacytoid dendritic cell; migDC, migratory dendritic cell.

Tables S1–S9 are provided online. Table S1 lists neuronal stress signature genes that were used for GSEA in Fig. 1. Table S2 lists datasets, identifiers, and fastq download links for datasets that were used for ARACNe. Table S3 includes Ensembl gene names of receptors that were used as input for ARACNe. Table S4 shows the neuronal receptor network output from ARACNe. Table S5 lists the results and the number of animals used in individual EAE experiments. Table S6 summarizes clinical data for brain specimens. Table S7 lists chemicals, Table S8 lists antibodies, and Table S9 lists primers and oligonucleotides that we used for creating overexpression constructs.

2 Introduction

2.1 Multiple sclerosis

Multiple sclerosis (MS) is an autoinflammatory disease of the central nervous system (CNS) and the most common non-traumatic cause of neurological disability in young adults[1]. It is estimated that 2.3 million people are suffering from MS worldwide and its prevalence has increased substantially in the last decades[2]. First symptoms usually manifest in the third or fourth decade of life and thus lead to great socioeconomic costs for lifelong support and treatment[3].

Three main subtypes of MS exist. The majority of patients, 85%, suffer from relapsing-remitting MS (RRMS) characterised by episodes with worsening of neurological symptoms followed by clinical remission. However, after several years of the disease, a considerable number of patients develop secondary progressive MS (SPMS), where neurological symptoms accumulate with very limited response to disease-modifying anti-inflammatory therapies. Notably, number of relapses does not correlate with disease progression in late stages. Approximately 15% of patients suffer from primary progressive MS with accumulation of neurological symptoms from disease onset[4].

The pathogenesis of MS has been attributed to a breakdown of the blood-brain barrier and subsequent infiltration of autoreactive T cells that secrete pro-inflammatory cytokines and initiate the formation of a chronic inflammatory and excitotoxic environment[5,6]. This is further maintained by activation of parenchymal glial cells and sub-meningeal lymphoid follicles[7]. Subsequently, demyelination and neurodegeneration occur that drive neurological symptoms in MS patients. Potent immunomodulatory drugs have been developed in recent years that suppress inflammatory relapses. Mechanistically, these drugs inhibit CNS infiltration (natalizumab[8]), proliferation of leukocytes (cladribine[9], alemtuzumab[10]) or repress the migration of immune cells from lymphoid organs (fingolimod[11]). Nonetheless, neurodegeneration is not halted sufficiently. Additionally, histopathological and transcriptomic studies of *post-mortem* tissues of MS patients identified neuronal stress responses and neurodegeneration in non-lesioned normal-appearing grey matter without immune cell infiltration[12–14]. Furthermore, regression analyses of neurological disabilities in large MS cohorts revealed that rather cortical volume than the number of inflammatory lesions are most predictive for disease progression[15]. However, treatment strategies that specifically reduce neurodegeneration are limited due to lack of knowledge about neuron-intrinsic molecular pathways that determine susceptibility or resilience to a chronic inflammatory environment.

Preclinical *in vivo* studies heavily rely on the mouse model of MS, experimental autoimmune encephalomyelitis (EAE). Here, mice are actively immunized against myelin-oligodendrocyte-glycoprotein (MOG) by subcutaneously injecting an emulsion of $MOG_{35–55}$ and complete Freund's adjuvant (CFA). Blood brain barrier leakage is induced by intraperitoneally injecting pertussis toxin. Thus, antigen presenting cells are primed against MOG, migrate to lymph nodes, and activate T cells that are specific for MOG. These T cells infiltrate the CNS and induce demyelination, synaptic loss and neuronal demise[16]. Pharmacological and genetic studies using EAE revealed several mechanisms that contribute to inflammatory neurodegeneration in MS. These include production of reactive oxygen and nitrogen species which lead to mitochondrial damage and energy failure[12,17]. Moreover, activation of the unfolded protein response[18,19], disturbance of the nucleocytoplasmic shuttle[19], somatic aggregation of synaptic proteins and disruption of the ion homeostasis further drive neuroaxonal

demise[20,21]. However, identification of druggable targets remains difficult due to lack of knowledge of injurious neuron-intrinsic pathways and respective regulators.

Therefore, to deepen our understanding of the neuronal stress response is of crucial importance for the development of novel neuroprotective strategies that guarantee fine-tuned modulation of neuronal maladaptive pathways and at the same time do not reduce neuronal functionality.

2.2 Glutamate excitotoxicity

A pathological driver of neuronal miswiring and loss that is shared between primary and secondary neurodegenerative diseases is glutamate excess[22,23]. Glutamate is the main excitatory amino acid in the CNS and thus, its tight regulation is crucial for neuronal function and communication. While synaptic glutamate release is promoting neuronal survival and is necessary for synaptic activity, glutamate excess can also lead to neuronal cell death[24]. The latter is common for acute neuronal challenges as observed during ischemic stroke[25] but also for chronic neuronal stress as present in primary neurodegenerative disorders[26]. Notably, glutamate excess is also found in MS. Here, glutamate levels in the cerebrospinal fluid as well as in the brain parenchyma of MS patients are elevated as measured by magnetic resonance spectroscopy imaging[27,28].

Glutamate binds to ionotropic (iGluR) and metabotropic glutamate receptors (mGluR) and thereby leads to a variety of downstream signalling. Whether glutamate enhances neuronal survival or promotes cell death has been attributed to its binding to different N-methyl-D-aspartate (NMDA) receptors, a subtype of iGluRs, with either synaptic (sNMDAR) or extrasynaptic (eNMDAR) location[29]. By systematically stimulating and inhibiting glutamate receptors in primary neuronal cultures, it has been shown that activation of eNMDAR as opposed to stimulation of sNMDAR results in somatic and nuclear calcium accumulation, transcriptional inhibition of anti-apoptotic and induction of apoptotic gene profiles[30]. This is mainly mediated by post-translational modifications of different transcription factors. For example, sustained activation of sNMDAR results in phosphorylation and subsequent nuclear translocation of the cyclic-AMP response element binding protein (CREB) that induces pro-survival genes that are also involved in several other processes such as neurogenesis, synaptic plasticity, and memory consolidation. This is in line with *in vitro* studies that have shown activity-dependent CREB phosphorylation (pCREB) and neuroprotection against oxidative and excitotoxic insults[31]. In contrast, activation of eNMDAR results in a shut-down of pCREB and activation endogenous apoptotic pathways by inhibiting the RAS-ERK1/2 pathway that physiologically increases CREB phosphorylation. Its activation also initiates nuclear translocation of the juxtasynaptic attractor of caldendrin on dendritic boutons protein (JACOB) that inhibits CREB phosphorylation[32,33].

