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Enhanced GABAergic tonic inhibition reduces intrinsic excitability of hippocampal CA1 pyramidal cells in experimental autoimmune encephalomyelitis

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Abstract

Cognitive impairment (CI), a debilitating and pervasive feature of multiple sclerosis (MS), is correlated with hippocampal atrophy. Findings from postmortem MS hippocampi indicate that expression of genes involved in both excitatory and inhibitory neurotransmission are altered in MS, and although deficits in excitatory neurotransmission have been reported in the MS model experimental autoimmune encephalomyelitis (EAE), the functional consequence of altered inhibitory neurotransmission remains poorly understood. In this study, we used electrophysiological and biochemical techniques to examine inhibitory neurotransmission in the CA1 region of the hippocampus in EAE. We find that tonic, GABAergic inhibition is enhanced in CA1 pyramidal cells from EAE mice. Although plasma membrane expression of the GABA transporter GAT-3 was decreased in the EAE hippocampus, an increased surface expression of $\alpha 5$ subunit-containing $GABA_A$ receptors appears to be primarily responsible for the increase in tonic inhibition during EAE. Enhanced tonic inhibition during EAE was associated with decreased CA1 pyramidal cell excitability and inhibition of $\alpha 5$ subunit-containing $GABA_A$ receptors with the negative allosteric modulator L-655,708 enhanced pyramidal cell excitability in EAE mice. Together, our results suggest that altered GABAergic neurotransmission may underlie deficits in hippocampal-dependent cognitive function in EAE and MS.

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Introduction

Cognitive impairment (CI) is a debilitating feature of multiple sclerosis (MS) that presents in both relapsing remitting and progressive MS subtypes (Ruano et al., 2016), and is associated with reduced quality of life due to impacts on employment and social engagement (Rao et al., 1991). Brain magnetic resonance imaging studies show atrophy within the hippocampus, disproportionately affecting the CA1 region (Sicotte et al., 2008). Importantly, both total and regional hippocampal atrophy (Sicotte et al., 2008, Longoni et al., 2015) are correlated with impairments in visuospatial/verbal learning, one of the primary cognitive domains affected by MS (Litvan et al., 1988, Denney et al., 2005, Ruano et al., 2016, for reviews see Chiaravalloti and DeLuca 2008, Guimaraes and Sa 2012). Therefore, the vulnerability of the hippocampus and CA1 subregion to pathologic processes during MS may contribute to the clinical presentation of CI.

Although the molecular mechanisms underlying hippocampal dysfunction in MS are unclear, findings from postmortem MS hippocampi indicate a downregulation of genes involved in excitatory synaptic transmission, including NMDA receptor (NMDAR) and AMPA receptor subunits, but an upregulation of genes involved in inhibitory synaptic transmission, including $\alpha5$, $\beta3$, and $\gamma2$ subunits of $GABA_A$ receptors (Dutta et al., 2011). Consistent with these findings, alterations in both excitatory (Centonze et al., 2009, Ziehn et al., 2012, Grasselli et al., 2013, Mandolesi et al., 2013) and phasic forms of inhibitory synaptic transmission (Rossi et al., 2011, Mandolesi et al., 2012, Nistico et al., 2013), have been reported in the MS model experimental autoimmune encephalomyelitis (EAE). Interestingly, $\alpha5/\beta3/\gamma2$ subunit-containing $GABA_A$ receptors give rise to an extrasynaptic pool of $GABA_A$ receptors that mediate a distinct form of inhibitory transmission, called tonic inhibition, in hippocampal CA1 pyramidal cells (Caraiscos et al., 2004; Zarnowska et al., 2015). Although studies of inhibitory synaptic dysfunction in MS have focused on alterations in phasic forms of inhibition (Mandolesi et al., 2015), changes in $GABA_A$ receptor subunit expression that occur in MS suggest that tonic forms of inhibition might be altered as well. By regulating neuronal excitability, tonic inhibition not only influences neuronal information processing (Mitchell and Silver 2003, Chadderton et al., 2004, Pavlov et al., 2009), but also opposes the induction of activity-dependent forms of synaptic plasticity involved in memory formation, such as long-term potentiation (LTP) (Martin et al., 2010, Whissell et al., 2013). Furthermore, pharmacological inhibition of $\alpha5$ subunit-containing $GABA_ARs$ ($\alpha5$-$GABA_ARs$) improves performance on hippocampus-dependent learning tasks (Atack et al., 2006; Etherington et al., 2017), and $\alpha5$ subunit null mutant mice exhibit enhanced spatial learning (Collinson et al., 2002). Therefore, an increase in tonic inhibition might contribute to deficits in LTP (Kim et al., 2012; Di Filippo et al., 2013; Novkovic et al., 2015; Di Filippo et al., 2016; Planche et al., 2016), as well as learning and

memory (Ziehn et al., 2010; Acharjee et al., 2013; Novkovic et al., 2015; Di Filippo et al., 2016), observed during EAE.

To determine whether alterations in GABAergic inhibition might contribute to disruption of hippocampal function in MS, we examined phasic and tonic inhibition in EAE. We find that both forms of inhibition are significantly enhanced in hippocampal CA1 pyramidal cells during EAE. We then examined how changes in plasma membrane levels of $\alpha5$-$GABA_A$Rs and GABA transporters might contribute to enhanced tonic inhibition in EAE and explored the functional effects of enhanced tonic inhibition on pyramidal cell intrinsic excitability and LTP induction.

Experimental Procedures

All experimental protocols used in this study were approved by UCLA's Chancellor's Animal Research Committee and are consistent with PHS Policy on Humane Care and Use of Laboratory Animals.

Active EAE induction

Active EAE was induced in C57BL/6 mice (Jackson labs) using myelin oligodendrocyte glycoprotein (MOG) amino acids 35–55 peptide (MOG35–55), as described (Itoh et al., 2018). Briefly, female mice at 8–15 weeks of age underwent subcutaneous injection of MOG35–55 (200 μg/animal, Mimotopes) emulsified in Complete Freund's Adjuvant, supplemented with Mycobacterium Tuberculosis H37Ra (200 μg/animal, Difco Laboratories), over two sites drained by left inguinal and auxiliary lymph nodes on day 0 (0.1 ml/mouse). A booster immunization was applied subcutaneously on day 7 over contralateral lymph nodes. Pertussis toxin (500 ng/mouse) (List Biological Laboratories) was injected intraperitoneally on days 0 and 2. Animals were monitored daily and scored based on a standard EAE 0–5 scale scoring system: 0, healthy; 1, complete loss of tail tonicity; 2, loss of righting reflex; 3, partial paralysis; 4, complete paralysis of one or both hind limbs; and 5, premoribund state. Whole-cell voltage-clamp recordings of phasic and tonic inhibitory transmission were performed 21–42 days post-induction (DPI). The induction of LTP by theta pulse stimulation was examined at both early (16–29 DPI) and later (starting at 40 DPI) time points post EAE induction. Whole-cell current-clamp recordings were used to examine CA1 pyramidal cell intrinsic excitability starting at 42 DPI and tissues for western blotting were collected at 40 DPI.

