Activation of calcineurin underlies altered trafficking of α2 subunit containing GABA_A receptors during prolonged epileptiform activity

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ABSTRACT

Fast inhibitory signalling in the mammalian brain is mediated by gamma-aminobutyric acid type A receptors (GABA_A Rs), which are targets for anti-epileptic therapy such as benzodiazepines. GABA_A Rs undergo tightly regulated trafficking processes that are essential for maintenance and physiological modulation of inhibitory strength. The trafficking of GABA_A Rs to and from the membrane is altered during prolonged seizures such as in Status Epilepticus (SE) and has been suggested to contribute to benzodiazepine pharmacoresistance in patients with SE. However, the intracellular signalling mechanisms that cause this modification in GABA_A Rs trafficking remain poorly understood. In this study, we investigate the surface stability of GABA_A Rs during SE utilising the low Mg^2+ model in hippocampal rat neurons. Live-cell imaging of super ecliptic pHluorin (SEP)-tagged α2 subunit containing GABA_A Rs during low Mg^2+ conditions reveals that the somatic surface receptor pool undergoes down-regulation dependent on N-methyl-D-aspartate receptor (NMDAR) activity. Analysis of the intracellular Ca^{2+} signal during low Mg^2+ using the Ca^{2+}-indicator Fluo4 shows that this reduction of surface GABA_A Rs correlates well with the timeline of intracellular Ca^{2+} changes. Furthermore, we show that the activation of the phosphatase calcineurin was required for the decrease in surface GABA_A Rs in neurons undergoing epileptiform activity. These results indicate that somatic modulation of GABA_A Rs trafficking during epileptiform activity in vitro is mediated by calcineurin activation which is linked to changes in intracellular Ca^{2+} concentrations. These mechanisms could account for benzodiazepine pharmacoresistance and the maintenance of recurrent seizure activity, and reveal potential novel targets for the treatment of SE.

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1. Introduction

GABA_A Receptors (GABA_A Rs) are ligand-gated chloride permeable ion channels which mediate both phasic (synaptic) and tonic (extrasynaptic) inhibitory neurotransmission in the central nervous system (Jacob et al., 2008; Luscher et al., 2011). They assemble from five subunits, the composition of which determines the receptors functional and pharmacological properties and the specific location on the neuronal membrane (Luscher et al., 2011; Jacob et al., 2008). GABA_A Rs containing the γ2 subunit mediate synaptic transmission (in contrast to extrasynaptic receptors located away from the synapse) and are a target for benzodiazepines (Pritchett et al., 1989).

The enrichment of GABA_A Rs in subcellular compartments such as the axon initial segment (AIS) has been reported for the α2 subunit and although both α1 and α2 subunits are found at the synapse in dendrites, a minority of GABA_A Rs in the AIS contain the α2 subunit (Panzanelli et al., 2011; Brüning et al., 2002). GABA_A Rs undergo dynamic movement within the cellular membrane. Lateral diffusion facilitates trafficking and assures the appropriate surface localisation of the receptor (Mukherjee et al., 2011), while trafficking to and from the membrane through exocytotic and endocytotic processes allows constant maintenance of the inhibitory synaptic receptor pool (Bogdanov et al., 2006; Kittler et al., 2000, 2004). Altered neuronal activity causes surface GABA_A Rs to undergo plasticity-induced trafficking changes. These are mediated by alterations in the activity of protein phosphatases and kinases which are linked to changes in intracellular Ca^{2+} (Muir et al., 2010; Bannai et al., 2009; Saliba et al., 2012; Luscher et al., 2011; Jacob et al., 2008; Petrini et al., 2014).

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SE evolves rapidly and dynamically, manifesting as a prolonged and self-sustaining seizure with significant morbidity and mortality (Lothman, 1990; Dodrill and Wilensky, 1990; Sutter et al., 2013). This distinct condition can occur in patients with previous epilepsy or may occur de novo as a result of acute neurological disorders (Trinka et al., 2012). As SE evolves, the patient's response to treatment with benzodiazepines decreases progressively which rapidly results in benzodiazepine pharmacoresistance. This may lead to refractory SE, a pathological state in which seizures are not stopped by first- or second-line anticonvulsant therapies.

