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Epileptic stimulus increases Homer 1a expression to modulate endocannabinoid signaling in cultured hippocampal neurons

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Abstract

Endocannabinoid (eCB) signaling serves as an on-demand neuroprotective system. eCBs are produced postsynaptically in response to depolarization or activation of metabotropic glutamate receptors (mGluRs) and act on presynaptic cannabinoid receptor-1 to suppress synaptic transmission. Here, we examined the effects of epileptiform activity on these two forms of eCB signaling in hippocampal cultures. Treatment with bicuculline and 4-aminopyridine (Bic+4-AP), which induced burst firing, inhibited metabotropic-induced suppression of excitation (MSE) and prolonged the duration of depolarization-induced suppression of excitation (DSE). The Homer family of proteins provides a scaffold for signaling molecules including mGluRs. It is known that seizures induce the expression of the short Homer isoform 1a (H1a) that acts in a dominant negative manner to uncouple Homer scaffolds. Bic+4-AP treatment increased H1a mRNA. A group I mGluR antagonist blocked the Bic+4-AP-evoked increase in burst firing, the increase in H1a expression, and the inhibition of MSE. Bic+4-AP treatment reduced mGluR-mediated Ca^{2+} mobilization from inositol trisphosphate-sensitive stores relative to untreated cells. Expression of H1a, but not a mutant form that cannot bind Homer ligands, mimicked Bic+4-AP inhibition of MSE and mGluR-mediated Ca^{2+} mobilization. In cells expressing shRNA targeted to Homer 1 mRNA, Bic+4-AP did not affect mGluR-mediated Ca^{2+} release. Furthermore, knockdown of H1a prevented the inhibition of MSE induced by Bic+4-AP. Thus, an epileptic stimulus increased H1a expression, which subsequently uncoupled mGluR-mediated eCB production. These results indicate that seizure activity modulates eCB-mediated synaptic plasticity, suggesting a changing role for the eCB system following exposure to aberrant patterns of excitatory synaptic activity.

Keywords

epilepsy; endocannabinoid; Homer proteins; metabotropic glutamate receptor

1. Introduction

Endocannabinoid (eCB) signaling is triggered on demand. Upon strong depolarization of the postsynaptic neuron and/or activation of metabotropic glutamate receptors (mGluRs), eCBs are synthesized and traverse the synapse in the retrograde direction to act on presynaptic cannabinoid receptor-1 (CB_1) (Kreitzer and Regehr, 2001; Maejima et al., 2001; Straiker and Mackie, 2007; Wilson and Nicoll, 2001). Activation of presynaptic CB_1 receptors

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inhibits adenylyl cyclase and voltage-gated Ca^{2+} channels, resulting in the inhibition of neurotransmitter release (Kano et al., 2009). The eCB system regulates processes ranging from appetite and emesis to mood and memory (Di Marzo et al., 2004).

The eCB system also influences neurodegenerative processes (Scotter et al., 2010). Activation of CB_1 receptors inhibits glutamate release affording protection from excitotoxicity *in vitro* (Shen and Thayer, 1998) and reducing brain damage in disease models with an excitotoxic component, such as cerebral ischemia (Parmentier-Batteur et al., 2002). The eCB system provides on-demand neuroprotection in *in vitro* epilepsy models (Marsicano et al., 2003). However, exogenous cannabinoids exert both anti- and pro-convulsive effects *in vivo* (Smith, 2005). Furthermore, epilepsy affects cannabinoid receptor levels (Maglóczy et al., 2010), although how other elements of the eCB system adapt to seizures remains unclear.

Homer proteins are molecular scaffolds that anchor various ligands to the postsynaptic density (PSD) via their N-terminal Ena/VASP homology domain 1 (EVH1 domain) (Worley et al., 2007). Long Homer isoforms contain a C-terminal coiled coil (CC) domain that oligomerizes with the other Homers, forming a mesh-like structure (Hayashi et al., 2009; Worley et al., 2007). Homer 1 holds group I mGluRs, phospholipase C β ($\text{PLC}\beta$) and inositol trisphosphate (IP_3) receptors in a signaling complex by EVH1-ligand binding (Nakamura et al., 2004) and it forms a complex with mGluR and diacylglycerol lipase (DGL), the enzyme that produces the eCB 2-AG (Jung et al., 2007). The long Homer isoforms, 1b/c, 2a/b, 3a/b are constitutively expressed in hippocampal neurons in culture (Shiraishi et al., 2004). It is believed that Homer 1 and 2 but not Homer 3 are involved in intracellular calcium signaling (Worley et al., 2007). The short Homer isoform 1a (H1a) lacks the CC domain and acts in a dominant negative manner to uncouple Homer scaffolds (Hayashi et al., 2009). H1a is an immediate early gene that is up-regulated in animal models of epilepsy (Potschka et al., 2002). H1a also participates in certain forms of homeostatic scaling (Hu et al., 2011) that might be relevant to changes in synaptic function associated with epilepsy. Furthermore, H1a regulates the induction mechanism of eCB mediated synaptic plasticity (Roloff et al., 2010). The effects of seizure-induced H1a expression on eCB signaling are not known.