Glutamate concentrations differ across different tissues and cell compartments. While intracellular glutamate concentrations in neurons are approximately 10 mM, measurements by microdialysis *in vivo* estimate that extracellular glutamate concentrations vary from 1 to 5 µM. Following action potential mediated synaptic release, glutamate levels in the synaptic cleft reach up to 1 mM for a few milliseconds and then return to less than 20 nM[34]. The concentration gradient of glutamate between the intra- and extracellular milieu is maintained by cellular compartmentalization and glia cells which immediately remove extracellular glutamate via excitatory amino acid transporters (EAATs). Thus, disruption of neuronal compartments such as synapses by hyperexcitation and glutamate spillover or disturbance of the neuronal soma integrity by uncontrolled activation of cell death pathways results in excessive glutamate release[35]. Furthermore, loss of glia cells contributes to increased

extracellular glutamate levels by decreased re-uptake[36,37]. In addition to neurons another source of glutamate constitutes its active secretion by infiltrating immune cells. For example, by leveraging genetically encoded fluorescent glutamate reporters it has been shown that T_H17 cells secrete glutamate in MS and EAE that contributes to neuronal demise[38]. This thesis focused on therapeutic strategies to counteract glutamate excitotoxicity in inflammatory neurodegeneration in MS.

2.3 Metabotropic glutamate receptors

Phylogenetically, metabotropic glutamate receptors (mGluR) are assigned to the glutamate-type metabotropic receptors. Additionally, four other groups of G-protein coupled receptors (GPCR; adhesion, secretin, rhodopsin, frizzled/Tas2) have been classified that consist of more than 350 non-olfactory metabotropic receptors, among them are more than 30% orphan receptors with unknown ligands and downstream signalling. Due to their often cell-specific expression and membranous location they have been attractive drug targets and consequently, more than 30% of FDA-approved drugs target metabotropic receptors[39,40].

Eight mGluRs have been characterized and can be subdivided into three groups. Whereas group one mGluRs (GRM1, 5) are $G\alpha_{q/11}$-coupled and thereby enhance glutamate excitation, group two (GRM2, 3) and three (GRM4, 6, 7, 8) negatively regulate cyclic adenosine monophosphate (cAMP) signalling by $G\alpha_i$-coupling, thereby inhibiting further glutamate release[41]. Accordingly, inhibition of GRM5 has been shown to be neuroprotective in rodent models of Parkinson's disease (PD), Alzheimer's disease (AD) and Fragile-X syndrome (FXS)[42–44] but failed to show effects in phase 2 clinical trials for FXS[45] and in EAE[46]. At the same time, positive modulation of group three metabotropic receptors also showed promising protective effects in neuronal stress assays. Indeed, activation of group three GRM4 has been reported to reduce EAE disease severity, but this was attributed to an effect on the immune response by directly blocking dendritic cell activation[47]. By contrast, the predominantly neuronally expressed group three GRM8 constitutes an attractive pharmacological target, which thus far has been mainly investigated in mouse models of anxiety and neuropathic pain[48,49]. *Grm8*$^{-/-}$ mice show increased anxiety and are more sensitive to neuropathic pain which is reversed by Grm8 activation[50,51]. Electrophysiologic recordings showed that these behavioural phenotypes are mediated by inhibiting excitatory synaptic transmission. Furthermore, demonstrating Grm8-dependent pre-pulse inhibition[49] and immunolabeling of Grm8 in olfactory bulbs and lateral perforant pathway[52,53] supported the notion of a presynaptic localisation. However, the lack of a specific antibody against GRM8 has made it difficult to investigate its localisation and regulation during CNS pathologies. In addition to the behavioural phenotypes, *Grm8*-deficiency in mice results in mild insulin resistance and weight gain[50]. Furthermore, *Grm8* is expressed in glutamatergic neurons of the enteric system and enhances intestinal motility[54]. Notably, gene variants of GRM8, among other iGluRs have been associated with MS progression in genome wide association studies[55,56].

Thus, the aim of my thesis was to disentangle the role of GRM8 in inflammatory neurodegeneration and its underlying molecular pathways.

3. Methods

To identify common transcriptional signatures of neurodegeneration in MS, in a first step we compared transcriptional responses of MS *post-mortem* tissues with *in vitro* mouse cultures that were exposed to defined stressors. Therefore, we analysed publicly available RNA bulk sequencing datasets that were obtained from Gene Expression Omnibus (GEO). By extracting differentially regulated genes of challenged neuronal cultures we defined transcriptional signatures for each stressor. We chose the top 100 differentially upregulated genes for subsequent analyses as defined by foldchange of the expression level. To obtain the MS-specific transcriptional stress signature we analysed two microarrays that compared brain tissues of healthy donors and MS patients by bulk RNA-sequencing[22,57]. Furthermore, we extracted neuronal transcriptional changes in MS by analysing two published single nucleus RNA sequencing (snRNA-seq) datasets of brains of healthy donors and MS patients[58,59]. To perform geneset enrichment analysis (GSEA) we created ranked gene lists by arranging the differential expression results from healthy donors and MS comparisons by *t*-statistics for microarray and Wald statistics for single-nucleus sequencing datasets. Finally, an enrichment analysis by hypergeometric testing of the neuronal stress signatures and the MS specific ranked gene lists was performed. Thus, we extracted neuronal stress responses from MS transcriptomes.

In the next step, we aimed to identify co-regulatory gene modules and their underlying master regulators that drive these neuronal stress responses and subsequent neuronal demise in MS. Therefore, we created a neuron-specific receptor interactome and compared the neuronal nuclei of MS patients with control donors. We first created correlative gene networks by retrieving raw read counts of 502 neuron-specific mRNA sequencing datasets of *in vitro* challenged neuronal cultures and *in vivo* mouse models of psychiatric, neurodegenerative, neuroinflammatory and metabolic diseases. Regulatory networks were reversely engineered by an algorithm for the reconstruction of gene regulatory networks (ARACNe)[60]. Next, we defined hub genes and calculated their underlying transcriptional network. We provided transmembrane receptors as defined as family member of the Gene Ontology identifier GO:0003700, “transmembrane receptor activity”, and its respective offspring as hub genes. Finally, we calculated perturbed receptor interactomes of neurons from brains of MS patients in comparison to healthy donors by Virtual Inference of Protein activity by Enriched Regulon (VIPER)[61].