To unravel the role of benzodiazepine pharmacoresistance associated with SE patients, studies have addressed whether the trafficking of GABAARs to and from the cellular membrane is altered during models of SE (Naylor et al., 2005; Goodkin et al., 2005; Blair et al., 2004). Interestingly, it has been suggested that GABAARs are subjected to subunit-specific trafficking during prolonged depolarisation. GABAARs containing the synaptic subunits β2/3 and γ2 undergo internalisation whereas those containing the extrasynaptic δ subunit remain unchanged (Goodkin et al., 2008). Despite recent studies, the temporal dynamics of GABAAR trafficking have not been investigated using live-cell imaging. Moreover, whether endocytosis occurs preferentially in distinct compartments such as dendrites or soma is unknown. It is not known which molecular pathways underlie this subunit-specific trafficking of GABAARs. Furthermore, it remains to be determined whether Ca2+- and its intracellular signalling cascades play a significant role in the modulation of GABAergic inhibition during SE.

To address the molecular mechanisms underlying altered GABAAR trafficking during SE, we used a live-cell imaging approach to examine the surface stability of GABAARs in hippocampal neurons. We induced prolonged epileptiform bursting activity in vitro by exposing neurons to artificial cerebrospinal fluid (aCSF) lacking Mg2+ (Mangan and Kapur, 2004; Sombati and Delorenzo, 1995). Using this model, we show a decrease in somatic surface GABAARs that is dependent on NMDAR activity and the Ca2+-dependent phosphatase, calcineurin. Furthermore, we show that epileptiform activity alters intracellular Ca2+ concentrations, which correlates with the decrease of GABAARs from the surface possibly contributing to pathological signalling during SE.

2. Materials and methods

2.1. Constructs

The N-terminally tagged GABAARs were a kind gift from S. Moss (Tufts University, Cambridge, MA) and has been described previously (Tretter et al., 2008).

2.2. Cell culture and transfection

All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. All efforts were made to minimise animal suffering and to reduce the number of animals used. Dissected hippocampi of P0 rat pups or E18 embryos were immediately placed in ice-cold dissection buffer (HBSS (Invitrogen), 5 mM sodium pyruvate, 5 mM glucose, 0.2 mM CaCl2 and 2.5 mM KCl, 1 mM MgCl2, 1 mM NaH2PO4, 5 mM Sodium Pyruvate) pre-equilibrated for 20 min with 95% O2 and 5% CO2 to establish a pH of 7.4. Temperature of the waterbath was constantly measured using a digital Thermometer (Hanna Instruments) and maintained at 37 °C. Any focus drift was corrected manually. Protocols were adapted to achieve minimal bleaching conditions. Imaging of SEP-tagged GABAARs was done for 60 min at a rate of one frame every 20 s (189 frames, 48.8 ms exposure, no averaging). Imaging of intracellular Ca2+ using fluo4 (1 μM, Molecular Probes, Invitrogen) hippocampal neurons were incubated for 30 min at 37 °C. After washing twice, fluo4-imaging was done for 60 min (720 frames, 5 ms exposure, no averaging) at 1 frame every 5 s.

2.3. Life-cell imaging

Live-cell imaging was performed on an upright Olympus microscope (BX51WI) coupled to an EM-CCD camera (Ixon, Andor). Cells were imaged with a water-immersion 60× objective (Olympus). Excitation was provided by an X-cite 120Q light source (Lumen Dynamics). Appropriate filters were used (in nm): Excitation: 470/40; Emission: 525/50; Dichroic: 495, long pass. The image pixel scale was calculated by dividing the camera pixel size (16 μm) by the lens magnification (60×).

yielding a pixel size of 0.27 μm. Before constant perfusion with a Cole–Parmer Master-Flex pump (-4 μl/min), aCSF (126 mM NaCl, 24 mM NaHCO3, 10 mM d-Glucose, 2.5 mM KCL, 2 mM CaCl2, 1 mM MgCl2, 1 mM NaH2PO4, 5 mM Sodium Pyruvate) was pre-equilibrated for 20 min with 95% O2 and 5% CO2 to establish a pH of 7.4. The temperature of the waterbath was constantly measured using a digital Thermometer (Hanna Instruments) and maintained at 37 °C. Any focus drift was corrected manually. Protocols were adapted to achieve minimal bleaching conditions. Imaging of SEP-tagged GABAARs was done for 60 min at a rate of one frame every 20 s (189 frames, 48.8 ms exposure, no averaging). Imaging of intracellular Ca2+ using fluo4 (1 μM, Molecular Probes, Invitrogen) hippocampal neurons were incubated for 30 min at 37 °C. After washing twice, fluo4-imaging was done for 60 min (720 frames, 5 ms exposure, no averaging) at 1 frame every 5 s.