In this study, we tested the hypothesis that H1a expression induced by an epileptic stimulus modulates eCB signaling. Our data indicate that bicuculline and 4-aminopyridine (Bic+4-AP) evoked epileptiform firing and increased H1a expression that subsequently depressed metabotropic-induced suppression of excitation (MSE) by uncoupling mGluRs from downstream effectors. Attenuating activity-induced expression of H1a using pharmacological or knockdown strategies prevented the depression of MSE.

2. Materials and Methods

2.1 Materials

Bicuculline methochloride, dihydroxyphenylglycine (DHPG), and 2- Methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP), were purchased from Ascent Scientific (Princeton, New Jersey). Dulbecco's Modified Eagle's Medium (DMEM) and sera were purchased from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, Missouri).

2.2 Cell culture

Rat hippocampal neurons were grown in primary culture as described previously (Waataja et al., 2008) in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Fetuses were removed on embryonic day 17 from maternal rats

euthanized by CO₂ inhalation. Hippocampi were dissected and placed in Ca²⁺ and Mg²⁺-free HEPES-buffered Hanks salt solution (HHSS), pH 7.45. HHSS contained the following (in mM): HEPES 20, NaCl 137, CaCl₂ 1.3, MgSO₄ 0.4, MgCl₂ 0.5, KCl 5.0, KH₂PO₄ 0.4, Na₂HPO₄ 0.6, NaHCO₃ 3.0, and glucose 5.6. Cells were dissociated by triturating through a 5 ml pipette and a flame-narrowed Pasteur pipette then re-suspended in DMEM without glutamine, supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively). Dissociated cells were then plated at a density of 60,000–120,000 cells/dish onto a 25-mm-round cover glass (#1) pre-coated with matrigel (200 µL, 0.2 mg/mL). Neurons were grown in a humidified atmosphere of 10% CO₂ and 90% air (pH 7.4) at 37 °C, and fed at days 1 and 6 by exchange of 75% of the media with DMEM, supplemented with 10% horse serum and penicillin/streptomycin. Cells used in these experiments were cultured without mitotic inhibitors for a minimum of 12 days.

2.3 Whole-cell patch-clamp recordings

The whole-cell voltage-clamp technique was used to record CNQX (6-cyano-2,3-dihydroxy-7-nitroquinoxaline)-sensitive excitatory postsynaptic currents (EPSCs) as previously described (Roloff et al., 2010). Electrodes were prepared using a horizontal micropipette puller (P-87, Sutter, Novato, CA) from glass capillaries (Narishige, Japan). The internal solution contained (in mM): 113 K-gluconate, 15 KCl, 6 MgCl₂, 10 BAPTA, 10 HEPES, 5 Na₂ATP, 6.85 CaCl₂, pH 7.3 (KOH), 290 mOsm for MSE experiments or 120 K-gluconate, 15 KCl, 6 MgCl₂, 0.2 EGTA, 10 HEPES, 5 Na₂ATP, pH 7.3 with KOH, 290 mOsm/kg for depolarization-induced suppression of excitation (DSE) recordings. A high BAPTA concentration in the internal solution does not prevent MSE but, is omitted in DSE recordings because DSE is triggered by Ca²⁺ influx (Maejima et al., 2001). Pipette resistance was 3–5 MΩ using these internal solutions. For whole-cell current-clamp recordings, pipettes were filled with the following (in mM): 135 K-Gluconate, 10 NaCl, 10 HEPES, 3 MgATP, pH 7.25 with KOH, 290 mOsm/kg. Recordings were performed in an extracellular solution containing the following (in mM): 140 NaCl, 5 KCl, 9 CaCl₂, 6 MgCl₂, 5 glucose, 10 HEPES, 0.01 bicuculline methochloride, 0.5% BSA, pH 7.4 with NaOH, 325 mOsm/kg. Solutions were applied by a gravity-fed superfusion system.

EPSCs were evoked with a bipolar platinum electrode (FHC, Bowdoin, Maine) placed close to the presynaptic neuron. Voltage pulses (0.1 ms) were applied at 0.5 Hz using a Grass S44 stimulator and a SIU-5 stimulus isolation unit (Astro-Med, West Warwick, RI). MSE was induced by superfusion of 1 µM DHPG for 2 min. DSE was induced by depolarizing the postsynaptic cell to 0 mV for 15 s.