Inhibitory Synaptic Transmission:

Mice were deeply anesthetized with isofluorane, decapitated, and the brain was rapidly removed and placed into ice-cold, oxygenated (bubbled with 100% O_2) cutting solution containing 135 mM N-methyl-D-glucamine, 10 mM D-glucose, 4.0 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.0 mM KCl, 1.2 mM KH_2PO_4, and 26 mM HEPES (pH = 7.4). Coronal brain slices (320 μm thick) containing the dorsal hippocampus and striatum were cut using a Campden 7000SMZ-2 Vibratome. Slices were then maintained in an interface-slice type chamber perfused at 2–3 ml/min with a warm (30 °C), oxygenated (95% 02/5% CO_2) artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 4.4 mM KCl, 25 mM $NaHC0_3$, 1.0

mM NaH$_2$P04, 2.0 mM CaCl$_2$, 1.2 mM MgS04, and 10 mM glucose (pH = 7.4). Slices were allowed to recover for at least 1 hour prior to electrophysiological recordings. For each experiment, slices were transferred to a submerged-slice type recording chamber (Warner Instruments) perfused with warm (30 °C), oxygenated ACSF and individual cells were visualized using infrared differential interference contrast microscopy (Zeiss Axio Examiner.D1 fitted with 40x water-immersion objective) and a CMOS camera (QImaging Rolera Bolt). Low-resistance electrodes (3–6 MΩ) filled with an internal solution containing 120 mM CsMeSO$_4$, 10 mM CsCl, 5 mM TEA-Cl, 1.5 mM MgCl$_2$, 10 mM HEPES, 0.1 mM EGTA, 2 mM Na-ATP, 0.5 mM Na-GTP, and 5 mM QX-314 (pH = 7.3, osmolarity = 275–285 mOsm) were used to record spontaneous inhibitory postsynaptic currents (sIPSCs) in CA1 pyramidal cells or medium spiny neurons in the striatum. Whole-cell voltage-clamp recordings were performed using a Multiclamp 700B amplifier and pClamp 10 (Molecular Devices) and series resistance and whole-cell capacitance were automatically compensated. Recordings were discontinued if series resistance increased by >20%. Pyramidal cells were voltage- clamped at the reversal potential for excitatory postsynaptic currents (+10 mV) to record sIPSCs. Bath application of 100 μM SR-95531 (Caymen Chemical) blocked sIPSCs, confirming that inhibitory synaptic currents were adequately isolated using this approach. Data analysis was performed using Clampfit (Molecular Devices). The EVAN LabView-based program developed by the Moody laboratory at UCLA was used for the detection, and measurement of peak amplitudes, 10–90% rise times, weighted decay time constants, and frequency of sIPSCs.

Tonic inhibition was measured by the change in the holding current needed to voltage-clamp cells at +10 mV induced by bath application of 100 μM SR-95531. In our initial experiments examining tonic inhibition in CA1 pyramidal cells (Fig 2A,B), slices were continuously bathed in ACSF containing 5.0 μM GABA to enhance tonic inhibitory currents. All other experiments were performed without supplemental GABA in the ACSF. In experiments examining the effects of the GABA$_A$R modulators L-655,708 and DS2 (Tocris) or the GABA transporter inhibitor nipecotic acid (Tocris) on tonic inhibition, drugs were bath applied until changes in holding current needed to clamp cells at +10 mV reached steady state and SR-95531 was then bath applied. The current needed to clamp cells in the presence of SR-95531 was then subtracted from the amplitude of the holding current before and after application of L-655,708, DS2, or nipecotic acid to determine drug effects on tonic inhibition.

Intrinsic Excitability:

Intrinsic excitability was examined using 400 μm thick dorsal hippocampal slices prepared using previously described techniques (Babiec et al., 2017) and maintained at 30 °C in interface-type chambers perfused with ACSF at 2–3 mL/min. Slices were transferred to a submerged-slice recording chamber perfused with ACSF and whole-cell current-clamp recordings from CA1 pyramidal cells were performed using patch electrodes (4–8 MΩ) containing 122.5 mM K-gluconate, 17.5 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 10 mM Na$_2$-phosphocreatine, 4 mM Mg-ATP, 0.3 mM Na-GTP (pH = 7.3, 290 mOsm). The number of action potentials elicited by 500 millisecond depolarizing current pulses (50 – 150 pA) was used to determine neuronal excitability and the peak change in membrane potential

elicited by a 500 millisecond long hyperpolarizing current pulse (−100 pA) was used to measure membrane input resistance. Reported membrane potentials have not been corrected for an estimated liquid junction potential of 13.4 mV.

LTP Induction Protocol:

Standard extracellular recording techniques (Babiec et al., 2017) were used to examine LTP in slices maintained in interface-slice recording chambers. In these experiments, a glass microelectrode filled with ACSF (resistance = 5 – 10 MΩ) was placed in stratum radiatum of the CA1 region to record field excitatory postsynaptic potentials (fEPSPs) evoked by single pulses of presynaptic fiber stimulation (0.2 msec in duration, stimulation rate = 0.02 Hz) delivered by a bipolar stimulating electrode placed in stratum radiatum. At the start of each experiment, the intensity of presynaptic fiber stimulation was varied to determine the maximum fEPSP amplitude and the stimulation strength was then set to evoke fEPSPs that were 50% of maximum. After a stable baseline recording of 20 minutes, LTP was induced by a 150 pulse train of theta- frequency stimulation (single stimulation pulses delivered at 5 Hz). fEPSPs were recorded for 45 minutes post-LTP induction. fEPSP slopes were normalized to the pre-5 Hz stimulation baseline and fEPSP slopes averaged over the final 5 minutes were used for statistical comparisons.

Plasma membrane protein isolation:

Dorsal portions of the hippocampus were dissected free from rest of the brain and immediately frozen on dry ice. Frozen hippocampi were pulverized using a mechanical tissue grinder, and further processed for either plasma membrane or whole lysate fractions. Plasma membrane fractions were prepared using a commercially available plasma membrane protein extraction kit (Abcam, ab65400) according to manufacturer's instructions. Briefly, pulverized tissue was resuspended in homogenization buffer containing 1:500 protease inhibitor cocktail, and homogenized using a glass Dounce homogenizer. Homogenates were centrifuged for 10 min at 700 rcf to remove debris and the nuclear fraction. The supernatants were then centrifuged for 30 min at 10,000 rcf, to separate the cytosol fraction from total cellular membrane protein. The pellets from this spin were resuspended in upper phase and lower phase solutions provided in the kit. The upper phase, containing plasma membrane proteins, was purified after three, 5 min centrifugation steps at 1000 rcf. The purified upper phase was then diluted in water (5x), and centrifuged for 10 min at 13,500 rpm. The resulting pellet was resuspended in modified RIPA (mRIPA) buffer, containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 10 mM EGTA, 10 mM EDTA, 25 mM sodium pyrophosphate, 10 μM cantharidin, and cocktails of both protease (Sigma- Aldrich) and protein phosphatase inhibitors (Phosphatase Inhibitor Cocktails 2 and 3 from Sigma-Aldrich). For whole lysate protein isolation, mechanically pulverized hippocampi were resuspended in mRIPA buffer and briefly homogenized with a handheld sonicator. Protein concentrations were determined by Bradford assay.

Western Blotting:

Whole hippocampal lysates and plasma membrane protein fractions were diluted in 2X loading buffer (National Diagnostics) and denatured by boiling for 5 minutes. Samples containing 10 μg of protein were separated by SDS-PAGE, using 4% and 12% acrylamide

stacking and resolving gels, respectively. Proteins were transferred onto a 0.2 μm nitrocellulose membrane and blocked for 1 hour at room temperature in 4% nonfat dry milk dissolved in Tris-buffered saline containing 0.05% Tween-20 (TBST). Nitrocellulose membranes were incubated overnight at 4°C in milk/TBST containing the antibodies: anti-α5-GABA$_A$R (Aviva Systems Biology, RRID:AB_2046033), anti-GAT3 (Abcam, RRID:AB_304437), anti-GAT1 (Abcam, RRID:AB_2189971), anti-Flotillin-1 (Santa Cruz Biotechnology, RRID:AB_2106567), and anti-GSTπ (Enzo Life Science, RRID:AB_2039146). Blots were then washed three times in TBST and incubated for 3 hours at room-temperature in milk/TSBT containing HRP-conjugated secondary antibodies (1:2000, GE Healthcare catalog no. NA934V, NA931V). To image blots, membranes were immersed in ProtoGlow ECL (National Diagnostics), and immunoreactive bands were detected using a 12-bit cooled CCD camera and Image Lab software (Bio-Rad). Densitometry analysis was performed using ImageJ (NIH). For quantification, density values from α5-GABA$_A$R, GAT1, and GAT3 bands were normalized to values for either the membrane-associated protein flotillin-1, for plasma membrane fractions, or βIII tubulin (Millipore, RRID:AB_309804), for whole lysate proteins, to control for loading variations. Levels of α5-GABA$_A$R and GAT1/3 were expressed as a percentage of control. To estimate molecular weights, MagicMark XP (Thermo Fisher Scientific) standard lanes were imaged together with sample lanes. To confirm enrichment of plasma membrane proteins, dorsal hippocampal lysates containing total protein from controls were run together with plasma membrane fractions during Western immunoblotting. In addition, immunoblots were probed with a glutathione S-transferase π (GSTπ) antibody to confirm the absence of significant cytosolic protein contamination in plasma membrane fractions.