2.4. Cell-attached recording

Cell-attached recordings were made on transfected hippocampal neurons at 13 DIV using an Axopatch 200B amplifier (Molecular Devices) and pClamp software. Cells were visualised using an upright Olympus BX50WI microscope equipped with a 40× water-immersion objective and infrared optics. Recording electrodes were pulled from standard-walled borosilicate glass capillaries (Warner Instruments) and filled with aCSF. Gigaseal cell attached recordings were made in voltage-clamp mode at −70 mV; the cells were constantly perfused with aCSF. To block currents during recording NBQX disodium salt (20 μM, Abcam) and DAPV (D(-)-2-Amino-5-phosphono pentanoic acid, 25 μM, Tocris) were added to the perfusion solution.

2.5. Low Mg2+ and drug treatments

To induce epileptiform bursting activity, aCSF without Mg2+ but 2 mM glycine (126 mM NaCl, 24 mM NaHCO3, 10 mM d-Glucose, 2.5 mM KCL, 2 mM CaCl2, 1 mM MgCl2, 1 mM NaH2PO4, 5 mM Sodium Pyruvate) was used. We confirmed previous studies (Robinson et al., 1993; Mangan and Kapur, 2004; DeLorenzo et al., 1998) that low Mg2+ results in cellular burst spiking that is dependent upon glutamatergic transmission (Sup. Fig. 2). Moreover, action potentials were associated with post-synaptic currents, indicating that the bursting was the result of network activity (Sup. Fig. 2). The mitochondrial substrate pyruvate was preincubated to supplement neuronal death (Kovac et al., 2012). Transfected hippocampal neurons were perfused with control aCSF for 3.3 min (10 frames, baseline), followed by either low Mg2+ treatment or continued perfusion with aCSF with Mg2+ (control) for 60 min. To block NMDAR activity during low Mg2+ treatment, the NMDAR blocker APV (25 μM, Tocris) was used continuously throughout the low Mg2+ treatment without preincubation. For low Mg2+ (NMDA treatment, NMDA (30 μM, Tocris) was added to the low Mg2+ medium and applied continuously for 60 min. To block the activity of the Ca2+-dependent phosphatase calcineurin, cells were pre-incubated with calcineurin autoinhibitory peptide (Triveda et al., 2003) (50 μM, Calbiochem) for 25 min at 37 °C and imaged (without application of the peptide during perfusion) either during control or low Mg2+ treatment (Muir et al., 2010).

2.6. Image analysis

Intensity analysis of specific regions of interests (ROIs: background, soma, diffuse, clustered) was done in ImageJ 1.43s which allowed the export of raw data to Matlab 2011b Software. Image correction was done in ImageJ software using the plugin StackReg macro (Thévenaz et al., 1998) which corrects for drift in (xy). Inverted average intensity projection was done in ImageJ by using the Z-stack application from frame 1–10 (0–3 min) and frame 30–60 (10–20 min). Analysis of SEP-imaging raw data was done using Matlab Software through a custom designed code. Background was subtracted from each frame, fluorescence intensity was normalised to the baseline (average value of t = 0–3.33 min) and averaged for each experimental group. The fluorescence intensity values for each specific ROI were analysed in individual loops which allowed separate analysis. Furthermore, the standard error of mean (SEM) was calculated for each time-point and the mean normalised values including error bars were plotted against time. Ca2+ imaging was analysed using ImageJ. Fluorescence intensity in the soma was extracted from one ROI per cell. Baseline for 60 min Ca2+ imaging was the average of the first 10 frames (t = 0–50 s), which corresponds to control conditions.