Neurons were held at –70 mV. Whole-cell currents and/or voltages were amplified using an Axopatch 200B (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz, and digitized at 11 kHz with a Digidata interface controlled by pClamp software (Molecular Devices). Analysis was performed off-line with Clampfit (Molecular Devices). Access resistance and leak currents were monitored throughout the recording, and cells with access resistances below 25 MΩ and leak currents below 100 pA were included in the analysis. Sweeps showing polysynaptic transmission were excluded from analysis. To calculate the percentage of MSE, the average peak current from the sweeps collected during the 1 min preceding DHPG application was compared with the average peak current during the last 1 minute of DHPG application. To calculate the percentage of DSE, the mean peak current of the first two sweeps immediately following the depolarization was compared to the mean peak current from the sweeps collected during the 30 sec before the depolarization.

2.4 Quantitative real-time reverse transcription-PCR

Total RNA was extracted using an RNA isolation kit (Zymo Research, Orange, CA) and quantitative real-time reverse transcription-PCR (Q-RT-PCR) was performed on 100 ng of isolated RNA using a SYBR Green Q-RT-PCR kit (Agilent Technologies, Santa Clara, CA). We used H1a primers (5'-CAA ACA CTG TTT ATG GAC TG-3' and 5'-TGC TGA ATT GAA TGT GTA CC-3') as previously described (Roloff et al., 2010). H1b/c (5'-GCT ATA TTC TCC GCG CAA CCT T-3' and 5'-GCA ACT CAA CGA GGC AGC CAA T-3') and H2a/b primers (5'-GAG TGG AAA GCG TGT GTG AG-3' and 5'-CGC ATT ACA GAA GCA AAC GGA G-3') were purchased from Open Biosystems/Thermo Fisher Scientific (Waltham, MA) as previously described (Kane et al., 2005). H3 primers (5'-TTG CAC TTC AGG ACA GCA AC-3' and 5'-TCT GGA TCT CCT GGT CCT TG-3') were purchased from RealTimePrimers (Elkins Park, PA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference control. QuantiTect primers (QIAGEN, Valencia, CA) were used for amplification of GAPDH mRNA. PCR cycling and detection was performed on an Mx3005P PCR system. Q-RT-PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

2.5 $[Ca^{2+}]_i$ imaging

$[Ca^{2+}]_i$ was recorded as previously described (Waataja et al., 2008) with minor modifications. Briefly, coverslips with cells were transferred to a recording chamber, placed on the stage of an Olympus IX71 microscope (Melville, NY) and viewed through a 40 x objective. Fura-2 was introduced using single cell electroporation (pipette concentration=250 μ M; stimulus =15 V, 150 ms) (Waataja et al., 2008). Excitation wavelength was selected with a galvanometer driven monochromator (8 nm slit width) coupled to a 75 W xenon arc lamp (Optoscan; Cairn Research Ltd, UK). $[Ca^{2+}]_i$ was monitored using sequential excitation of fura-2 at 340 and 380 nm. Fluorescence images, 510/40 nm were projected onto a cooled charge-coupled-device camera (Cascade 512B; Roper Scientific Inc.) controlled by MetaFluor software (Molecular Devices Corporation, Union City, CA, USA). After background subtraction, the 340 and 380 nm image pairs were converted to $[Ca^{2+}]_i$ by using the formula $[Ca^{2+}]_i = K_d \beta (R - R_{min}) / (R_{max} - R)$ where R is 340/380 nm fluorescent intensity ratio. The dissociation constant used for fura-2 was 140 nM, and β was the ratio of fluorescence intensity acquired with 380 nm excitation measured in the absence and presence of Ca^{2+} . R_{min} , R_{max} and β were determined in a series of calibration experiments on intact cells by applying 10 μ M ionomycin in Ca^{2+} -free buffer (1 mM EGTA) and saturating Ca^{2+} (5 mM Ca^{2+}). Values for R_{min} , R_{max} and β were 0.354, 3.10 and 5.34, respectively. These calibration constants were applied to all experimental recordings.

2.6 Transfection and DNA constructs

Rat hippocampal neurons were transfected between 10 and 13 days *in vitro* using a modification of a calcium phosphate protocol described previously (Waataja et al., 2008). Briefly, hippocampal cultures were incubated for at least 20 min in DMEM supplemented with 1 mM kynurenic acid, 10 mM $MgCl_2$, and 5 mM HEPES, to reduce neurotoxicity. A DNA/calcium phosphate precipitate containing 1 μ g plasmid DNA per well was prepared, allowed to form for 30 min at room temperature and added to the culture. After a 90 min incubation, cells were washed once with DMEM supplemented with $MgCl_2$ and HEPES and then returned to conditioned media, saved at the beginning of the procedure. Transfection efficiency ranged from 5–10 %.