To assess the impact of Grm8 activation on inflammatory neurodegeneration, we used experimental autoimmune encephalomyelitis (EAE), the mouse model of MS. All EAE experiments were conducted with mice on C57BL/6J background. Adult mice (6-20 weeks) from both sexes were used and mice were sex- and age-matched. Mice were immunized subcutaneously with 200 µg $MOG_{35–55}$ peptide (Schafer-N) in Complete Freund’s Adjuvant (CFA; Difco, cat. no. DF0639-60-6) containing 4 mg × ml^{-1} *Mycobacterium tuberculosis* (Difco, cat. no. DF3114-33-8). In addition, 200 ng pertussis toxin (Calbiochem, cat. no. CAS70323-44-3) was injected intraperitoneally on the day of immunisation and 48 hours later. Animals were scored daily for clinical signs by the following system: 0, no clinical deficits; 1, tail weakness; 2, hind limb paresis; 3, partial hind limb paralysis; 3.5, full hind limb paralysis; 4, full hind limb paralysis and fore limb paresis; 5, premorbid or dead. Animals reaching a clinical score ≥ 4 were euthanized according to the regulations of the local Animal Welfare Act. We compared Wildtype and *Grm8*$^{-/-}$ mice[50] that were treated with DMSO vehicle control or the positive allosteric modulator (PAM) of Grm8 AZ12216052 (AZ; Tocris; cat. no.

4832)[51]. Where indicated in the respective figures, animals were injected intraperitoneally with 1 mg kg^{-1} body weight AZ starting from day of disease onset.

We analysed neuronal loss and immune cell infiltration following *Grm8* deletion by immunohistochemistry of spinal cord sections of respective animals that were perfused with 4% paraformaldehyde (PFA) and embedded in paraffin. The exact procedures as well as the primary and secondary antibodies used in this study are provided in the methods section of the publication. Fluorescent imaging was performed with confocal microscopy (Zeiss LSM 700).

To quantify transcripts of *GRM8* in human post–mortem samples of healthy controls and MS patients, we performed RNAscope fluorescent *in situ* hybridization using the RNAscope Fluorescent Multiplex Kit V2 (Advanced Cell Diagnostics). Images were acquired with a Panoramic 250 FLASH II (3DHISTCH) Digital Slide Scanner.

Mechanistic studies were conducted in primary cortical cultures. These were prepared from cortices of mouse embryos at Theiler's embryonic stage 16.5. Cortices were collected, dissociated, and plated at a density of 10^5 per 1 cm^2. Subsequently, cells were treated with 1 µM cytarabine at 1 day in vitro (div) to inhibit glial proliferation. We carried out experiments after div 14 to 23.

We assessed cell viability by luminescence-based assays. We applied the RealTime-Glo assay (Promega) to measure the intracellular redox potential as approximation of cell viability. The assay was performed as provided in the manufacturer's protocol. Treatments were added after 5 hours of equilibration. Luminescence was recorded with a Spark 10M multi-mode microplate reader (Tecan) at 37°C and 5% CO_2 every 30 minutes for an overall time of 24 hours. For statistical analysis of the time series data, we assessed area under the curve (AUC). Furthermore, for end point measurements the CellTiter-Glo assay (Promega) that monitors ATP levels in cell lysates was performed according to manufacturer's protocol 24 hours after stimulation.

For cytosolic calcium imaging we seeded primary neurons on Ibidi 60 µ-Dish Quad (Ibidi). To visualize calcium levels, we infected neuronal cultures with an adeno-associated virus (AAV) serotype 7 containing the genetically encoded fluorescent calcium indicator pAAV-hSyn-GCamp6f-WÜRE-SV40[62] at 8-12 div with a 10,000–20,000-fold multiplicity of infection. Metabotropic glutamate receptor-dependent calcium activity was isolated by inhibiting all glutamate receptors except for mGluRs. Therefore, we applied 25 µM bicuculline (gamma-aminobutyric acid $(GABA)_A$-R blocker), 2 µM CGP 55845 ($GABA_B$-R) blocker, 50 µM D-2-Amino-5-phosphonopentanoic acid (APV; NMDAR blocker), 20 µM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX; alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and Kainat-R blocker) and 20 µM DL-threo-beta-benzyloxyaspartate (DL-TBOA; blocker of excitatory amino acid transporters) and subsequently mGluRs were activated by adding 20 µM glutamate. To analyse nuclear calcium levels, we incubated neuronal cultures with 5 µM Fluo-4 acetoxymethyl ester for 30 minutes at 37°C and 5% CO_2 and subsequently, pictures were acquired in an imaging buffer that contained 10 mM glucose, 140 mM $NaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and 2 mM $CaCl_2$, pH 7.4. We electrically silenced cultures by adding 1 µM tetrodotoxin (Ttx) for at least 1 hour. Since Gcamp6f and Fluo-4 do not allow ratiometric estimations of calcium levels we added 10 µM ionomycin at the end of each recording to induce the maximum cellular calcium response that was used for normalisation in the analysis. We performed live cell calcium imaging with a confocal LSM 700 laser scanning microscope (Zeiss) every 0.48 seconds with 20-fold magnification in an imaging chamber maintaining 37°C and 5% CO_2.

To measure intracellular levels of cAMP, we made use of a transgenic mouse line that allowed Förster resonance energy transfer (FRET) based measurements of cAMP. Neuronal cultures from pregnant FVB/NRJ Epac1-PLN mice[63,64] were prepared and cultured on 25-mm-diameter coverslips. The imaging details have been described in detail elsewhere[65]. Coverslips were washed in the imaging buffer and subjected to the mGluR isolation protocol (see above). As a viability control, we added 50 µM forskolin and 50 µM 3-isobutyl-1-methylxanthine at the end of each recording. FRET measurements were recorded with an inverted fluorescent microscope (Nikon Ti) and Fiji software. The FRET donor CFP was excited at 440 nm using a CoolLED light source and 10 ms exposure time.

Neuronal nuclei were isolated according to the Nuclei Isolation Kit (Sigma Aldrich) following the manufacturer`s protocol. We visualised nuclei with propidium iodide staining and neurons by NeuN fluorescent labelling. Nuclei were sorted using a BD Aria III cell sorter (BD Biosciences).

RNA was reverse transcribed with the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher) according to the manufacturer`s protocol and gene expression was analysed by real-time PCR in an ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems). All TaqMan gene Expression Assays (ThermoFisher) are provided in the methods section of the manuscript.

To quantify the impact of Grm8 modulation on the immune system the CNS infiltrating immune cells from EAE animals during acute inflammation 12–17 days after immunisation were isolated and quantified as previously described. Furthermore, an antigen-specific recall assay was performed. Here, immune cells were isolated from draining inguinal lymph nodes in preclinical EAE animals nine days after immunization and were stimulated *ex vivo* with vehicle control, $MOG_{35–55}$ or CD3 with CD28 for in total 72 hours. To quantify proliferation cells were pulsed with bromodeoxyuridine (BrdU) for 16 hours. The exact protocol, concentrations, used antibodies and gating strategies are provided in the methods section of the publication.