2.7. Statistical analysis

All experiments were performed on neurons from at least three individual preparations. The software GraphPad Prism was used for statistical tests and to generate bar charts. Data sets were tested to determine if they were normally distributed (KS normality test) before undertaking further statistical analysis. For low Mg2+ only and low Mg2+/NMDA treatments, p values were determined using a Student’s t test (two-tailed). Repeat measures ANOVA (for normally distributed data) or Friedman test was used to analyse significance of low Mg2+/Ca2+ induced effects during Ca2+ imaging, low Mg2+/dAPV and low Mg2+/CAIP experiments since there were more than two experimental groups. Appropriate post-hoc tests such as Tukey’s for normally distributed data or Dunn’s multiple comparison for non-normally distributed data were used. Values are given as mean ± SEM. Error bars represent SEM.
3. Results

3.1. Surface stability of somatic GABAARs in hippocampal neurons is altered during Low Mg²⁺ treatment

To examine the influence of SE on GABAAR stability and clustering in vitro, we mimicked the characteristic repetitive epileptiform bursting activity of SE by removal of Mg²⁺ from the extracellular medium of cultured hippocampal rat neurons transfected with SEP-tagged GABAAR α₂ subunit (α₂SEP) (Sombati and Delorenzo, 1995). Surface GABAARs were imaged via the SEP-tag on the α₂ subunit (Muir et al., 2010) (which allows visualisation through high fluorescence in neutral pH, Sup. Fig.1) for 60 min. α₂SEP fluorescence was analysed in 3 distinct regions of interests (ROIs): soma, diffuse (extrasynaptic compartment in dendrites) and clusters. At t = 20 min, somatic fluorescence of α₂SEP-containing GABAARs was significantly decreased (control F/F₀: 0.997 ± 0.02, low Mg²⁺ F/F₀: 0.85 ± 0.02; p = 0.02) indicating that internalisation of GABAARs at the somatic level increases during low Mg²⁺ treatment (Fig. 1C,F). This could account for a decrease in hippocampal GABAergic inhibition during epileptiform activity. However, α₂SEP fluorescence intensity at the soma was not found to be significantly changed at t = 60 min (control F/F₀: 0.92 ± 0.03, low Mg²⁺ F/F₀: 1.01 ± 0.04; p = 0.09) suggesting a biphasic regulation of surface

Fig. 1. Somatic surface α₂SEP-GABAARs decrease upon low Mg²⁺ treatment. (A) Representative average intensity projection of α₂SEP expression in control and low Mg²⁺ treated neurons over time (0–3 min and 10–20 min). Scale bar, 10 μm. (B) Kymograph (a line scan vertically projected over time) showing somatic (left; scale bar, 5 μm) and clustered (right; arrow heads indicate clusters; scale bar, 2 μm) α₂SEP-fluorescence intensity over the movie in control (aCSF) conditions and in the presence of low Mg²⁺ (grey bar). Red bar on the right indicates a decrease in somatic fluorescence intensity upon low Mg²⁺ treatment. (C) Average fluorescence intensity of somatic α₂SEP-GABAAR F/F₀: control (green, n = 9 cells) and low Mg²⁺ (blue, n = 7); (D) Time course of diffuse α₂SEP-GABAAR F/F₀: control (green, n = 9 cells); low Mg²⁺ (purple, n = 7). (E) Time course of α₂SEP-GABAAR clusters F/F₀: control (green, n = 9 cells) and low Mg²⁺ (light blue, n = 7). (F) Bar graph of ROIs’ F/F₀: soma (left), diffuse (middle) clusters (right). Significant loss of fluorescence in the soma compared to control at 20 min following low Mg²⁺ treatment (p = 0.02). Diffuse fluorescence is not altered at 20 min (p = 0.36) after low Mg²⁺ treatment. Fluorescence intensity of α₂SEP-GABAAR clusters is unaltered following low Mg²⁺ treatment (t = 20 min; p = 0.28) compared to control. (F) At 60 min after low Mg²⁺ treatment somatic (p = 0.09), diffuse (p = 0.85) and clustered (p = 0.42) fluorescence intensity are not significantly altered.*p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
GABARs during low Mg²⁺ treatment (Fig. 1C,F'). Interestingly, α₂SEP-GABAR clusters (t = 20 min; control F/F₀: 1.02 ± 0.02 low Mg²⁺/F₀: 0.96 ± 0.07; p = 0.36) and diffuse (t = 20 min; control F/F₀: 1.01 ± 0.02, low Mg²⁺/F₀: 0.95 ± 0.05; p = 0.28) fluorescence intensity in the neuronal dendrites during low Mg²⁺ treatment showed only a minor, non-significant decrease. Our data thus indicates compartmental specificity of low Mg²⁺ induced decrease of GABARs from the surface (Fig. 1D,E), with GABARs primarily endocytosed from the cell soma surface.