Statistical Analyses

All values are reported as mean ± SEM. Student's t tests as well as two-way and three-way ANOVAs (followed by Bonferroni t-tests for specific comparisons) were used to determine statistical significance. All statistical analyses were performed using Prism 6 (GraphPad Software) or SigmaPlot (SPSS).

Results

Phasic inhibitory synaptic transmission in CA1 pyramidal cells is enhanced in EAE

Previous studies have found that the frequency of sIPSCs in striatal medium spiny neurons (Rossi et al., 2011), cerebellar Purkinje cells (Mandolesi et al., 2012), and hippocampal CA1 pyramidal cells (Nistico et al., 2013) is significantly reduced during early stages of EAE (20–25 DPI). Thus, to determine how inhibitory transmission in hippocampal CA1 region is altered at later stages of EAE, we first examined sIPSCs in CA1 pyramidal cells from healthy, age-matched control mice and EAE mice 21–42 days post-induction (Fig. 1A,B). Strikingly, at these time points post-EAE induction, sIPSC frequency was significantly higher in CA1 pyramidal cells from EAE mice (Fig. 1B,C) There was no difference, however, in the amplitude, rise time, or decay time course of sIPSCs in cells from EAE and control mice (Fig. 1C). Although our findings, along with those of Nistico et al (2013), suggest that phasic inhibition in the hippocampal CA1 region undergoes distinct changes during EAE, phasic inhibitory synaptic transmission in striatal medium spiny neurons is

significantly reduced at both early (20 DPI) and later stages of EAE (up to 90 DPI) (Rossi et al., 2011). Thus, the increase in sIPSC frequency in CA1 pyramidal cells during EAE suggested that there might be regional differences in how inhibitory synaptic transmission is altered in EAE. To examine this possibility we next recorded sIPSCs in striatal medium spiny neurons from both EAE (21–42 DPI) and control mice. Consistent with previous findings (Rossi et al., 2011), the frequency of sIPSCs was significantly reduced in medium spiny neurons from EAE mice (Fig 1D,E). Together, these results suggest that there are significant time and brain region-dependent effects of EAE on phasic inhibitory synaptic transmission.

Increased surface expression of α5-GABA_ARs contributes to enhanced tonic inhibition in EAE

To examine whether tonic inhibition is altered during EAE, we measured the decrease in the steady-state current needed to voltage-clamp CA1 pyramidal cells at +10 mV induced by the GABA_A receptor antagonist SR-93551 (gabazine) (Fig. 2A). As shown in figure 2B, tonic inhibition in cells from EAE mice was approximately 2-fold larger compared to control cells. Tonic inhibition in CA1 pyramidal cells is primarily mediated by α5 subunit-containing receptors (Caraiscos et al., 2004; Zarnowska et al., 2015). Thus, to determine whether differences in the expression of extrasynaptic GABA_ARs might contribute to the enhanced tonic inhibition in EAE, we measured plasma membrane levels of α5 subunits in hippocampal samples from control and EAE mice. Interestingly, plasma membrane levels of α5 subunits were significantly increased in EAE compared to normal (Fig 2C,D). In a separate series of experiments, we found that total levels of GABA_AR α5 subunit expression were also significantly elevated in hippocampal lysates prepared from EAE mice (levels in samples from EAE mice were $131 \pm 11\%$ of control, n = 5 EAE and 6 control mice, t(9) = 2.39, p = 0.04). This suggests that that the increased plasma membrane levels of α5 subunits is due, at least in part, to increased total levels of α5 subunit protein. To test whether enhanced α5- GABA_ARs expression is responsible for increased tonic inhibition during EAE, we examined the effects of L-655,708, an inverse-agonist of the α5 subunit-containing GABA_ARs (Quirk et al., 1996) on tonic inhibition in CA1 pyramidal cells from EAE and control mice. Following bath application of L-655,708 (5 μM) tonic inhibition was reduced to a greater extent in cells from EAE mice than from control mice (Fig 2E). Moreover, in the presence of L-655,708, tonic inhibition in cells from EAE mice was not significantly different from control cells (t(26) = 1.792, p = 0.08). Because δ subunit- containing GABA_ARs also contribute to tonic inhibition in CA1 neurons (Scimemi et al., 2005), we examined the effects of DS2, a positive allosteric modulator of 5 subunit- containing GABA_ARs (Wafford et al., 2009) on tonic inhibition in EAE and control cells. The increase in tonic inhibition produced by bath application of DS2 (2 μM) was the same in control cells ($26.1 \pm 3.8\%$, n = 14 cells from 3 mice) and cells from EAE mice ($22.5 \pm 4.0\%$, n = 11 cells from 3 mice, t(23) = 0.653, p = 0.52). Together, these results indicate that an increase in plasma membrane expression of α5 subunit-containing GABA_ARs has an important role in enhanced tonic inhibition during EAE.

Plasma membrane expression of the GABA transporter GAT-3 is reduced in EAE

The GABA transporters GAT-1 and GAT-3 are the primary neuronal and astrocytic GABA transporters, in hippocampal pyramidal cells (Chembrowski et al., 2016) and astrocytes (Chai et al., 2017). Moreover, both of these transporters are thought to have important roles in regulating tonic inhibition by controlling extracellular levels of GABA (Nusser and Mody, 2002; Jensen et al., 2003; Semyanov et al., 2003; Kersante et al., 2013). In contrast, the GAT-2 GABA transporter is not expressed in hippocampal pyramidal cells or astrocytes (Durkin et al., 1995; Evans et al., 1996; Cembrowski et al., 2016; Chai et al., 2017). Thus, to investigate whether deficits in GABA uptake might also contribute to enhanced tonic inhibition during EAE, we examined whether the expression of GAT-1 and/or GAT-3 is altered during EAE. Both plasma membrane (Fig. 3A) and total GAT-1 levels in whole hippocampal lysates from EAE mice were not significantly different from controls (total levels in lysates from EAE mice were $129 \pm 14.3\%$ of control, n = 6 EAE and 6 control mice, t(10) = 1.437, p = 0.181). In contrast, GAT-3 plasma membrane expression was significantly decreased in the EAE hippocampus (Fig 3B). There was no difference, however, in total levels of GAT-3 in lysates from EAE and control mice (levels were 96 $\pm$ 6.7% of control in EAE lysates, n = 6 EAE and 6 control mice, t(10) = 0.371, p = 0.718). This suggests that the decrease in plasma membrane GAT-3 levels during EAE is due to an alteration in GAT-3 membrane trafficking. To address the functional significance of decreased GAT-3 membrane expression during EAE, we next determined whether the increase in tonic inhibition induced by GAT-1 and GAT-3 inhibitor nipecotic acid (Pandit et al., 2013) was blunted in CA1 pyramidal cells from EAE mice (Fig 3C). As shown in figure 3D, bath application of nipecotic acid (10 μM) induced a robust increase in tonic inhibition in both control and EAE cells. Though this finding does not rule out a potential role for deficits in GABA transporter function in the enhancement of tonic inhibition in EAE, it does suggest that an increase in α5 subunit-containing $GABA_AR$ is likely to have a more important role.