All constructs were co-transfected with an expression plasmid for DsRed2 (pDsRed2-N1) from Clontech (Mountain View, CA) or TagRFP (pTagRFP-N) from Evrogen (Moscow, Russia). After 48 hrs, transfected cells were identified by red fluorescence (excitation=543

nm, emission > 605 nm). All plasmids were propagated in *Escherichia coli* DH5 α strain (Invitrogen) and isolated using Maxiprep kits (Qiagen, Valencia, CA). Knockdown of Homer 1 was accomplished using shRNA expression vectors (H1-shRNA) obtained from Open Biosystems/Thermo Fisher Scientific (Waltham, MA). Knockdown was accomplished by transfecting with 3 shRNA constructs for Homer1 in combination (pLKO.1 vector; sense sequences #1 CCTGTCTATTATAGAAGGAAT; #2 GCATGCAGTTACTGTATCTTA; #3 TGACCCGAACACAAAGAAGAA). Using multiple shRNAs targeted to different regions of the same mRNA increases knockdown (Ji et al., 2003). All knockdown experiments were compared with cells transfected with non-silencing shRNA (NS-shRNA) obtained from Open Biosystems/Thermo Fisher Scientific (Waltham, MA).

To confirm knockdown of H1a we used the following approach. Hippocampal neurons were grown on micro-islands by coating a coverglass with a small droplet (30 μ L) of matrigel. This enabled cells to be plated at the same density used for all the physiology experiments and resulted in a small network of 200–300 neurons. We then used single cell electroporation to transfect neurons on the micro-island with either H1-shRNA or NS-shRNA. Approximately 90% of the neurons were electroporated. A glass pipette (3–5 M Ω , when filled with saline) was filled with 100 μ L of a solution containing plasmid DNA (12 ng/ μ L total) encoding H1-shRNA (3 ng/ μ L each) or NS-shRNA (9 ng/ μ L) plus Tag-RFP (3 ng/ μ L) and a fluorescent dye to confirm successful electroporation (250 μ M fura-2 pentapotassium salt), in water at 18–22°C. The pipette was positioned next to but not touching the soma, then a 1 s, 150 Hz train of 1 ms, 15 V pulses was applied via a Grass stimulator (S44 with stimulus isolation unit). 18–24 h after transfection, the cells were treated with Bic+4-AP for 4 h, then RNA was harvested and Homer 1a mRNA quantified by Q-RT-PCR. We replicated this approach on 3 separate cultures and found that H1a mRNA in H1-shRNA transfected islands was reduced by 72 $\pm$ 8% relative to that measured in cells transfected with NS-shRNA.

All data were presented as mean $\pm$ S.E.M.. Significance was determined using Student's *t* test or ANOVA with the Tukey post test for multiple comparisons. Exponential functions were fitted to plots of EPSC amplitude after depolarization using a nonlinear, least-squares curve-fitting algorithm (Origin 8.1 software). Experiments in which the correlation (r^2) of the fitted curve was <0.8 were excluded from analysis.

3. Results

3.1 An epileptic stimulus induces burst firing

Application of the GABA-A receptor antagonist bicuculline and the K⁺ channel blocker 4-aminopyridine (Bic+4-AP) evoked epileptiform activity in cultured hippocampal neurons. We superfused hippocampal cultures with Bic (30 μ M)+4-AP (50 μ M) and recorded synaptically-driven action potentials using whole-cell current-clamp recording. Upon application of Bic+4-AP, paroxysmal bursting characteristic of epileptiform activity was evoked (Fig. 1). CNQX (10 μ M), an AMPA/Kainate receptor antagonist, blocked Bic+4-AP induced firing. In contrast, blocking NMDA receptors with MK801 (10 μ M), did not affect the firing pattern. MPEP (100 μ M), an antagonist for group I mGluRs, blocked the epileptiform activity (Fig. 1), consistent with previous reports that found mGluR agonists evoked and antagonists blocked epileptiform activity (Lee et al., 2002). Thus, Bic+4-AP treatment induces epileptiform activity that requires the activation of group I mGluRs and AMPA/Kainate receptors in cultured hippocampal neurons.

3.2 Bic+4-AP application inhibits MSE and slows the recovery of DSE

We hypothesized that inducing epileptiform activity might modulate eCB-mediated synaptic plasticity. Activation of postsynaptic mGluRs or depolarization of the postsynaptic cell