To visualise Grm8 localization, we inserted an EGFP at the N-terminal extracellular domain next to its 33–amino acid–long signal peptide. Sequentially, EGFP, Grm8 signal peptide and murine *Grm8* without signal peptide were inserted into a temporary backbone and finally inserted into a pAAV backbone with a neuron specific hSynapsin-promoter. Primary cortical neurons were transfected with 500 ng DNA with Lipofectamine 2000 according to the manufacturer`s protocol.

To visualise the membranous localisation and distribution of Grm8, surface staining of GFP and GFP-*Grm8* transfected neurons was performed. Therefore, primary antibody against GFP was added to living neurons and incubated for 30 min at 4°C. Subsequently, cultures were fixed with 4% PFA and blocked with 10% Normal Donkey Serum (NDS) and secondary antibody was added for 30 min. Thus, only surface GFP-Grm8 was labelled. After washing the fixed cultures were permeabilised and intracellularly fixed with 10% NDS and 0.1% Triton. Afterwards primary antibody against GFP was added again and labelled with a different fluorescent secondary antibody. Thus, we labelled total GFP-Grm8 and membranous GFP with different colours.

We differentiated human-induced pluripotent stem cell-derived neurons (hiPSC-neurons) as previously published[66–69]. We performed assays after 18–20 weeks of incubation. Medium was changed twice weekly. To analyse the impact of GRM8 modulation on cell viability, we incubated hiPSC-neurons with vehicle or 1 µM AZ for 1 hour and subsequently stimulated cultures with 200 µM glutamate, 100 ng mL^{-1} IFN–γ and 50 ng mL^{-1} TNF–α for 24 hours. This was followed by measurement of cell viability by applying the cell impermeable nuclear dye DAPI for 10 minutes on unfixed

cultures. Subsequently, DAPI uptake as marker of cell membrane damage was quantified by immunofluorescence. To measure the impact of positive GRM8 modulation on ionotropic potassium currents, somatic whole-cell voltage-clamp and current-clamp recordings were performed with hiPSC-neurons that were incubated in artificial cerebrospinal fluid (ACSF). All compounds were added for 4–8 seconds and immediately washed out afterwards. The buffer recipes and different stimulations are provided in detail in the methods section of the publication.

All statistical comparisons and visualisations were conducted within the R environment (version 1.2.5001) on a Mac OS X. The R packages that were used in this thesis are provided in the methods section of the publication. Flow cytometric data was analysed with FlowJo Software (FlowJo LLC), confocal images were analysed with Fiji software (NIH), patch-clamp experiments were analysed with Fitmaster (HEK Elektronik) and Igor Pro 6.03 (Wavemetrics). Unless stated otherwise in the respective figure legends of the publication the data are presented as mean ± standard error mean. Differences between two groups were analysed by an unpaired two-tailed Student's *t* test with FDR correction for multiple comparisons if applicable. Clinical scores of EAE animals were compared by applying a non-parametric Mann–Whitney *U* test to the AUC of the score of each animal. The exact number of experiments and biological replicates are provided in the figure legends of the publication.

4. Results

The following section describes the results of the research paper “Neuronal metabotropic glutamate receptor 8 protects against neurodegeneration in CNS inflammation” that was published in the journal “Journal of Experimental Medicine” (Woo et al. 2021) and is provided in chapter one. All figure references pertain to this publication.

First, we assessed transcriptional signatures of neuronal demise in MS. Therefore, we set out to compare neuronal stress responses that are induced by defined stressors with transcriptional signatures of MS. In a first step, published mRNA-sequencing datasets of primary neurons that were exposed to defined challenges were analysed for differentially expressed (DE) genes, thereby generating stimuli-specific transcriptional signatures. Subsequently, we extracted transcriptomes and DE genes of bulk sequencing datasets of cortices of MS patients[22,70] in comparison to healthy controls and of neuronal nuclei of single-nucleus mRNA sequencing datasets (snRNA-seq) of white and grey matter of MS patients[58,59]. These MS specific transcriptional signatures of CNS tissue or isolated neuronal nuclei were compared with the stimuli-specific transcriptional signatures of *in vitro* challenged primary neurons by gene set enrichment analysis. Here, transcripts indicative of glutamate excitotoxicity and viral infection showed the strongest enrichment across whole CNS and neuronal nuclei of MS patients in all different neuronal populations (cf. Fig. 1A–C, supplemental Fig. 1 A–C)[71]. Subsequently, we aimed to find determinants of glutamate excitotoxicity in MS. Thus, we focused on glutamate receptors that have been previously associated with disease progression and brain volume loss in MS patients[55,56]. We assessed their expression in neuronal nuclei of publicly available snRNA-seq datasets and observed that these receptors were not differentially expressed in MS patients compared to healthy donors (cf. Fig. 1D, supplemental Fig. 1D)[71]. Since receptor activity is not transcriptionally determined, we inferred the respective receptor activity by analysing their transcriptional downstream networks. We leveraged the reverse engineering by an algorithm for the reconstruction of gene regulatory networks (ARACNe) algorithm[60,72] to create receptor interactomes of 502 publicly available neuronal transcriptomes of murine disease models and challenged primary neurons. Subsequently, we compared differentially perturbed receptor activities in neuronal nuclei of MS patients. Of all identified neuronal subtypes, we found that excitatory pyramidal neurons were most severely affected by dysregulated receptor interactome with ionotropic glutamate receptor and inhibitory G-protein coupled receptor (GPCR) signalling being highest upregulated whereas adenylate cyclase-activating GPCR signalling and networks that regulate excitatory postsynaptic potential were downregulated. Notably, the receptor interactomes of *GRM8* and *GRIK4* were upregulated in MS. We decided to focus on *GRM8* since its downstream network consisted of genes that are crucial for synaptic plasticity, electric transmission and responses to axonal injury as assessed by GO term analysis (cf. Fig. 1E–H, supplemental Fig. 1E–F)[71].

To further characterise Grm8, we first analysed its distribution throughout the CNS and found high expression in the cortex and spinal cord of mice. Next, we isolated and separated neuronal nuclei from the spinal cord by fluorescent immunolabeling with the pan-neuronal marker NeuN and subsequent flow cytometric sorting and compared *Grm8* expression in $NeuN^+$ neuronal and $NeuN^-$ non-neuronal nuclei. We found 15-fold enrichment of *Grm8* transcripts in $NeuN^+$ nuclei underlining its predominant neuronal expression (cf. Fig. 2A, supplemental Fig. 2A–D)[71]. Since commercially

available antibodies showed unspecific staining, we constructed an overexpression plasmid and inserted an EGFP between the N-terminal signalling peptide and the rest of *Grm8*. Since the N-terminus is extracellularly located we were able to visualise its surface localisation by fluorescent staining. We detected its expression in pre- and post-synapses as well as at the surface of neuronal soma. Interestingly, it was located in the synaptic cleft as well as in its close proximity at the synaptic shaft (cf. Fig. 2B–E, supplemental Fig. 2E–F)[71].