Decreased CA1 pyramidal cell excitability and LTP deficits in EAE

Tonic inhibition acts as a form of shunting inhibition that can potently reduce neuronal excitability. Thus, to begin to explore the impact of enhanced tonic inhibition in EAE on hippocampal function we first examined whether intrinsic excitability of CA1 pyramidal cells is reduced in EAE. Although resting membrane potentials in cells from control and EAE mice were similar (Fig. 4A), membrane input resistance was significantly lower in cells from EAE mice (Fig. 4B). Consistent with previous findings (Novkovic et al., 2015), there was also a robust, right-shift in action potential firing-to- current injection curves in cells from EAE mice, indicating a significant decrease in intrinsic excitability (Fig. 4C,D). To determine whether the decrease in pyramidal cell excitability in EAE is due to enhanced tonic inhibition, we next examined resting membrane potential, input resistance, and excitability in cells continuously bathed in ACSF containing 5.0 μM L-655,708. Although L-655,708 had no effect on resting membrane potential (Fig. 4E), membrane input resistance in cells from control and EAE mice were no longer significantly different (Fig. 4F). Moreover, L-655,708 significantly enhanced depolarization-induced action potential firing in cells from EAE mice but had no effect on excitability in cells from control mice (Fig

4.G,H), indicating that the decrease in CA1 pyramidal cell excitability in EAE is due to enhanced tonic inhibition.

Because enhanced inhibition and decreased excitability can oppose the postsynaptic depolarization needed for activation of NMDARs and LTP induction, we next examined whether enhanced tonic inhibition is responsible for LTP deficits seen in EAE. Compared to slices obtained from healthy controls, the induction of LTP by a brief train of theta-pulse stimulation (150 pulses at 5 Hz) was significantly reduced in slices obtained from EAE mice at both early and later time points post-EAE induction (16–29 and > 40 days, respectively) (Fig 5A,B). However, reducing tonic inhibition with L- 655,708 failed to enhance LTP induction in slices obtained from mice >40 day post-EAE induction (Fig. 5B), a time point post-EAE induction where L-655,708 strongly enhances CA1 pyramidal cell excitability (Fig. 4G,H). L-655,708 also had no effect on theta-pulse stimulation-induced LTP induction in slices obtained from healthy control mice (in the presence of L-655,708, fEPSPs in were potentiated to $143 \pm 7\%$ of baseline, n = 7 slices from 5 mice, t(16) = 0.595, p = 0.56 compared to vehicle controls). Thus, the LTP deficits that occur during EAE are not simply due to changes in tonic inhibition but instead likely involve additional pathophysiological changes in excitatory synaptic function.

DISCUSSION

In this study, we assessed the role of GABAergic transmission in modifying hippocampal function in EAE. We found that CA1 pyramidal cells exhibit both enhanced phasic and tonic currents during EAE. Although decreased surface expression of the GAT-3 GABA transporter may contribute, our results indicate that enhanced tonic inhibition during EAE is mediated primarily by increased surface expression of $\alpha 5$- GABA$_A$Rs. Importantly, tonic inhibition regulates neuronal excitability and thus can strongly influence information transmission through neural circuits (Semyanov et al., 2004). For example, in the cerebellar cortex, GABA$_A$-mediated tonic inhibition is the main inhibitory influence regulating the transfer of information at mossy fiber synapses onto granule cells, and is thought to have a crucial role in the sparse coding of sensory information in the cerebellar cortex (Hamann et al., 2002, Chadderton et al., 2004). Tonic inhibition also regulates the excitability of hippocampal pyramidal cells (Glykys and Mody, 2006, Bonin et al., 2007, Pavlov et al., 2009, Groen et al., 2014). Thus, alterations in network activity and information processing due to increased tonic inhibition and decreased neuronal excitability may contribute to cognitive disability in MS.

Although our study is the first to identify changes in tonic inhibition in EAE, previous studies have found that phasic inhibitory synaptic transmission is also altered in EAE (Rossi et al., 2011, Mandolesi et al., 2012, Nistico et al., 2013). Interestingly, although sIPSPC frequency in CA1 pyramidal cells is reduced relatively soon after EAE induction (Nisticò et al., 2013), we find that the frequency of sIPSCs is enhanced at later time points post-EAE induction. Although differences in EAE induction protocols might contribute to this difference, our results, along with those of Nisticò et al. (2013), suggest that phasic inhibitory synaptic transmission in the hippocampus undergoes distinct, time-dependent changes during the course of EAE. In contrast to the changes in phasic inhibitory synaptic

transmission that occur in hippocampal CA1 pyramidal cells during EAE, sIPSC frequency is strongly reduced in striatal medium spiny neurons at both early and later time points post-EAE induction (Rossi et al., 2011). Thus, EAE may lead to brain region-specific alterations in phasic inhibition, perhaps due to region and/or cell type-specific effects on expression of distinct GABA$_A$R subtypes. Indeed, there are brain region differences in EAE-induced changes in astrocyte gene expression (Itoh et al., 2018). Notably, loss of parvalbumin (PV) positive inhibitory interneurons occurs in both MS (Clements et al., 2008) and EAE (Ziehn et al., 2010; Rossi et al., 2011; Nisticò et al., 2013). Although loss of PV-positive interneurons is thought to underlie the reduction in sIPSC frequency in medium spiny neurons (Rossi et al., 2011), we observed an increase is sIPSC frequency in CA1 pyramidal cells even though the number of PV-positive interneurons is also reduced in hippocampal CA1 region during EAE (Ziehn et al., 2010; Nisticò et al., 2013). Thus, the mechanisms responsible for increased sIPSCs in CA1 pyramidal cells during EAE are unclear. It is interesting, however, that PV-positive, basket and bistratified interneurons in the CA1 region form inhibitory synaptic connections onto both pyramidal cells and other inhibitory neurons (Bezaire and Soltesz, 2013). For example, nearly 30% of all inhibitory synapses onto PV-positive interneurons in the CA1 region arises from other, PV-positive cells (Gulyás et al., 1999, for review see Hu et al., 2014). Thus, the increase in sIPSC frequency we observed during EAE may reflect disinhibition of inhibitory interneuron networks due to partial loss of PV-positive interneurons.

Tonic inhibition in hippocampal CA1 pyramidal cells is primarily mediated by α5β3γ2 subunit-containing GABA$_A$Rs (Caraiscos et al., 2004; Zarnowska et al., 2015), although α5β2 subunit-containing receptors may also be involved (Ju et al., 2009). Thus, although our results are consistent with the notion that up-regulation of α5β3γ2 subunit-containing receptors has an important role in the increase in tonic inhibition in EAE, additional experiments are needed to better define how the expression of β3/2 and γ2 subunits changes during EAE. The molecular mechanisms responsible for increases in α5-GABA$_A$Rs during EAE are also unclear. However, our finding that α5- GABA$_A$R-mediated tonic inhibition is enhanced in the hippocampal CA1 during EAE parallels other studies suggesting that inflammatory molecules acting on GABA$_A$Rs can effect cell excitability, excitatory neurotransmission and synaptic plasticity. For example, the pro-inflammatory cytokine interleukin-1beta (IL-1β) induces a rapid increase in GABA$_A$R surface expression in hippocampal neurons (Serantes et al., 2006) and mediates the increase in α5-GABA$_A$Rs surface expression and tonic inhibition in CA1 pyramidal cells following systemic inflammation induced by the bacterial endotoxin lipopolysaccharide (Wang et al., 2012). Although other inflammatory cytokines, such as tumor necrosis factor alpha and interleukin 6, can regulate inhibitory synaptic transmission (Stellwagen et al., 2005, Garcia-Oscos et al., 2012), these cytokines have little, if any, effect on tonic inhibition in cultured hippocampal neurons (Wang et al., 2012). Interestingly, extrasynaptic α5-GABA$_A$R expression and tonic inhibition are also strongly increased in dentate gyrus granule cells in the 5xFAD model of Alzheimer's disease (Wu et al., 2014). Moreover, enhanced α5-GABA$_A$R-mediated tonic inhibition in cortical pyramidal neurons found within the peri-infarct region contributes to reduced cortical plasticity and motor recovery in a rodent stroke model (Clarkson et al., 2010). Together with these findings, our results suggest that enhanced α5-GABA$_A$R-

mediated tonic inhibition may be a common pathological response that occurs during neurodegeneration and brain injury.