induces 2-AG synthesis, which will reduce the amplitude of EPSCs via the retrograde action of 2-AG on presynaptic CB₁ receptors (Kano et al., 2009). Application of the group I mGluR agonist DHPG (1 μ M; 2 min) reduced the amplitude of EPSCs by $52 \pm 5\%$ in naïve cells ($n=6$, Fig. 2A, E). This inhibition was completely blocked in the presence of the CB₁ antagonist rimonabant. EPSC amplitude increased by $8 \pm 5\%$ ($n=3$) when DHPG was applied in the presence of 100 nM rimonabant. An epileptic stimulus was applied to the culture by adding Bic+4-AP to the media and the cells returned to the incubator. After 4 h, the cells were placed in recording media without Bic+4-AP. DHPG reduced the amplitude of EPSCs by only $16 \pm 6\%$ in cells treated with Bic+4-AP for 4 h ($n=7$, $p<0.01$, compared to naïve cells, Fig. 2B, E). Bic+4-AP treatment did not produce a tonic synaptic inhibition that could potentially occlude MSE because mean EPSC amplitudes recorded from untreated and Bic+4-AP treated cultures were 189 ± 22 pA ($n=23$) and 181 ± 12 pA ($n=25$), respectively. In untreated cultures, depolarizing the postsynaptic cell for 15 s reduced the EPSC amplitude by $46 \pm 3\%$ ($n=10$, Fig. 2C, E). DSE was completely blocked in the presence of rimonabant. EPSC amplitude increased by $9 \pm 7\%$ ($n=3$) when the cell was depolarized in the presence of 100 nM rimonabant. After treating cells with Bic+4-AP for 4 h, depolarization reduced the amplitude of EPSCs by $48 \pm 4\%$ ($n=9$, Fig. 2D, E). Thus, even though both MSE and DSE are mediated by CB₁ receptors, the epileptic stimulus inhibited MSE while the amplitude of DSE was not affected.

The duration of DSE was prolonged following treatment with Bic+4-AP. We fitted the EPSC amplitude following depolarization with a single exponential function. The decay was dramatically slowed in Bic+4-AP-treated cells ($\tau=62 \pm 11$ s, $n=6$) relative to naïve cells ($\tau=28 \pm 8$ s, $n=7$, $p<0.05$) (Fig. 2F). Thus, epileptiform activity slowed the recovery of EPSC amplitude from depolarization-induced inhibition, although the magnitude of inhibition was not affected.

3.3 Epileptic stimulus induces H1a expression

Seizures induce the expression of the immediate early gene H1a (Potschka et al., 2002). Previous studies have shown that H1a gates the induction for eCB-mediated synaptic plasticity (Roloff et al., 2010). Thus, we hypothesized that Bic+4-AP would increase H1a expression in the hippocampal cultures studied here. Hippocampal cultures were treated with Bic+4-AP for 2 to 24 h, then RNA was extracted and Q-RT-PCR performed. We used GAPDH as an internal control. As shown in Figure 3A, H1a mRNA increased by 2 h, peaked after 4h and then declined by 6 h with return to basal levels by 24h. H1a mRNA is rapidly translated (Wang et al., 2012); thus, all subsequent studies were performed after 4 h of Bic+4-AP treatment. Treatment with Bic+4-AP increased H1a mRNA 14-fold ($n=9$) compared to naïve cells (Fig. 3B). mRNA levels for the long Homer isoforms H1b/c, H2a/b and 3a/b were not significantly changed by the epileptiform stimulus (Fig. 3B). The level of GAPDH showed no change after 4 h treatment with Bic+4-AP. The C_t (threshold cycle) value for GAPDH was 19.1 ± 0.3 ($n=10$) for the naïve group and 19.6 ± 0.3 ($n=10$) for the Bic+4-AP treated group. MPEP significantly inhibited Bic+4-AP-induced H1a expression (2 ± 1 fold, $n=7$, $p<0.01$, Fig. 3C) indicating that activation of group I mGluRs was required. DHPG (100 μ M), an agonist for group I mGluRs that is pro-convulsive (Alexander and Godwin, 2006), increased H1a mRNA 5 fold. This increase was blocked by MPEP (100 μ M) (0.6 ± 0.4 fold, $n=4$, $p<0.05$, Fig. 3C). Thus, Bic+4-AP induced epileptiform activity and H1a expression required the activation of group I mGluRs. A scheme describing the potential interactions that underlie Bic+4-AP induced epileptiform activity and H1a expression is shown in Fig. 3D.

3.4 MPEP blocks the inhibition of MSE induced by Bic+4-AP

If Bic+4-AP induced bursting activity and H1a expression require mGluR activation, then treatment with the mGluR antagonist MPEP should prevent inhibition of MSE following Bic+4-AP. Thus, we applied 100 μ M MPEP with Bic+4-AP. MPEP inhibited Bic+4-AP-induced expression of H1a in the Q-RT-PCR experiments (Fig. 3C). After treatment with Bic+4-AP for 4 h, DHPG inhibited EPSC amplitude by $16 \pm 6\%$ ($n=7$, Fig. 4A). In cultures treated with 100 μ M MPEP prior to and during Bic+4-AP, DHPG inhibited EPSCs by $40 \pm 8\%$ ($n=5$, $p<0.05$, compared to the cells treated with Bic+4-AP, Fig. 4B). Thus, blocking mGluRs prevented the reduction in MSE induced by Bic+4-AP.