3.2. Activity of NMDA receptors induces the down-regulation of somatic GABAAR receptors from the surface during Low Mg²⁺ treatment

Low extracellular Mg²⁺ induces epileptiform activity which is abolished by application of the NMDAR antagonist dAPV (Coan and Collingridge, 1985, 1987; Tancredi et al., 1990; Albowitz et al., 1997; Westerhoff et al., 1995; Gulyás-Kovács et al., 2002; Mangan and Kapur, 2004). Therefore, we tested whether inhibition of NMDAR activity during low Mg²⁺ treatment blocks the somatic down-regulation of GABARs (Fig. 2). Low Mg²⁺ alone induced a significant decrease of α₂SEP-GABAR fluorescence intensity at t = 20 min whereas this loss was inhibited by the co-application of dAPV (t = 20 min; control F/F₀: 0.97 ± 0.05, low Mg²⁺/F₀: 0.65 ± 0.03 (p < 0.001), low Mg²⁺/dAPV F/F₀: 0.96 ± 0.07 (p < 0.05); one-way ANOVA test, Tukey’s multiple comparison post test), confirming that the down regulation of surface GABARs was dependent on the activation of NMDARs (Fig. 2C,C').

3.3. Epileptiform activity evokes intracellular Ca²⁺ changes that correspond to the temporal dynamics of somatic surface GABAAR decrease

To further explore the mechanisms of NMDAR-driven decrease in surface GABARs during low Mg²⁺ treatment, we applied the fluorescent Ca²⁺ indicator fluo4 in low Mg²⁺ treated hippocampal neurons. This allowed us to investigate intracellular Ca²⁺ transients evoked by low Mg²⁺ treatment. Hippocampal neurons perfused with control aCSF exhibit small Ca²⁺ transients reflecting spontaneous activity, whereas low Mg²⁺ perfusion significantly altered intracellular Ca²⁺ throughout the timeline of 60 min (Fig. 3B,B'). Fluo4 imaging reported intracellular Ca²⁺ increases rapidly upon early perfusion with low Mg²⁺ (10–20 min F/F₀: 375.8 ± 28.4; p < 0.001, Friedman test and Dunn’s multiple comparison post test) and at t = 60 min (F/F₀: 215.2 ± 28.8; p < 0.05, Friedman test and Dunn’s multiple comparison post test) in comparison to baseline (t = 100–150 s; F/F₀: 104.5 ± 4.4). (Fig. 3C). This indicates that low Mg²⁺ treatment induces an intracellular Ca²⁺ rise, which is likely to be caused by activation of NMDARs. Interestingly, intracellular Ca²⁺ concentration drops significantly during the timeline (10–20 min, F/F₀: 375.8 ± 28.4; 50–60 min, F/F₀: 215.2 ± 28.8; p < 0.05, Friedman test and Dunn’s multiple comparison post test) showing that intracellular Ca²⁺ concentration undergoes alteration on a similar timescale to that of somatic surface GABAAR decrease (Fig. 3C).