Because GABAergic inhibition potently regulates the induction of LTP at glutamatergic synapses (Wigstrom and Gustafsson, 1983, Davies et al., 1991, Arima- Yoshida et al., 2011), we investigated if the enhanced tonic inhibition in EAE leads to LTP deficits in the hippocampal CA1 region. Consistent with previous findings showing that LTP is reduced in EAE (Kim do et al., 2012, Di Filippo et al., 2013, Novkovic et al., 2015), the induction of LTP by a theta-frequency stimulation protocol was significantly reduced in hippocampal slices from EAE mice. However, reducing tonic inhibition with L-655,708 had little effect on theta frequency stimulation-induced LTP. This suggests that the LTP deficits that occur during EAE are not simply due to enhanced tonic inhibition but instead are more likely to reflect alterations in the NMDAR-dependent signaling pathways involved in the induction and/or maintenance of LTP. Notably, there is considerable controversy regarding how LTP induction is altered in EAE. Although the deficits in theta-frequency stimulation-induced LTP observed in our experiments are consistent with deficits in high-frequency stimulation-induced LTP seen in previous studies (Kim et al., 2012, Di Filippo et al., 2013, Novkovic et al., 2015), other studies have found either no change (Ziehn et al., 2010; Prochnow et al., 2013) or even an enhancement of LTP in EAE (Nisticò et al., 2013). A number of experimental variables, such as differences in the pattern of synaptic stimulation used to induce LTP and methods for EAE induction, might contribute to these disparate findings. Interestingly, although we find significant deficits in the induction of LTP by relatively modest theta pulse stimulation protocols at early time points following EAE induction, recent findings indicate that the induction of LTP by stronger, high-frequency synaptic stimulation in the hippocampal CA1 region becomes progressively impaired as disease progresses (Novkovic et al., 2015). Moreover, LTP induction in the dentate gyrus is impaired early in EAE when high-frequency stimulation-induced LTP in CA1 is intact, suggesting that hippocampal dysfunctions may extend through the trisynaptic loop as the disease progresses (Planche et al., 2016). These findings parallel the clinical observation that cognitive deficits accumulate with aging and progression of other MS disabilities (Ruano et al., 2016). Thus, future studies examining how deficits in the induction of LTP by different patterns of synaptic stimulation and how alterations in tonic inhibition evolve with time during EAE should provide important insights into the synaptic and molecular mechanisms underlying CI in MS.

Acknowledgments:

Abbreviations:

ACSF	artificial cerebrospinal fluid
CI	cognitive impairment
DPI	days post-induction
EAE	experimental autoimmune encephalomyelitis
fEPSP	field excitatory postsynaptic potential
IPSC	inhibitory postsynaptic current
LTP	long-term potentiation
MS	multiple sclerosis
NMDAR	NMDA receptor
PM	plasma membrane
sIPSC	spontaneous inhibitory postsynaptic current

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Highlights

- Tonic GABAergic inhibition in the hippocampal CA1 region is enhanced in experimental autoimmune encephalomyelitis (EAE).

- Plasma membrane expression of $\alpha 5$-GABA$_A$ receptors that mediate tonic inhibition in CA1 pyramidal cells is enhanced in EAE.

- EAE is also associated with decreased membrane expression of the GABA transporter GAT-3.

- Enhanced tonic inhibition does not appear to underlie deficits in LTP that occur in EAE.

- Alterations in both inhibitory synaptic transmission and LTP may importantly contribute to cognitive impairment in MS.
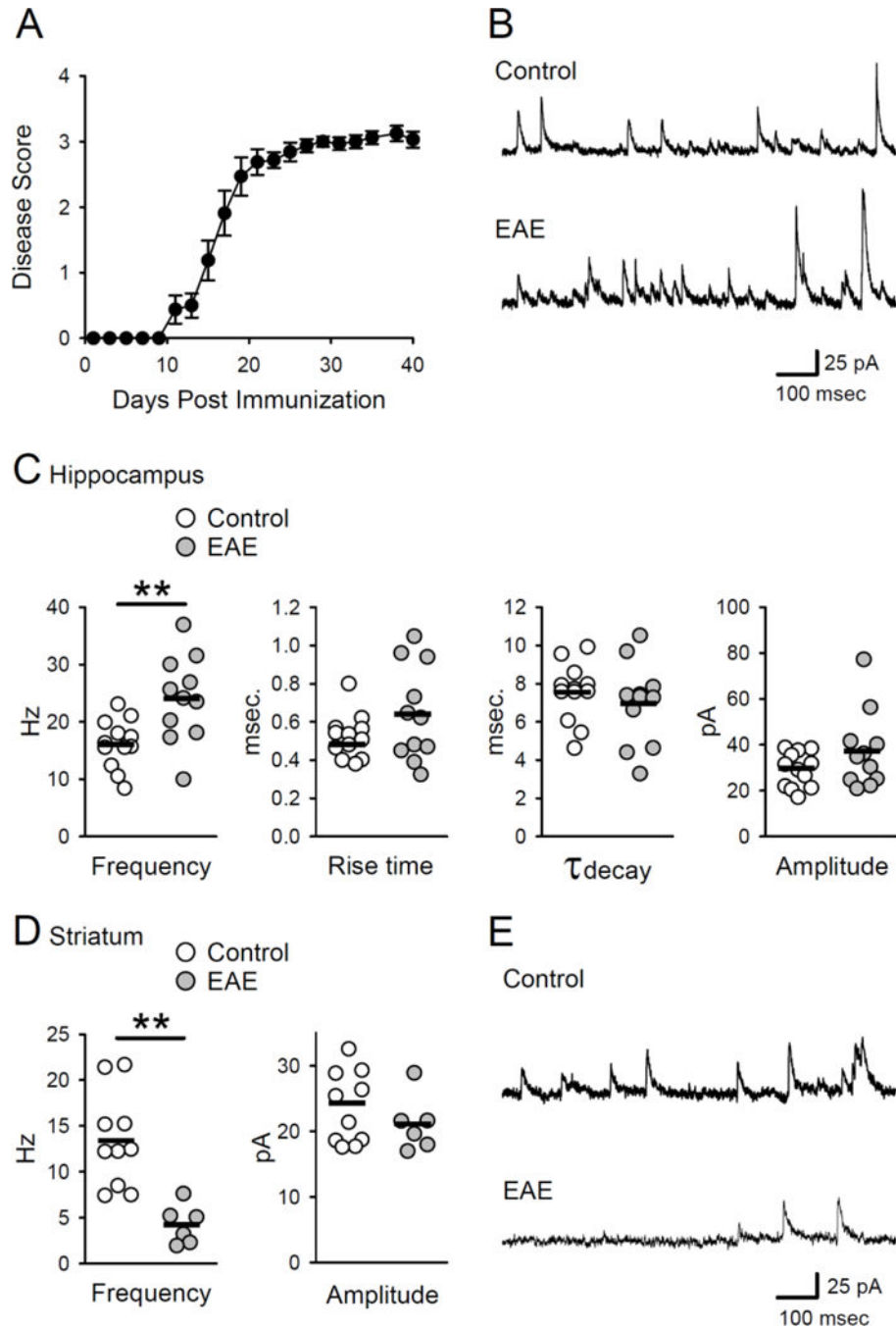
Figure 1.
Phasic inhibition is altered in EAE. **A,** Disease scores for EAE animals used in experiments investigating phasic and tonic inhibition (N = 17). Disease scores were averaged across all animals, yielding a mean disease score per group. **B,** Examples of sIPSCs in CA1 pyramidal cells in slices from control and EAE mice. **C,** Frequency, amplitude, rise time, and decay time of sIPSCs in cells from healthy control and EAE mice. Plots show results from all cells, bars indicate mean values. The frequency of sIPSCs in cells from EAE mice (24 ± 2.3 Hz, n = 12 cells from 4 mice) was significantly higher than the sIPSC frequency in cells from