3.5 H1a expression mimics and H1a knockdown prevents Bic+4AP induced uncoupling of mGluR-mediated Ca^{2+} release

mGluR5 mediated mobilization of IP_3 -sensitive Ca^{2+} stores requires an intact Homer scaffold; thus, expression of H1a uncouples this signaling pathway (Kammermeier and Worley, 2007). In hippocampal pyramidal neurons, DHPG evokes Ca^{2+} release from IP_3 -sensitive Ca^{2+} stores in the proximal dendrite that spreads in a wave to the soma and distal dendrites (Nakamura et al., 2000). DHPG evoked a similar Ca^{2+} response in the cultured hippocampal neurons studied here (Fig. 5A–C). Following an initial depolarization-induced Ca^{2+} increase to uniformly load endoplasmic reticulum (ER) stores, DHPG increased the $[\text{Ca}^{2+}]_i$ in proximal dendrites from a basal level of 52 ± 5 nM to peak at 344 ± 77 nM. The response initiated in the proximal dendrites and subsequently spread throughout the cell (Fig. 5C). If H1a uncouples mGluR-mediated signaling by binding to mGluR and displacing it from the scaffold maintained by long Homer proteins, then expression of H1a should inhibit DHPG induced $[\text{Ca}^{2+}]_i$ increases. As shown in Fig 5D–E, cells expressing H1a exhibited significantly ($p<0.01$) reduced DHPG-evoked $[\text{Ca}^{2+}]_i$ increases relative to cells expressing RFP. In contrast, expressing a form of H1a that cannot bind ligands because of a point mutation in the EVH-1 domain, the tryptophan at position 24 was substituted with alanine (H1aW24A), failed to affect DHPG-induced $[\text{Ca}^{2+}]_i$ responses. If Bic+4-AP-induced expression of H1a was responsible for blocking MSE, then this treatment should also inhibit DHPG-induced $[\text{Ca}^{2+}]_i$ increases. As shown in Figures 5F and G, 4 h treatment with Bic+4-AP inhibited the DHPG-induced response by 93%, consistent with our hypothesis. The inhibition did not result from depletion of Ca^{2+} stores because $[\text{Ca}^{2+}]_i$ increases evoked by 30 μ M cyclopiazonic acid (CPA), which releases ER Ca^{2+} by acting directly on the store Ca^{2+} ATPase independent of receptor activation, were not significantly different in naive (21 ± 2 nM; $n=8$) relative to Bic+4-AP (17 ± 1 nM; $n=5$) treated cells. To determine whether H1a expression was required for Bic+4-AP-induced inhibition of mGluR-mediated responses we used an shRNA strategy to knockdown H1a expression. Hippocampal cultures were transfected with 3 expression plasmids encoding shRNA targeted to H1 mRNA in combination (H1-shRNA) or an equivalent amount of plasmid that encodes non-silencing shRNA (NS-shRNA). In validation experiments, we found that 18–24 h after transfection, Bic+4-AP (4 h) induced up-regulation of H1a mRNA was inhibited by $72 \pm 8\%$ ($n=3$) in H1-shRNA expressing cells, relative to cells transfected with NS-shRNA as determined by Q-RT-PCR (see Methods). DHPG-evoked $[\text{Ca}^{2+}]_i$ increases in H1-shRNA expressing cells were not significantly different from control responses in the absence of the epileptic stimulus. Because the H1-shRNA was not specific for the 1a splice variant, Homer1b and 1c are probably knocked down in addition to H1a. However, hippocampal neurons express multiple long Homer isoforms that compensate (Kammermeier, 2008). Because basal $[\text{Ca}^{2+}]_i$ and the DHPG response were unaffected we conclude that H1-shRNA did not adversely affect mGluR signaling. We did note that after 3–4 days, cells expressing H1-shRNA showed signs of excitotoxicity such as somatic swelling, vacuoles and thinning dendrites, which were not observed in NS-shRNA-expressing cells. Thus, we performed these experiments within 2 days of transfection. In cells expressing H1-shRNA and

subsequently treated with Bic+4-AP, DHPG evoked a $[Ca^{2+}]_i$ increase that was over 10-fold larger than that observed in NS-shRNA-expressing cells treated with Bic+4-AP (Fig. 5F and G). Thus, H1 knockdown prevented the Bic+4-AP-evoked uncoupling of mGluR-mediated Ca^{2+} mobilization.