Next, we aimed to analyse how Grm8 modulation affects neuronal viability in an excitotoxic environment. Therefore, we used primary neurons of *Grm8*$^{-/-}$ mice[50] and tested the effect of the positive allosteric modulator of Grm8 AZ12216052 (AZ)[51] on survival. First, we treated *Grm8*$^{-/-}$ and WT neuronal cultures with the vehicle and AZ and subsequently glutamate and observed that Grm8 activation was neuroprotective whereas genetic deletion resulted in more deleterious effects of glutamate (cf. Fig. 3A)[71]. Notably, AZ did not affect glutamate-induced toxicity in *Grm8*$^{-/-}$ neuronal cultures underlining the compound's specificity. Since calcium accumulation in neurons is a major effector of glutamate excitotoxicity, we measured nuclear and cytosolic calcium levels. Consistent with our cell viability results, we observed that *Grm8*$^{-/-}$ neurons were more prone to glutamate induced calcium accumulation, an effect that could be rescued with AZ pre-treatment (cf. Fig. 3B–E, supplemental Fig. 2G–I)[71]. Notably, baseline cell viability and glutamate receptor expression were similar between *Grm8*$^{-/-}$ and WT neurons (cf. supplemental Fig. 2J–L)[71]. Subsequently, we aimed to identify the source of the glutamate-induced neuronal calcium elevations that is modulated by Grm8. Since Grm8 is an inhibitory GPCR we hypothesized that its activation negatively regulates calcium release from internal stores, especially the endoplasmic reticulum (ER). Thus, we prevented calcium release from the ER by emptying its calcium stores. This was achieved by either inhibiting calcium reuptake or by activating ryanodine receptors. Furthermore, we inhibited calcium release by blocking IP3-receptors and subsequently, applied glutamate. We observed that preventing calcium release from the ER strongly reduced glutamate-induced calcium increase in neurons. Accordingly, IP3R-inhibitors protected neurons from glutamate excitotoxicity (cf. Fig. 3F–G)[71].

To further elucidate the role of mGluR signalling in glutamate excitotoxicity, we established a protocol to specifically activate mGluRs and monitor the downstream calcium activation in neurons. This was dependent on IP3R-singalling since the IP3R-inhibitor 2-Aminoethoxydiphenylborane (2-APB) completely abolished the mGluR-dependent calcium activation. Notably, *Grm8*$^{-/-}$ showed enhanced mGluR-dependent calcium release from the ER that could be reversed by AZ treatment in WT neurons but not in *Grm8*$^{-/-}$ neurons underlining its specificity. Since Grm8 is an inhibitory GPCR we next analysed the role of the second messenger cAMP in mGluR-induced calcium release of the ER. Therefore, we stimulated neuronal cultures with forskolin which led to acute increase in cAMP levels. This resulted in enhanced vulnerability of neurons to glutamate challenge and exacerbated the mGluR-specific calcium response. Next, we used primary neurons of a FRET-based cAMP reporter mouse and observed that mGluR activation resulted in robust cAMP increase which was inhibited by Grm8 activation but not by 2-APB indicating that the cAMP signalling acts upstream of the IP3R activation. Notably, we found that the cAMP-dependent IP3R-sensitisation is independent of protein kinase A (PKA) activation implying direct regulation of IP3R sensitivity by cAMP. In conclusion, we found that Grm8 activation limits glutamate-induced calcium accumulation by inhibiting IP3R-dependent calcium release from the ER (cf. Fig. 4, supplemental Fig. 3)[71].

To translate our *in vitro* findings, we induced EAE, the mouse model of MS that strongly affects the motoneurons of the spinal cord. Murine motoneurons are a good model system since they show tight resemblance with human excitatory pyramidal neurons which we observed when performing transcriptional enrichment analysis of human neuronal cell types and motoneuron-specific transcriptomes of the spinal cord of mice. Thus, we conducted EAE with WT and *Grm8*$^{-/-}$ mice and found that Grm8 deficiency resulted in increased disability in EAE. Histopathologic analyses revealed less neuronal loss and less axonal damage in WT animals. Importantly, immune cell infiltration was similar as analysed by immunohistochemistry and flow cytometry. (cf. Fig. 5, supplemental Fig. 4)[71]. In a next step, we induced EAE in WT and *Grm8*$^{-/-}$ mice and treated them with AZ starting from the day of disease onset and induced EAE. Here we observed that AZ protects WT mice from clinical disability but not *Grm8*$^{-/-}$ mice underlining its specificity *in vivo*. Immunohistochemistry showed less neuronal loss but similar inflammatory activity indicating neuroprotective potential of pharmacological positive Grm8 modulation (cf. Fig. 6, supplemental Fig. 5A–I)[71].

Finally, we set out to investigate whether positive GRM8 modulation reduces glutamate excitotoxicity in hiPSC neurons. First, we analysed an established marker of glutamate excess: the phosphorylation of CREB at serine 133 (pCREB) in *post-mortem* tissue of MS patients and found robust neuronal upregulation in normal appearing grey matter and in lesions. This was also observed in hiPSC neurons that were exposed to glutamate and was inhibited by pre-treatment with the NMDAR-inhibitor APV, 2-ABP and AZ indicating that GRM8 activation and subsequent desensitization of IP3R counteract glutamate-induced pCREB upregulation in human neurons (cf. Fig. 7A–E, supplemental Fig. 5J–P)[71]. Notably, we did not observe differences in whole-cell patch-clamp recordings in vehicle and AZ pre-treated neurons that were exposed to glutamate indicating that GRM8 does not act by directly modulating the activity of ionotropic glutamate receptors (Fig. 7F)[71]. Last, we set out to translate our findings in the animal model and investigate whether positive GRM8 modulation acts neuroprotective in human neurons. Thus, we treated hiPSC neurons with vehicle and AZ and subsequently exposed them to cytokines and glutamate to mimic the inflammatory and excitotoxic environment in MS. Notably, AZ treatment reduced neurotoxicity (Fig. 7G, supplemental Fig. 5Q)[71], underlining the neuroprotective properties of GRM8 in human neurons and translatability of our findings.