controls (16.2 ± 1.2 Hz, n = 11 cells from 3 mice, t(21) = 3.139, **p = 4.96 × 10^{-3}). Peak sIPSC amplitudes were 29.2 ± 2.2 pA in control cells and 37.3 ± 5.1 pA in EAE cells (t(21) = 1.503, p = 0.148). Rise times (1090% of peak amplitude) were 0.52 ± 0.03 msec in control cells and 0.64 ± 0.08 msec in EAE cells (t(21) = 1.503, p = 0.148). Decay time constants derived from single exponential fits to the decaying phase of sIPSCs were 7.5 ± 0.5 msec in control cells and 6.9 ± 0.7 msec in EAE cells (t(21) = 0.767, p = 0.45). **D,** Frequency and amplitude of sIPSCs in striatal medium spiny neurons. The frequency of sIPSCs in cells from EAE mice (4.2 ± 0.9 Hz, n = 6 cells from 3 mice) was significantly reduced compared to control cells (13.4 ± 1.6 Hz, n = 10 cells from 3 mice, t(14) = 4.11, **p = 0.001). Peak sIPSC amplitudes were 23.7 ± 1.8 pA in control cells and 21.0 ± 1.7 pA in cells from EAE mice (t(14) = 0.96, p = 0.353). **E,** Examples of sIPSCs in medium spiny neurons in slices from control and EAE mice.
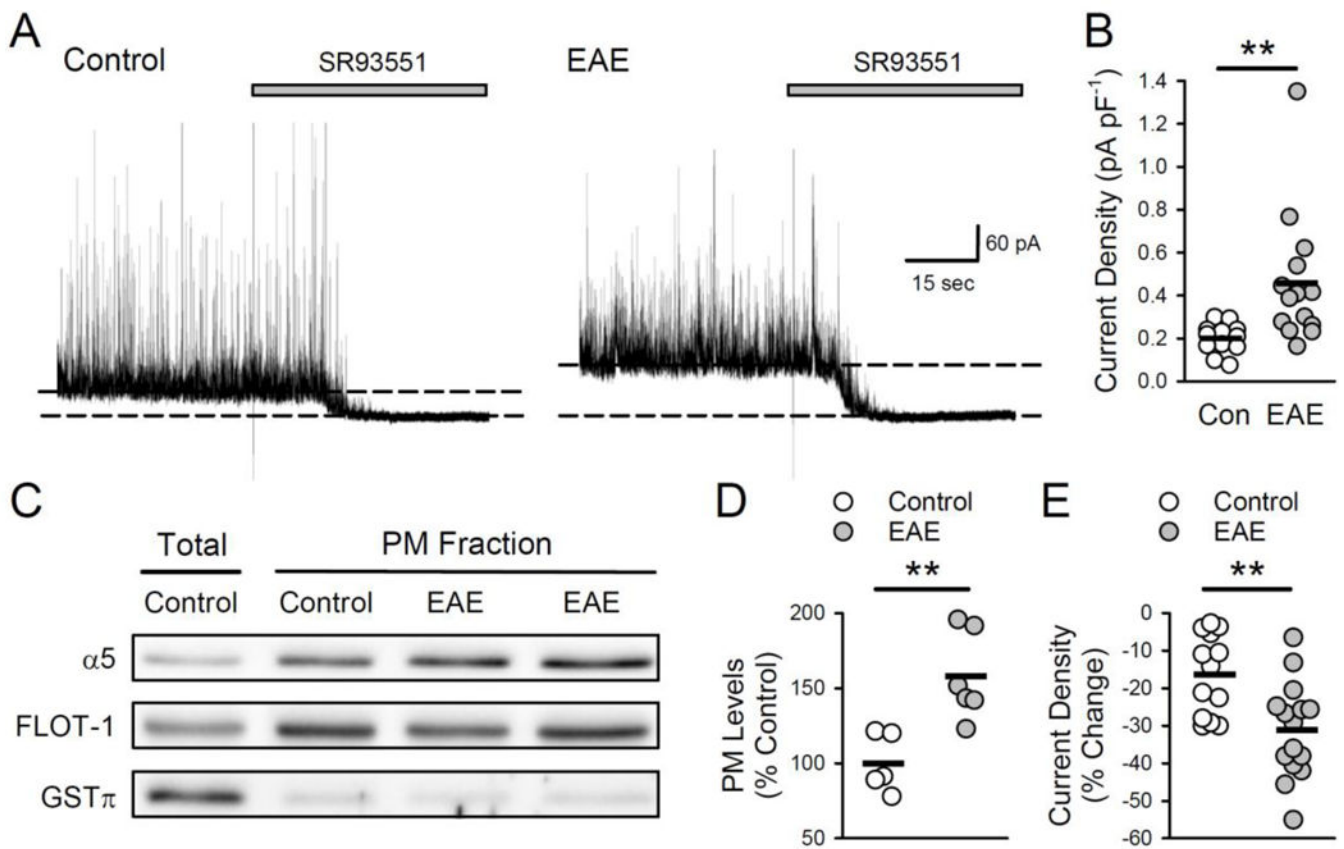
Figure 2.
Tonic inhibition and plasma membrane levels of α5 GABA$_A$R subunits are increased in EAE. **A,** Examples of tonic inhibition in cells from control and EAE mice (slices were continuously bathed in ACSF containing 5.0 μM GABA). Tonic inhibition is revealed by the change in the holding current needed to voltage-clamp cell at +10 mV (indicated by the dashed lines) before and after blocking GABA$_A$ receptors with bath application of SR93551 (100 μM, indicated by the bar). **B,** Tonic inhibition is significantly enhanced in CA1 pyramidal cells from EAE mice. Tonic inhibitory current density was 0.2 ± 0.02 pA/pF in control cells (n = 12 cells from 4 mice) and 0.46 ± 0.08 pA/pF in EAE cells (n = 14 cells from 4 mice, t(24) = 2.845, $**p = 8.93 \times 10^{-3}$). Plot shows results from all cells, bars indicate mean values. **C,** Immunoblots showing total and plasma membrane (PM) levels of α5 subunit levels in samples prepared from control and EAE hippocampi. Comparison of total and plasma membrane fractions shows enrichment in the membrane fraction while GSTπ levels show extent of cytosolic protein contamination in membrane fractions. Levels of α5 subunits were normalized to FLOT-1 levels. **D,** Plot shows results from all experiments, bars indicate mean values. Plasma membrane levels of α5 GABA$_A$R subunits in hippocampal homogenates prepared from EAE mice (n = 6) were $157.9 \pm 12.0\%$ of levels in homogenates from control animals (n = 5, t(9) = 3.753, $**p = 0.005$). **E,** A 10 minute bath application of L-655,708 (5 μM) reduced tonic inhibition to a greater extend in pyramidal cells from EAE mice compared to controls. Plot shows results from all cells, bars indicate mean values. Tonic inhibition was reduced by $31.2 \pm 3.3\%$ in cells from EAE mice (n = 15 cells from 3