3.6 H1a but not H1aW24A inhibits MSE

If H1a mediates the attenuation of MSE induced by epileptiform activity then expression of H1a should mimic Bic+4-AP treatment and block MSE. As shown in Figure 6B–C, in cells expressing H1a, MSE was significantly reduced ($9 \pm 4\%$, $n=6$) relative to cells expressing RFP ($57 \pm 4\%$, $n=6$; $p<0.001$). If H1a prevents MSE by acting in a dominant negative manner, binding to mGluRs but not the Homer scaffold, then expressing an H1a mutant that cannot bind mGluRs would be predicted to leave MSE intact. As shown in Figures 6A and C, MSE was comparable to control (RFP) in H1aW24A expressing cells ($59 \pm 3\%$, $n=6$).

3.7 Epileptic stimulus gates eCB production via H1a expression

To further test the hypothesis that Bic+4-AP treatment inhibited MSE by increasing the expression of H1a, we expressed shRNA targeted to H1 mRNA to knock down H1a expression. For cells expressing NS-shRNA, DHPG inhibited the amplitude of EPSCs by $37 \pm 3\%$ ($n=6$, Fig. 7A, D). After treating NS-shRNA-expressing cells with Bic+4-AP for 4 h, DHPG inhibited the amplitude of EPSCs by $21 \pm 3\%$ ($n=7$, $p<0.01$, compared to untreated NS-shRNA-expressing cells, Fig. 7B, D). In contrast, MSE in cells expressing shRNA for H1 was not reduced by Bic+4-AP treatment; DHPG inhibited EPSC amplitude by $43 \pm 3\%$ ($n=6$, $p<0.01$, compared to NS-shRNA-expressing cells treated with Bic+4-AP, Fig. 7C, D). Thus, H1a expression induced by an epileptic stimulus attenuated MSE in cultured hippocampal neurons.

4. Discussion

Here we demonstrate that an epileptic stimulus increases expression of H1a, a short isoform of the Homer family of scaffolding proteins that modulates eCB signaling in hippocampal cultures. Bic+4-AP evoked burst firing and activated group I mGluRs to increase expression of H1a. Treatment with Bic+4-AP inhibited MSE and prolonged DSE. Expression of H1a, but not a mutant form that cannot bind Homer ligands, mimicked the effects of epileptiform activity on MSE. H1a knock down or pharmacological blockade of H1a expression prevented the attenuation of MSE by the epileptic stimulus. Therefore, an increase in H1a expression induced by epileptiform activity inhibits eCB production via an mGluR pathway but spares eCB signaling triggered by depolarization-induced Ca^{2+} influx.

4.1 Epileptic stimulus increased H1a expression

Bic+4-AP application rapidly intensified excitatory synaptic activity and induced an epileptiform firing pattern. Bic+4-AP has been used previously as a stimulus to model epileptic firing and to study activity-induced gene expression (Brückner et al., 1999; Leveille et al., 2010). We found that activation of AMPA receptors and group I mGluRs, but not NMDA receptor activity, was required for Bic+4-AP induced burst firing, consistent with previous studies of hippocampal slices (Lee et al., 2002; Perreault and Avoli, 1991). This result contrasts with the NMDA receptor-dependence of synchronized paroxysmal activity induced by Bic+4-AP in a developing thalamocortical network (Golshani and Jones, 1999).

Bic+4-AP-induced H1a expression required the activation of group I mGluRs. Activation of group I mGluRs is a strong driver of epileptic activity (Lee et al., 2002). DHPG produced a strong induction of H1a mRNA. Activation of group I mGluRs results in robust

phosphorylation of extracellular signal-regulated kinase (ERK) (Gallagher et al., 2004) and the MAPK/ERK cascade stimulates H1a expression (Sato et al., 2001). Thus, our observation that Bic+4-AP induced H1a expression through activation of mGluRs is consistent with previously reported mGluR activation of H1a transcription via ERK signaling pathways.

Glutamate increases H1a expression in cerebellar granule cells, but this action required Ca^{2+} influx via NMDA receptors (Sato et al., 2001) in contrast to our results in which MK801 had no effect on Bic+4-AP-induced epileptiform activity. Bic+4-AP treatment reduces inhibitory tone and depolarizes both neurons and astrocytes to drive burst firing (Bordey and Sontheimer, 1999; Brückner et al., 1999). In hippocampal slices, astrocytes amplify and promote seizure initiation and prolong ictal discharges evoked by 4-AP (Gómez-Gonzalo et al., 2010). Astrocytes express 4-AP-sensitive K^{+} channels and are depolarized by treatment with Bic+4-AP (Bordey and Sontheimer, 1999), which evokes glutamate release via both secretion and reversal of glutamate uptake (Paluzzi et al., 2007). Bic+4-AP likely produces a prolonged elevation of glutamate sufficient to activate mGluRs and thus induce H1a expression.