5. Discussion

To date no MS therapy exists which directly prevents neuronal loss and the immunomodulatory and -suppressive drugs which are available cannot prevent secondary or primary progression sufficiently. One reason is that neuron-intrinsic (mal-)adaptations to an inflammatory and excitatory environment remain poorly understood[5]. Here, we investigated neuron-intrinsic mechanisms of neurodegeneration in MS and identified glutamate excitotoxicity as its major driver. Excessive extracellular levels of glutamate have been proposed to be involved in several primary and secondary neurodegenerative diseases such as PD, AD and Huntington's disease (HD)[32]. Sources of glutamate are the release of intracellular stores from dying cells, spillover from hyper-excitatory synapses as well as active secretion by immune and glia cells. Here, it is unclear, whether elevated levels of glutamate in the CNS of MS patients and their CSF occur as by-product of acute inflammatory activity or whether neurons are exposed to a sustained excitatory environment. We used pCREB as a marker of glutamate activation[33,73,74] and found it strongly induced in glutamate-exposed hiPSC neurons and in MS lesions suggesting prolonged exposure to excessive glutamate levels. Notably, pCREB levels were also increased in normal-appearing grey matter indicating that glutamate excitotoxicity promotes chronic neuronal stress independent of inflammatory lesions. This might be explained by a subtle global inflammation in the CNS or an imbalance of neuronal excitation and inhibition that is triggered by local inflammatory damages[75]. Intriguingly, the widespread neuronal stress response might explain brain atrophy and cognitive deficits that cannot be solely explained by the spatial pattern of lesions[76–78].

Mechanistically, *in vitro* studies in primary neuronal cultures and organotypic slice cultures have demonstrated that excessive extracellular levels of glutamate is toxic by activation of eNMDAR whereas stimulation of sNMDARs acts neuroprotective. eNMDAR activation results in ionic imbalance, the production of reactive oxygen species and mitochondrial damage by excessive cytosolic and nuclear calcium accumulation. Thus far, this has been mostly attributed to calcium influx through ionotropic glutamate receptors by activation of direct glutamate binding and subsequent activation of voltage-dependent calcium channels[79–81]. However, here we demonstrated that eNMDAR induced calcium accumulation strongly depends on IP3R-controlled calcium release from the ER. We found that inhibiting calcium release by blocking IP3R protected against glutamate excitotoxicity, whereas enhancing glutamate-induced calcium release from the ER by simultaneously activating ryanodine receptors was more deleterious for neurons. The ER is the main internal calcium store and extends throughout the entire neuron[82]. Accordingly, missense mutations of *Itpr1*, the gene encoding for IP3R1, have been found in patients with spinocerebellar ataxia and deletion of *Itpr1* in cerebellar neurons results in severe ataxia and synaptic loss. Moreover, IP3R-induced calcium release is promoting neurodegeneration in AD and HD, indicating the importance of ER calcium for neuronal health[83–85]. In our experiments IP3R-induced calcium release from the ER was strongly enhanced by elevation of cytosolic cAMP levels and could be reduced by decreasing cytosolic cAMP concentration. There are several possible explanations how cAMP might regulate IP3R dynamics: (I) cAMP-dependent activation of the proteinkinase A and subsequent phosphorylation of IP3R1 and IP3R2[86]. (II) Engaging to low-affinity cAMP binding sites that shift the cytosolic calcium-concentration dependent IP3R opening probability[87]. As we observed immediate increase of IP3R-dependent calcium release from the ER after pharmacologically stimulating cAMP production, and as this was independent of PKA inhibition, we suggest that our observed effects are mediated

by sensitisation of IP3Rs by direct cAMP binding. This mechanistic link has been shown in HEK cells and osteoblasts, although the binding site is currently unknown[87]. However, our data indicate that direct IP3R sensitisation by cAMP is a widespread mechanism throughout different cell-types that can be modulated by pharmacologically modifying GPCRs.

Thus, we focused on pharmacological activation of inhibitory GPCRs as neuroprotective strategy in MS to reduce glutamate-triggered calcium release from the ER. We investigated GRM8 since its downstream receptor interactome was upregulated in neurons of MS patients and GWAS studies found it significantly associated with brain volume loss and disease progression in MS[55]. Interestingly, we found that in MS mainly excitatory pyramidal neurons were affected by dysregulated receptor interactomes that were driven by glutamate excess. This may indicate selective neuronal vulnerability of excitatory pyramidal neurons to an excitotoxic and inflammatory environment. Histopathological studies that identified predominant loss of pyramidal layer V neurons in MS support this hypothesis[13,14]. In addition to identification of pathologic receptor signalling in neurons of MS patients, the novel neuron-specific receptor interactome is a powerful tool to study possible GPCR drug candidates in other diseases. For this purpose, we included transcriptional signatures of healthy and challenged neurons *in vitro* and *in vivo* as supposed to publicly available interactomes that only used transcriptomes of different brain regions of healthy mice. This is important since recent studies have demonstrated transcriptional responses in neurons that are specifically induced upon challenges to respective stressors. For example, we and others have shown that neurons exhibit type I and type II interferon responses in mouse models of neuroinflammation that are absent in steady-state neurons[18,88]. Thus, *in silico* analyses of other neurological diseases by leveraging our receptor interactome framework holds the promise to identify novel druggable master regulators of neurodegeneration.

To counteract the receptor disbalance and consecutive neuronal loss in MS, we focused on the inhibitory group III mGluR GRM8 as it was associated with disease severity and progression and as we found its downstream regulatory network – that was associated with neuroaxonal repair and synaptic plasticity – significantly upregulated in pyramidal neurons of MS patients. Studies have shown that inhibition of stimulatory mGluRs GRM1 and GRM5 did not affect disease course in EAE[46]. Moreover, the other inhibitory mGluRs GRM4 and GRM7 are highly expressed in non-neuronal cells whereas GRM6 is exclusively expressed in retinal ON-bipolar cells[52,89,90]. Thus, other mGluRs do not constitute attractive pharmacological targets to confer neuroprotection. For example, *Grm4*-deficiency primarily affects the activation of dendritic cells and subsequently, the differentiation of T_H17 cells[47]. In contrast, GRM8 is an attractive pharmacological target since it is mainly expressed in neurons, and it has been reported that its activation confers neuroprotection in neuronal cell lines against the neurotoxin $MPP4^+$, indicating broader neuroprotective properties of GRM8 signalling[91]. Additionally to MS, GRM8 has been associated with various psychiatric disorders such as major depression and schizophrenia[92,93] and behavioural studies revealed that *Grm8*-deficient mice suffer from higher levels of anxiety[50].