mice) compared to $16.3 \pm 3.0\%$ in control cells (n = 13 cells from 3 mice, t(26) = 3.284, **p = $2.93{\times}10^{-3}$).
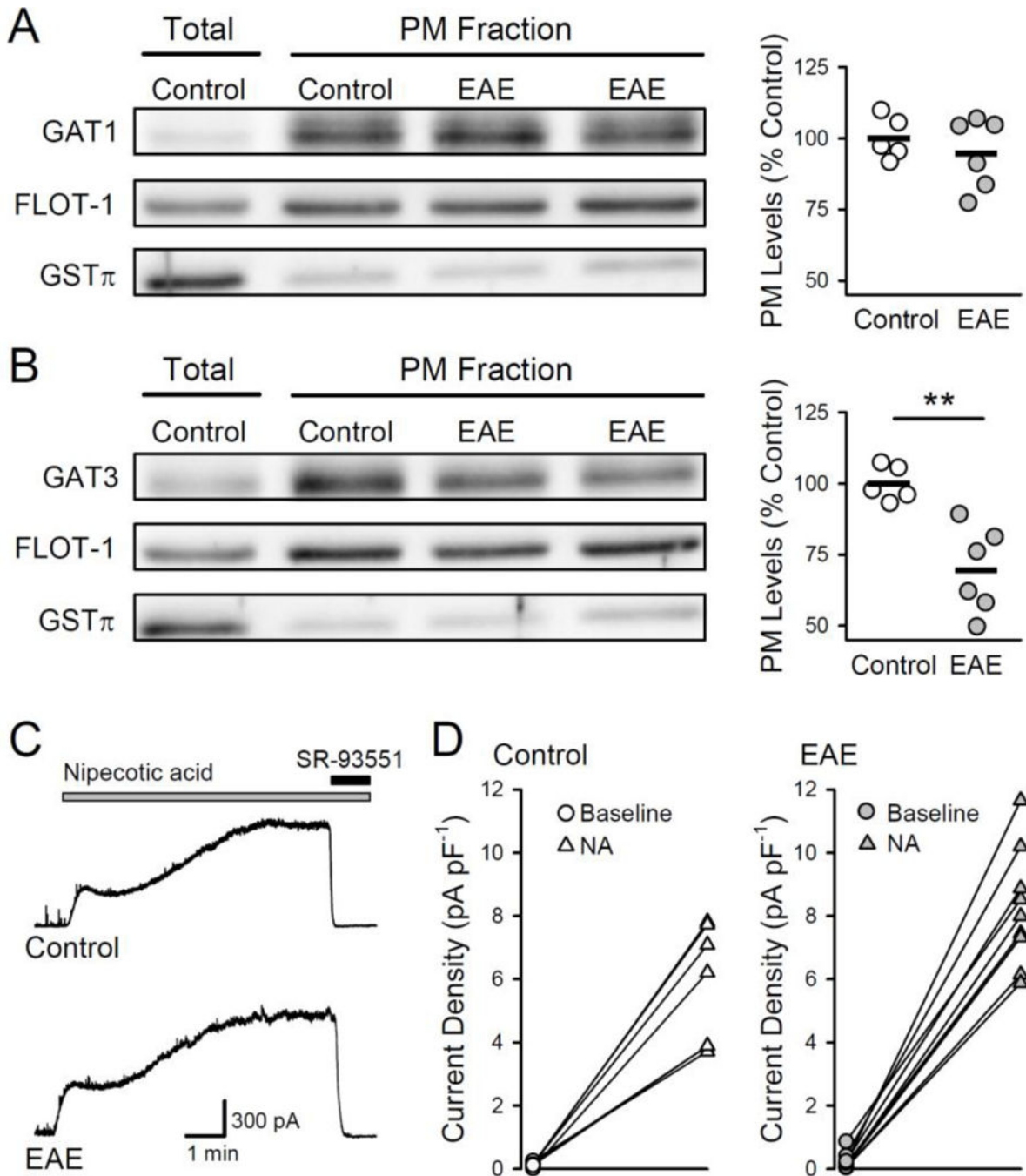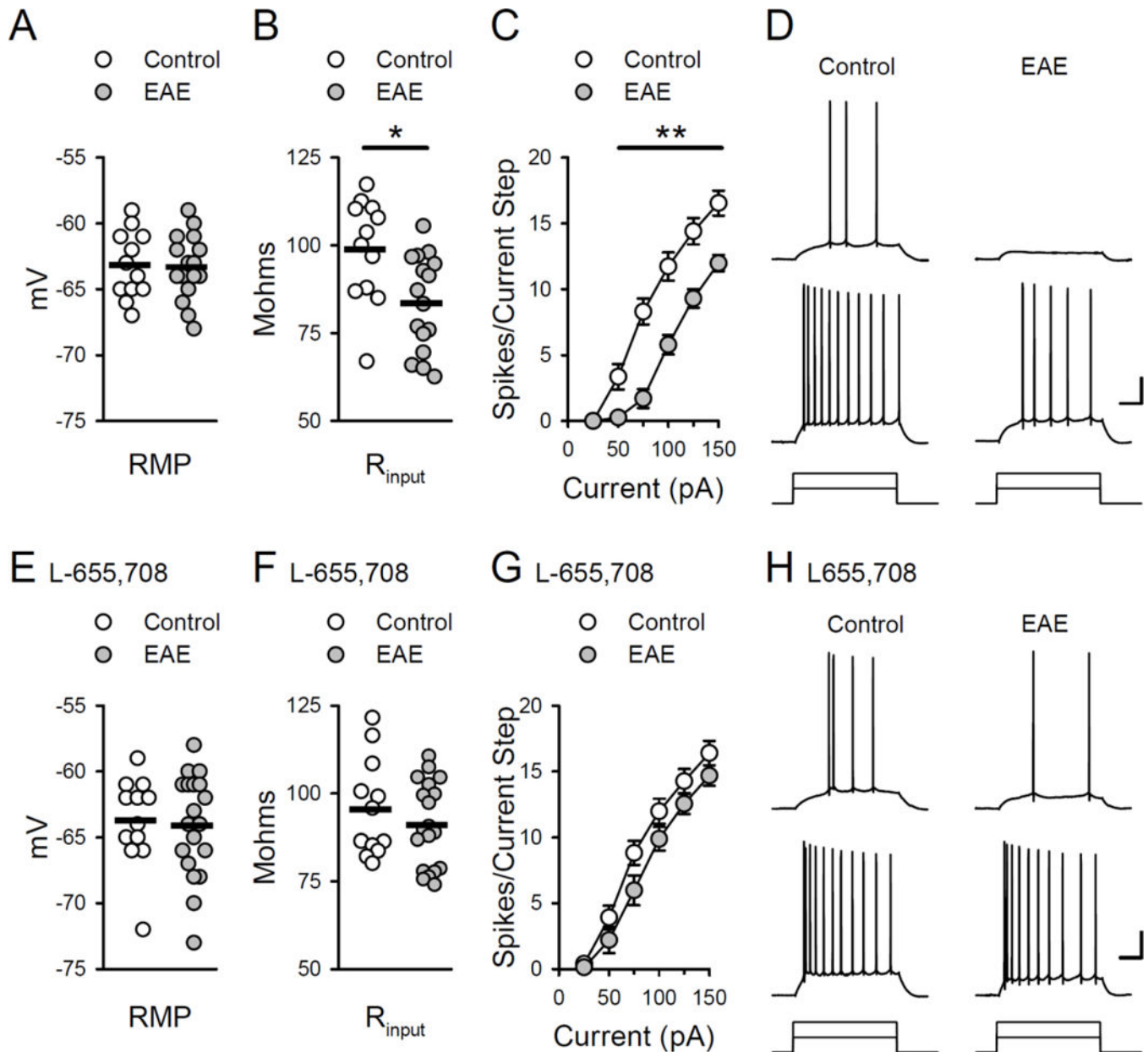
Figure 3.
Plasma membrane levels of the GABA transporter GAT-3 are reduced in EAE. **A,B,** Plasma membrane (PM) levels of GAT-1 (A) and GAT-3 (B) in hippocampal samples from control (n = 5) and EAE mice (n = 6). GAT-1 and GAT-3 levels were normalized to levels of plasma membrane protein FLOT-1. Total protein levels in hippocampal lysates were used to confirm enrichment in the plasma membrane fraction, and GSTπ levels were used to monitor the extent of cytosolic contamination. Plots show results from all experiments, bars indicate mean values. Although plasma membrane levels of GAT-1 were not altered (levels were 95

± 5% of control, t(9) = 0.827, p = 0.43), membrane levels of GAT-3 were significantly reduced in samples from EAE mice (levels were 69 ± 6% of control, (t(9) = 4.192, **p = 0.002). **C,** Examples of outward currents induced by bath application of the GAT-1/3 inhibitor nipecotic acid (10 μM, indicated by the bar) in CA1 pyramidal cells from control and EAE mice (V_{hold} = +10 mV). The contribution of GABA$_A$Rs to the outward current (tonic inhibition) was determined by bath application of 100 μM SR-93551 at the end of the recording (indicated by the bar). **D,** Increase in tonic inhibition induced by nipecotic acid (NA) in CA1 pyramidal cells from control (n = 7 cells from 2 mice) and EAE mice (n = 9 cells from 2 mice).

Figure 4.
Enhanced tonic inhibition reduces CA1 pyramidal cell excitability in EAE. **A,** Resting membrane potential (RMP) in cells from EAE and control mice. Resting membrane potentials were −63.3 ± 0.6 mV in 16 cells from 7 EAE mice and −63.2 ± 0.7 mV in 12 cells from 5 control mice. **B,** Membrane input resistance (Rin) was significantly lower in cells from EAE mice (83.6 ± 3.4 MΩ) compared to controls (98.9 ± 4 .2 MΩ, *p < 0.01, Bonferroni t-test comparison). Plots in A and B show results from all cells, bars indicate mean values. **C,** Action potential firing induced by somatic current injections is significantly reduced in cells from EAE mice (n = 17 cells from 7 EAE mice and 12 cells from 5 control mice, **p < 0.001, Bonferroni t-test comparison). **D,** Traces show examples of action potentials elicited by 50 and 100 pA current injections in cells from control and EAE mice.