3.4. Dispersion of clustered GABAAR receptors is induced by NMDA receptor activation

It has been reported that the dispersal of surface GABAAR clusters in neuronal processes is regulated through Ca²⁺ influx via NMDARs (Muir et al., 2010). Therefore we tested whether further increasing the activation of NMDARs by co-application of low Mg²⁺ and the agonist NMDA would trigger dispersion of surface GABAAR clusters in proximal dendrites. Indeed, the activation of NMDARs with low Mg²⁺ in addition to application of the agonist NMDA (low Mg²⁺/NMDA) caused a loss of α₂SEP-GABAR fluorescence intensity in dendritic clusters (Fig. 4A,B,B') at t = 20 min (control F/F₀: 1.01 ± 0.04, low Mg²⁺/NMDA F/F₀: 0.799 ± 0.03; p < 0.01) suggesting that surface stability of GABARs corresponds with the potency of NMDAR activation (Fig. 4C,F). Interestingly, during low Mg²⁺/NMDA perfusion diffuse (control F/F₀: 1.02 ± 0.03, low Mg²⁺/NMDA F/F₀: 0.95 ± 0.09, p = 0.58) and total (data not shown) fluorescence intensity in neuronal processes remains unaltered at t = 20 min (Fig. 4D,F). This indicates dispersion of surface GABAAR
Fig. 3. Low Mg²⁺ treatment induces intracellular Ca²⁺ accumulation. (A) Representative average intensity projection of fluo-4 loaded neurons (coloured squares indicate individual cell bodies). (B) Raw fluorescence intensity of spontaneous Ca²⁺ transients in a neuron (bottom) and correlating kymograph (top; segmented line through somatic injection of increases in fluorescence intensity during low Mg²⁺ treatment and is of similar size compared to low Mg²⁺/NMDA. Somatic GABAAR fluorescence intensity is significantly decreased at t = 10 min after low Mg²⁺/NMDA (control F/Fl0: 1.00 ± 0.02, low Mg²⁺/NMDA F/Fl0: 0.77 ± 0.059, p < 0.01) treatment, however it is not significantly altered at t = 20 min (control F/Fl0: 0.98 ± 0.03, low Mg²⁺/NMDA F/Fl0: 0.85 ± 0.11, p = 0.34) (Fig. 4 E). Although the decrease in somatic surface GABAARs fluorescence intensity during low Mg²⁺/NMDA also occurs rapidly after treatment and is of similar size compared to low Mg²⁺ only treatment, the biphasic recovery phase is shorter in the low Mg²⁺/NMDA treatment.

3.5. Calcineurin mediates the decrease of GABAAR receptors from the surface during low Mg²⁺ induced bursting activity

We next investigated the signalling mechanisms involved in NMDAR mediated GABAAR surface decrease during epileptiform bursting activity. Calcineurin is implicated in activity-dependent regulation of GABAergic inhibition and hence could play an important role in Ca²⁺ mediated signalling, we therefore analysed its role in GABAAR stability during low Mg²⁺ bursting (Lu et al., 2000; Wang et al., 2003; Chen and Wong, 1995; Muir et al., 2005). Cells undergoing epileptiform activity showed decreases in somatic GABAAR fluorescence intensity compared to control. We found that treating cells with a calcineurin autoinhibitory peptide did not significantly affect somatic GABAAR intensity (control F/Fl0: 1.07 ± 0.10, control/CAIP F/Fl0: 0.95 ± 0.03; p > 0.05, one-way ANOVA test and Tukey’s multiple comparison post test) showing that calcineurin had no effect at t = 20 min in control conditions (Fig. 5C,C’). However, blocking calcineurin activity, inhibited the low Mg²⁺-induced decrease of surface GABAARs at the soma at t = 20 min significantly (low Mg²⁺/F0: 0.75 ± 0.06, low Mg²⁺/CAIP F/Fl0: 1.043 ± 0.06; p < 0.05, one-way ANOVA test and Tukey’s multiple comparison post test) (Fig. 5C,C’). These results suggest that calcineurin activation upon Ca²⁺ influx through NMDARs is directly involved in the decrease of surface GABAARs triggered by epileptiform bursting activity.

4. Discussion

GABAARs are a target for a variety of drugs including benzodiazepines, which are of high clinical relevance for first-line treatment of SE. Therefore it is likely that the modulation of surface stability of GABAARs induces or supports benzodiazepine pharmacoresistance in patients with SE. To identify potential mechanisms facilitating GABAAR internalisation, we performed live-imaging on SEP-tagged GABAARs. The key finding of this study shows epileptiform activity induces activation of calcineurin leading to a decrease in the number of surface GABAARs in the soma. This activity-dependent alteration of inhibitory strength is mediated by activation of NMDARs and is paralleled by an increase in intracellular Ca²⁺ which in turn is likely to activate calcineurin. These data identify a signalling mechanism underlying surface GABAAR decrease during SE and supports studies that report NMDAR activation regulates GABAergic inhibition (Muir et al., 2010; Rannai et al., 2009; Stelzer et al., 1987).