Our receptor interactome revealed that GRM8 is continuously activated in CNS inflammation that might be interpreted as endogenous countermeasure to adapt to a chronic inflammatory and excitotoxic environment. In accordance, *Grm8*-deficient neuronal cultures were more prone to glutamate excitotoxicity, whereas treatment with AZ, the positive allosteric modulator of GRM8 conferred neuroprotection in mouse and human neuronal cultures. Notably, this was translatable into the preclinical mouse model of MS since *Grm8*-deficient mice were more sensitive to inflammation-induced

neurodegeneration whereas daily intraperitoneal injections with AZ were neuroprotective. We chose to utilise a positive allosteric modulator because it is mechanistically advantageous in comparison to direct orthosteric agonists[40,94]. First, instead of directly stimulating GRM8, we increased the sensitivity of GRM8 activation and therefore, supported physiologic signalling. Second, PAMs allow for more specific drug designs because in contrast to orthosteric agonists they are directed against receptor-specific binding sites and not against the active zone. The latter requires high structural similarity to the ubiquitous natural ligand glutamate and consequently orthosteric agonists are more prone to unspecific binding. Therefore, unphysiological receptor activity as well as unspecific binding and subsequent adverse effects are minimized. Nevertheless, when considering GRM8 modulation as pharmacological strategy some potential adverse effects need to be taken into account. GRM8 is expressed in glutamatergic neurons of the enteric nervous system and enhances intestinal motility. Additionally, *Grm8*-deficient mice show mild weight gain and increased insulin resistance in comparison to wildtype mice[50,54]. Furthermore, we found *Grm8* to be expressed in plasmacytoid dendritic cells in mice. However, we did not observe differences in immune cell infiltration and activation in EAE when pharmacologically or genetically modifying GRM8 activation. Hence, our data indicate that GRM8 activation confers neuroprotection by specifically increasing neuronal resilience.

Mechanistically, it has been proposed that GRM8 signalling acts as a presynaptic negative feedback loop to limit glutamate transmitter release. The notion of presynaptic localisation is derived from electrophysiological recordings that identified GRM8-dependent pre-pulse inhibition and immunolabeling in the olfactory bulbs and lateral perforant pathway[49,52]. Nevertheless, its precise neuronal localisation was unknown. By expressing *Grm8* with an extracellular fluorescent Tag we visualised its surface localisation at pre-and post-synapses as well as at surfaces of neuronal somata. Although, overexpression might result in unphysiological localisations, the proximity to excitatory synapses might allow to closely control extracellular glutamate levels. Although, electrophysiological recordings in the *stria terminalis* have shown that Grm8 limits synaptic transmission[49], its exact mode of action and potential neuroprotective properties have remained unknown.

We found that GRM8 limits glutamate-induced calcium accumulation by desensitising IP3R open probabilities. This was mediated by inhibiting cAMP production which is in line with its proposed function as inhibitory GPCR. Notably, we did not observe interference with glutamate-induced currents, indicating that the neuroprotective properties did not result from modulating ionotropic NMDARs or AMPARs. In contrast, we identified that glutamate-induced ER calcium release is carefully balanced by mGluR signalling. Genetic deletion of GRM8 strongly enhanced mGluR-mediated calcium release from the ER that together with iGluR induced calcium influx eventually resulted in toxic cytosolic and nuclear calcium accumulation. On the other hand, increasing GRM8 activity selectively decreased glutamate-induced calcium release from the ER and thereby, conferred neuronal resilience. Our data show that the calcium release probability from the ER is a crucial constituent of neuronal integrity and is directly modulated by the druggable GRM8. The interplay between mGluR signalling and internal calcium stores emerges as crucial determinant of neuronal viability and is of interest for other neurodegenerative diseases.

In summary, this thesis identified GRM8 signalling as a neuroprotective negative feedback loop that counteracts glutamate-induced calcium accumulation. Mechanistically, GRM8 decreases cAMP production that desensitizes IP3Rs and thus, reduces calcium release from the ER. Notably, GRM8 activation conferred

neuroprotection in mouse and human neurons *in vitro* as well as in the preclinical mouse model of MS *in vivo* underlining the translatability of these findings. This commend GRM8 as novel and valuable therapeutic target to counteract inflammatory neurodegeneration.

6 Bibliography

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7 Synopsis

Introduction: Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) and the most common non-traumatic cause for neurological disability in young adults. Initially, patients suffer from inflammatory relapses with transient neurological deficits and recovery. During later disease stages, disability often accumulates which is apparent in progressive brain volume loss. Although, inflammation can be effectively halted, treating neurodegeneration is the major unmet clinical need and remains difficult due to a lack of knowledge about neuronal adaptations to inflammation. Chronic inflammation results in excessive extracellular glutamate that induces neuronal loss by calcium accumulation. Thus, glutamate levels are carefully balanced by ionotropic as well as stimulatory and inhibitory metabotropic glutamate receptors (GRM). The inhibitory GRM8 has been mostly investigated in psychiatric disorders. Its activation reduces electric transmission and protects cell lines from apoptosis. Notably, GRM8 has been associated with disease progression in MS. However, its role in neuroinflammation has been unclear.

Methods: We analysed the transcriptional signatures of neurons that were exposed to defined stressors and aligned them to transcriptomes of whole CNS tissues or isolated neuronal nuclei of MS patients by gene set enrichment analysis. We built receptor interactomes by reverse engineering by an algorithm for the reconstruction of gene regulatory networks (ARACNe) of 502 publicly available neuronal transcriptomes of disease models and challenged primary neurons. We pharmacologically enhanced Grm8 activation by applying the positive allosteric modulator AZ12216052 (AZ) and prevented by using *Grm8*$^{-/-}$ mice. We used murine primary cortical neurons and human induced pluripotent stem cells derived neurons (hiPSC-neurons) for *in vitro* studies. Calcium activity was measured by infecting cultures with the genetically encoded fluorescent calcium sensor Gcamp6f or by applying the calcium dye Fluo-4. cAMP levels were quantified in a transgenic mouse. We measured cell viability with luminescent based RealTimeGlo and CellTiterGlo assays. Immunofluorescent staining was used to visualise our GFP-*Grm8* construct. We daily treated WT and *Grm8*$^{-/-}$ mice with experimental autoimmune encephalomyelitis (EAE) with vehicle or AZ. Flow cytometry was used to analyse immune cell infiltration and immunohistochemistry was applied to quantify inflammatory lesions, neuroaxonal damage in EAE and pCREB in *post-mortem* CNS tissue of controls and MS patients.

Results: Transcriptional signatures of glutamate excitotoxicity were strongly enriched in neurons of MS patients. Dysregulated glutamate receptor interactomes in pyramidal neurons of MS patients confirmed this finding. Especially, the downstream network of GRM8 was upregulated. To analyse its localisation, we transfected neurons with GFP-*Grm8* and found it localised in proximity to synapses. Pharmacological activation of Grm8 by AZ protected neurons from glutamate excitotoxicity by inhibiting cytosolic and nuclear calcium accumulation, whereas *Grm8*-deficient neurons were more prone to glutamate. Grm8 reduced cytosolic cAMP levels and thus, limited glutamate induced calcium release from the ER that depend on IP3R activity. Accordingly, *Grm8*$^{-/-}$ mice were more prone to neuronal loss in EAE that was limited by treatment with AZ in WT but not *Grm8*$^{-/-}$ mice underlining its specificity. Notably, immune cell infiltration and activation were not modulated by Grm8. Finally, we identified pCREB upregulation as maker of prolonged glutamate exposure in *post-mortem* MS brains and counteracted glutamate-induced pCREB upregulation and toxicity in hiPSC-neurons by AZ.