Calibration: 20 mV, 100 ms. **E,** Resting membrane potentials in cells from EAE and control mice in the presence of 5.0 μM L-655,708. Resting membrane potentials were −63.8 ± 0.9 mV in 12 cells from 5 control mice and −64.2 ± 0.9 mV in 19 cells from 6 EAE mice. A two-way ANOVA analysis of resting membrane potentials in cells from EAE and control mice in the presence and absence (panel A) of L-655,708 revealed no effect of group (EAE vs. control), $F_{(1,55)} = 0.086$, $p = 0.77$, or L- 655,708, $F_{(1,55)} = 0.652$, $p = 0.423$, and no group x L-655,708 interaction, $F_{(1,55)} = 0.156$, $p = 0.903$. **F,** Membrane input resistance in cells from EAE and control mice in the presence of L-655,708. Input resistance was 91.1 ± 2.8 MΩ in cells from EAE mice and 95.5 ± 3.9 MΩ in cells from control mice. A two-way ANOVA analysis of membrane input resistance in cells from EAE and control mice in the presence and absence (panel B) of L-655,708 revealed a significant difference between cells from EAE and control mice, $F_{(1,55)} = 7.582$, $p = 0.0008$. Post-hoc Bonferroni t-tests: cells from EAE vs. control mice in the absence of L-655,708, $t = 2.971$, $p = 0.004$; cells from EAE vs. control mice in the presence of L-655,708, $t = 0.887$, $p = 0.379$. Plots in E and F show results from all cells, bars indicate mean values. **G,** Depolarization-induced action potential firing in cells from control and EAE mice in the presence of L-655,708 (n = 13 cells from 5 control mice and 19 cells from 6 EAE mice). A three-way ANOVA analysis of the action potential firing-to-current injection curves in the presence and absence (panel C) of L-655,708 revealed a significant effect of group (EAE vs. control) $F_{(1,342)} = 81.705$, $p < 0.001$), L-655,708 ($F_{(1,342)} = 25.081$, $p < 0.001$), and a significant group x L-655,708 interaction ($F_{(1,342)} = 20.521$, $p < 0.001$). Post-hoc Bonferroni t-tests: EAE vs. control cells in the absence of L-655,708, $t = 9.38$; $p < 0.001$), L-655,708 had no effect on excitability in control cells ($t = 0.307$, $p = 0.759$) but significantly enhanced excitability in cells from EAE mice ($t = 7.441$, $p < 0.001$). **H,** Traces show examples of action potential firing in response to 50 and 100 pA current injections in cells from control an d EAE mice in the presence of L-655,708. Calibration: 20 mV and 100 ms.
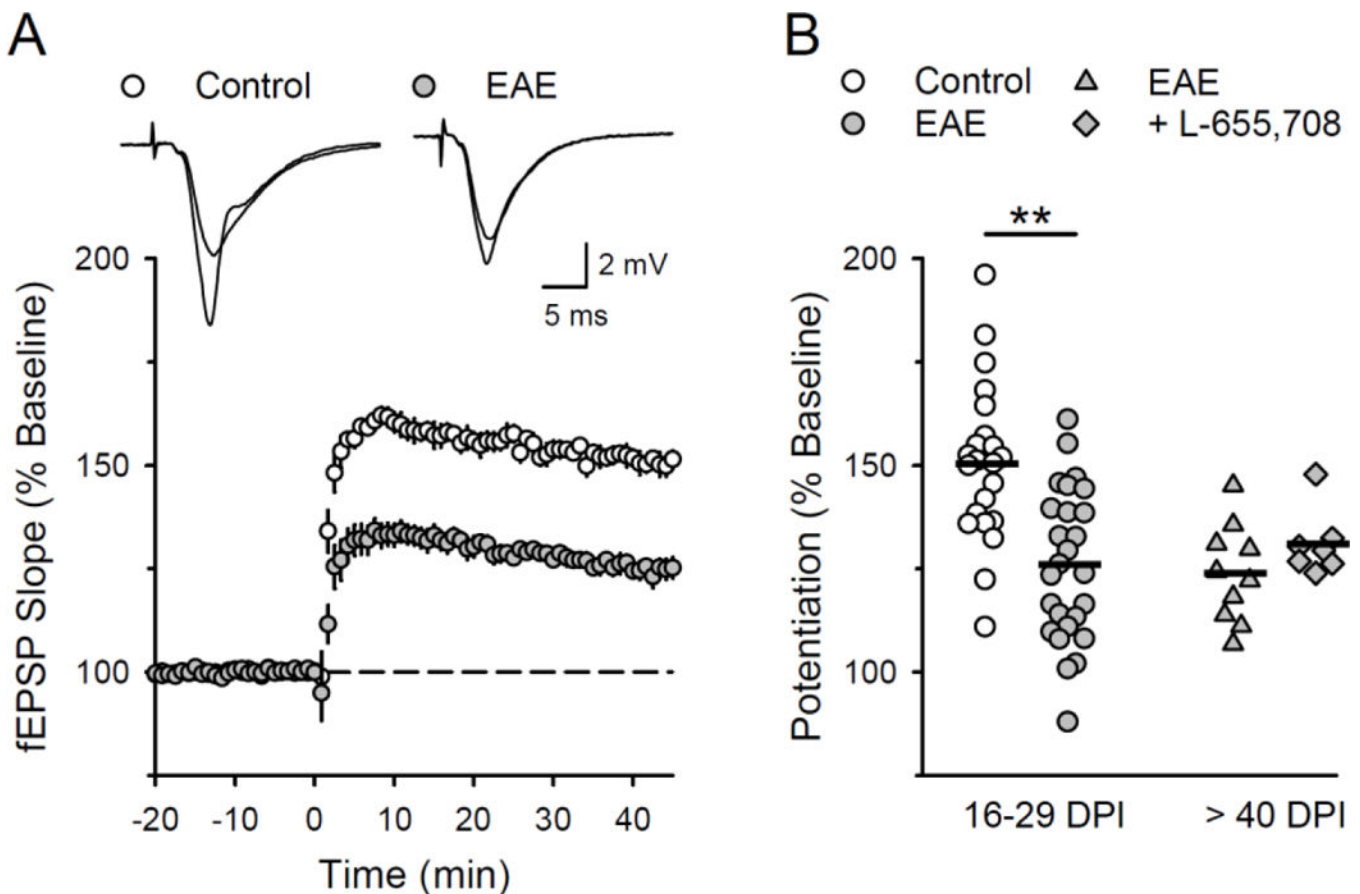
Figure 5.
LTP is impaired in the CA1 region of the hippocampus during EAE. **A,** LTP was induced by theta-frequency stimulation (150 presynaptic fiber stimulation pulses at 5 Hz, delivered at time = 0) in the CA1 region of hippocampal slices obtained from healthy control and EAE mice (16 to 29 days post EAE induction). Traces show superimposed fEPSPs recorded during baseline and 45 minutes post-LTP induction. **B,** Plot shows results from all LTP experiments, bars indicate mean values. In hippocampal slices obtained from EAE mice 16–29 days post induction (DPI), fEPSPs were potentiated to $125 \pm 4\%$ of baseline in slices from EAE mice (26 slices from 7 mice) and to $150 \pm 4\%$ of baseline in slices from healthy controls (22 slices from 5 mice, $t(46) = 4.498$, **$p = 4.64 \times 10^{-5}$). In experiments where hippocampal slices from EAE mice (>40 days post induction) were continuously bath in ACSF containing 5.0 μM L-655,708 fEPSPs were potentiated to $131 \pm 3\%$ of baseline (n = 7 slices from 5 mice) compared to $124 \pm 4\%$ of baseline in vehicle control experiments (0.1% DMSO, n = 10 slices from in 7 mice, $t(15) = 1.39$, $p = 0.185$).