It is known that internalisation of GABAARs containing α₂, γ₂ or β₁₃ subunits is increased during epileptiform activity in vitro using low Mg²⁺ or high KCl media (Blair et al., 2004; Goodkin et al., 2008, 2005). Furthermore, it has been demonstrated that GABAARs are internalised in chemooconvulsant models of SE in vivo (Naylor et al., 2005; Nishimura et al., 2005). Our data supports the hypothesis that GABAAR internalisation occurs during prolonged seizures, by demonstrating that GABAARs which contain the α₂ subunits are decreased during low Mg²⁺ treatment. In the majority of cells this decrease in surface GABAARs was biphasic possibly indicating an adaptional switch in inhibitory strength. Interestingly, we observe that this effect occurs preferentially in the soma but less in dendrites. Compartmental internalisation of GABAARs during SE has not been reported and the mechanisms underlying this differential regulation are unknown, but there are a number of possibilities. Firstly, it remains to be investigated to what extent intracellular Ca²⁺ buffering systems such as endoplasmic reticulum or mitochondria are contributing to surface stability of GABAARs in neurons undergoing epileptiform activity. Expression of signalling proteins in specific subcellular compartments could explain this effect. Secondly, since this study makes use of experiments based on overexpression of GABAARs, this could account for increased inhibition which could suppress a decrease of GABAARs in dendrites specifically. Omitting Mg²⁺ in the extracellular medium of cultured hippocampal neurons triggers a sequence of events leading to neuronal death (Kovac et al., 2012; Yoon et al., 2010). To significantly reduce neuronal cell death, mitochondrial substrate sodium pyruvate was added in our experiments. Although we control for this substitution, it would be interesting to test whether mitochondrial ATP production affects GABAAR trafficking during prolonged seizures. Thirdly, correlating the amount of intracellular ATP to the trafficking of GABAARs could contribute to the understanding of compartmentalised trafficking of GABAAR during low Mg²⁺ treatment.

A comprehensive body of literature describes that low Mg²⁺ induced epileptiform activity is dependent on increased NMDAR
activity (Coan and Collingridge, 1985, 1987; Tancredi et al., 1990; Albowitz et al., 1997; Westerhoff et al., 1995; Gulyás-Kovács et al., 2002; Mangan and Kapur, 2004), therefore we tested whether the somatic surface GABA<sub>R</sub> decrease was mediated by NMDAR activity. Our experiments confirm this hypothesis by reporting that the decrease in surface GABA<sub>R</sub> was blocked by application of NMDAR blocker dAPV. To our knowledge this is the first study showing a direct regulation of GABA<sub>R</sub>s by NMDAR activation.
during low Mg$^{2+}$ induced epileptiform activity. However, whether the same process underlies the regulation of GABA ARs during epileptiform activity induced by different approaches (Goodkin et al., 2008; Laurénc et al., 2005) remains to be determined. The observation that NMDAR activation and rises in internal calcium are common to all models of SE (Raza et al., 2004; Rice and DeLorenzo, 1998; Mazarati and Wasterlain, 1999) would suggest that this is a universal mechanism. 

Interestingly, the direct activation of NMDARs with low Mg$^{2+}$ and NMDA induces a potent change in surface stability of GABAAR clusters. The mechanisms underlying this modulation need further investigation, however dephosphorylation of GABAAR $\gamma$2 subunits could play a role (Muir et al., 2010). Our data suggest that potency of NMDAR activation correlates with the extent of GABAAR modification, and may explain the synergistic effect of benzodiazepines and NMDAR antagonists in the treatment of SE (Rice and DeLorenzo, 1999; Martin and Kapur, 2008).