Outlook: This thesis (i) shows neuroprotective properties of GRM8 in inflammatory neurodegeneration, (ii) dissects its underlying molecular pathways and (iii) provides a neuronal receptor interactome. I commend that prospective studies should further develop strategies to enhance GRM8 activity to induced neuronal resilience in MS.

Einleitung: Multiple Sklerose (MS) ist eine entzündliche Erkrankung des zentralen Nervensystems (ZNS) und die häufigste nicht-traumatische Ursache für neurologische Behinderung im jungen Erwachsenenalter. Betroffene leiden in der Regel unter einem schubförmigen Verlauf mit transienter neurologischer Symptomatik gefolgt von Remission. Im Verlauf kommt es häufig zu einer zunehmenden Behinderung und Hirnvolumenverlust. Trotz Behandlung der Entzündung kann Neurodegeneration wegen fehlender Kenntnisse über neuronale Stress-Antworten nicht aufgehalten werden. Vermehrte Glutamat-Exposition induziert Nervenzellverluste durch Calcium-Akkumulation. Daher werden Glutamat-Konzentrationen streng durch ionotrope und metabotrope Glutamat-Rezeptoren (GRM) reguliert. Der inhibitorische GRM8 wurde bisher in psychiatrischen Erkrankungen untersucht, da dessen Aktivierung neuronale Aktivität moduliert. Zudem sind genetische Varianten des *GRM8* Gens mit der MS Progression assoziiert. Die Rolle für neuronale Entzündung blieb jedoch bisher unklar.
Methoden: Wir analysierten transkriptionelle Signaturen von Nervenzellen, die definierten Stressoren ausgesetzt waren und verglichen diese mit den Transkripten von Gehirnen oder neuronalen Zellkernen von MS-Patient:innen durch eine „Gene set enrichment“ Analyse. Rezeptorinteraktome wurden durch reverses Engineering mithilfe des ARACNe Algorithmus aus 502 Neuronen-spezifischen Datensätzen von Mauskrankheitsmodellen und gestressten Neuronen erzeugt. Wir aktivierten Grm8 durch den positiven allosterischen Modulator AZ12216052 (AZ) und dessen Inhibition erfolgte durch eine transgene Knockout Maus. Für *in vitro* Studien wurden primäre Neuronen aus Mäusen und humane Neurone, die aus induzierten pluripotenten Stammzellen differenziert wurden, verwendet. Calciumaktivität wurde durch den genetisch kodierten Calcium Sensor Gcamp6f oder durch den calciumbindenden Farbstoff Fluo-4 gemessen. cAMP wurde mithilfe einer transgenen Mauslinie gemessen. Zellviabilität wurde mit den Assays RealTimeGlo und CellTiterGlo bestimmt. Immunofluoreszenz-Färbungen wurden angefertigt, um unser GFP-*Grm8* Konstrukt zu visualisieren. WT und *Grm8*$^{-/-}$ Mäuse mit experimenteller autoimmuner Enzephalomyelitis (EAE) wurden täglich mit Vehikel oder AZ behandelt. Durchflusszytometrie wurde verwendet, um Immunzellinfiltration zu quantifizieren und durch Immunohistochemie wurden entzündliche Läsionen und neuronaler Schaden in EAE und pCREB in *post-mortem* Gewebe von Kontrollen und MS gemessen.
Ergebnisse: Die transkriptionellen Signaturen von Glutamat-Toxizität waren in Neuronen von MS-Patient:innen überrepräsentiert. Dies zeigte sich auch in den dysregulierten Glutamat-Rezeptor-Interaktomen in deren pyramidalen Neuronen. Vor allem das GRM8-Netzwerk war hochreguliert. In transfizierten Neuronen mit einem GFP-*Grm8* Konstrukt konnten wir Grm8 in enger Nähe zu Synapsen darstellen. Grm8-Aktivierung durch AZ schützte Neurone vor Glutamat-Toxizität durch Reduktion der Calcium-Akkumulation, wohingegen *Grm8*$^{-/-}$ Neurone anfälliger für Glutamat-Stress waren. Grm8 reduzierte cAMP Konzentrationen und limitierte dadurch Glutamat-induzierten IP3R-abhängige Calcium-Einstrom aus dem ER. *Grm8*$^{-/-}$ Mäuse zeigten einen vermehrten Verlust von Nervenzellen in EAE, welcher durch Behandlung mit AZ in WT, aber nicht in *Grm8*$^{-/-}$ Mäusen verringert werden konnte, was dessen Spezifität unterstreicht. Infiltration von Immunzellen und deren Aktivierung wurde nicht von Grm8 moduliert. Zudem identifizierten wir pCREB als Marker für chronischen Glutamat-Stress in *post-mortem* MS-Gehirnen und konnten Glutamat-induzierte pCREB-Hochregulation und -Toxizität durch AZ-Behandlung in hiPSC-Neuronen vermindern.
Outlook: Diese Arbeit belegt (i) das neuroprotektive Potenzial von GRM8 in entzündlicher Neurodegeneration, (ii) dessen molekulares Netzwerk und (iii) erstellt ein Neuronen-spezifisches Rezeptor-Interaktom. Zukünftige Studien sollten pharmakologische GRM8-Aktivierung zur Neuroprotektion in MS weiterentwickeln.

8 Declaration of personal contribution

The following section will demonstrate my contributions towards the research paper “Neuronal metabotropic glutamate receptor 8 protects against neurodegeneration in CNS inflammation**”** published in the journal “The Journal of Experimental Medicine” (Woo et al.**,** 2021):

Most experiments were performed by me alone or with the aid of one of the co-authors, including mouse experiments, live cell imaging, immunohistochemistry, immunocytochemistry, flow cytometric analysis, quantitative real-time PCR, generation of overexpression constructs and cell viability assays. RNAScope *in situ* hybridization and immunohistochemistry of *post-mortem* MS tissue were performed by Giovanni Di Liberto, Kristof Egervari, Ingrid Wagner and Doron Merkler (all University of Geneva). Electrophysiological patch-clamp recordings were performed by Sönke Hornig (University Medical Centre Hamburg-Eppendorf). hiPSC-neurons were provided by Ole Pless and Undine Haferkamp (Frauenhofer Institute for Translational Medicine and Pharmacology). Grm8$^{-/-}$ mice were provided by J. Raber and R.M. Duvoisin. Data and bioinformatic analysis were done by me. The study was jointly designed by Manuel A. Friese, Friederike Ufer and me. Friederike Ufer helped with organisation of EAE experiments. Most *in vitro* experiments were designed and planned by me. The publication was jointly written by Manuel A. Friese and me, with contributions and corrections of all co-authors. All figures were created by me.

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10 Curriculum vitae

Der Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

11. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....

Marcel Seungsu Woo