Ca$^{2+}$ influx from the extracellular environment through NMDARs can alter the stability of inhibitory neurotransmitter receptors (Bannai et al., 2009; Muir et al., 2010). Bannai et al. showed that diffusion dynamics of GABAARs too are tuned by Ca$^{2+}$ entry from the extracellular space. Indeed, here we report an increase in

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**Fig. 5.** Calcineurin mediates the decrease of somatic surface GABAARs during low Mg$^{2+}$ treatment. (A) Representative images of $\alpha_2$SEP GABAAR fluorescence in control, control with CAIP (control/CAIP), low Mg$^{2+}$ and low Mg$^{2+}$/CAIP treated neurons as an average intensity projection over time (0–3 min and 10–20 min). Scale bar, 10 µm. (B) Kymographs showing somatic (scale bar: 5 µm) fluorescence intensity over the movie (duration: 30 min) in control conditions, control/CAIP and in the presence of low Mg$^{2+}$ and low Mg$^{2+}$/CAIP (grey bar). Red bar on the right indicates decrease in somatic fluorescence intensity upon low Mg$^{2+}$ treatment. (C) Average fluorescence intensity of $\alpha_2$SEP GABAAR F/F$_0$ control (light green, n = 6 cells); low Mg$^{2+}$ (dark blue, n = 7 cells); control/CAIP (dark green, n = 6); low Mg$^{2+}$/CAIP (purple, n = 6). (C') Bar graph showing quantification of $\alpha_2$SEP GABAAR F/F$_0$ F/F$_0$ at t = 20 min. Somatic fluorescence intensity of low Mg$^{2+}$ treated cells is significantly decreased compared to control at t = 20 min (dark blue bar, $p < 0.05$). Treatment of low Mg$^{2+}$ perfused cells with a calcineurin autoinhibitory peptide prevents the change in fluorescence intensity (dark green bar, $p < 0.05$). Treatment with calcineurin autoinhibitory peptide alone does not significantly alter the fluorescence intensity of GABAAR $\alpha_2$SEP (magenta bar, $p > 0.05$). *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
intracellular Ca²⁺ concentration during low Mg²⁺ treatment. Moreover, Ca²⁺ levels correlate with the temporal dynamics of the low Mg²⁺ induced effect on GABAARs. This indicates that fast Ca²⁺-signalling could indirectly be altering GABAARs surface stability. Although technically challenging, it would be of major interest to simultaneously dual record Ca²⁺ dynamics and GABAARs surface stability to better address the relationship of low Mg²⁺ induced spiking activity and GABAAR trafficking on a single cell level.

Surface stability of GABAARs is regulated by multiple processes which are facilitated by direct or indirect interaction with trafficking proteins (Marsden et al., 2007; Jacob et al., 2008). Our experiments further emphasise the relationship of indirect signalling via intracellular Ca²⁺ sensing proteins to stabilise neurotransmitter receptors. Downstream effects of Ca²⁺ sensing proteins such as calmodulin orchestrate a number of target proteins and can trigger selective effects on surface GABAAR stability. It is known that increased phosphorylation of GABAARs contributes to surface stability (Saliba et al., 2012). Terunuma et al. demonstrated deficits in GABAAR phosphorylation during SE mediated by protein kinase C (Terunuma et al., 2008). Opposing, dephosphorylation induces declustering and increases the diffusion dynamics of GABAARs (Muir et al., 2010). Calcinurin has been shown to interact with GABAARs via the γ₇ subunit and it modulates neuronal inhibition. Interestingly, basal and maximal activity of calcineurin is increased (Kurz et al., 2001) and subcellular distribution is altered (Kurz et al., 2003) in SE in vivo. However, it has been poorly investigated whether calcineurin mediates the decrease in surface GABAARs (Wang et al., 2009). We identify calcineurin as a mediator of the decrease in surface GABAAR and therefore, provide a mechanism of inhibitory modulation during SE, and a potential target for therapy. Moreover, this study is the first to demonstrate a decrease in surface GABAARs by live-imaging in hippocampal neurons during SE. The identification of the underlying trafficking mechanism could account for resistance to benzodiazepines and could have additive effects on duration or frequency of seizure activity. Further research is needed to develop more effective therapeutic strategies against SE to which this study will contribute.

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Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2014.09.014.

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