4.2 Activity-induced H1a expression inhibited MSE and prolonged DSE

Long Homer isoforms anchor PLC β and DGL, the enzymes that produce 2-AG, close to mGluRs (Jung et al., 2007; Worley et al., 2007). Increased H1a expression uncouples mGluR 5 from postsynaptic effectors (Kammermeier and Worley, 2007). Thus, our data indicating that mGluR-mediated Ca^{2+} mobilization from IP3-sensitive stores was reduced after Bic+4-AP treatment are consistent with established roles for Homer proteins. In cells expressing shRNA targeted to H1 mRNA, Bic+4-AP did not significantly affect mGluR-mediated Ca^{2+} release or MSE, suggesting that other long Homer isoforms expressed in hippocampal neurons such as H2a/b compensate for any reduction in H1b/c. Furthermore, the H1-shRNA was very effective in reducing the up-regulation of H1a, the expression of which correlates with the biological effects on Ca^{2+} release and MSE, in contrast to the long Homer isoforms that are constitutively expressed (Shiraishi et al., 2004). Additionally, expression of H1a but not H1aW24A, mimicked the effects of Bic+4-AP treatment. H1a binding to type 1 mGluRs will induce constitutive, ligand-independent activation of the receptor (Ango et al., 2001). Elevated eCB tone did not appear to occlude MSE because we would have expected DSE to be inhibited as well, which was not the case. Furthermore, EPSC amplitudes were not affected by Bic+4-AP treatment. Ligand-independent activation could lead to desensitization of mGluRs uncoupling the receptors from downstream effectors. Therefore, the most likely explanation for the inhibition of MSE induced by an epileptic stimulus is that H1a uncouples mGluRs from the downstream enzymes required for 2-AG synthesis because of either receptor desensitization or disruption of the Homer scaffold.

DSE decayed much slower in Bic+4-AP treated cells relative to naïve cells (Fig. 2). It is possible that the degradation of 2-AG by monoacylglycerol lipase (Dinh et al., 2002) was impaired. However, the level of monoacylglycerol lipase expression was unchanged in the epileptic human hippocampus (Ludanyi et al., 2008). The possibility that 2-AG synthesis was prolonged following H1a expression could also account for the slowed decay of DSE. PLC isoforms that differ in their coupling to G-proteins and Ca^{2+} sensitivity are preferentially activated by mGluR versus depolarization-induced Ca^{2+} influx (Hashimoto et al., 2005), suggesting that separate pathways mediate MSE and DSE. Perhaps releasing PLC and DGL α from the Homer scaffold disperses these enzymes prolonging their activation by depolarization-induced Ca^{2+} influx.

Exposure to an epileptic stimulus gated eCB production via H1a expression. Knock down of H1a, or inhibiting H1a expression by MPEP, prevented the inhibition of MSE induced by Bic+4-AP. Knock down of H1a also prevented the inhibition of Ca^{2+} release from IP_3 -sensitive stores induced by Bic+4-AP. The shRNA for Homer1 was not specific for the 1a splice variant. Thus, Homer1b and 1c are probably knocked down in addition to H1a. Since hippocampal neurons express multiple long Homer isoforms, it is likely that redundant alternative isoforms compensate to maintain mGluR signaling (Kammermeier, 2008). The baseline Ca^{2+} concentration was unchanged following Homer1 knockdown (Fig. 5) and DHPG evoked Ca^{2+} mobilization was not significantly affected by H1-shRNA expression, indicating that mGluR signaling was intact. The slight decrease in the amplitude of the DHPG-evoked Ca^{2+} response observed in H1-shRNA expressing cells does not imply a role for H1b/c in the Bic+4-AP induced effects because H1-shRNA preserved DHPG induced Ca^{2+} mobilization following treatment with the epileptiform stimulus. Thus, changes in the expression of long Homer isoforms did not complicate interpretation of the shRNA experiment.

4.3 Physiological consequences of H1a expression and MSE inhibition after seizure

H1a is rapidly up-regulated by excitatory synaptic activity. Ample evidence points to H1a as anticonvulsant (Potschka et al., 2002), since its expression reduces neural excitability. H1a not only uncouples mGluR5 from effectors such as those required for eCB synthesis, but it also induces an agonist independent activation of group I mGluRs that is required for homeostatic scaling, a non-hebbian form of plasticity that helps maintain neural excitability (Hu et al., 2011). Furthermore, H1a regulates neural excitability by modifying dendritic spine morphology. Long Homer isoforms and shank form a mesh-like matrix structure that provides a platform for other postsynaptic density proteins and determines the size of the postsynaptic density (Hayashi et al., 2009). H1a over expression uncouples these structures to reduce the density of dendritic spines (Hayashi et al., 2009). All these findings support the idea that increased H1a levels, initiated by intense synaptic activity, participate in feedback regulation to reduce neural excitability.

eCB signaling serves as an endogenous neuroprotective system that provides feedback inhibition to attenuate seizure activity by inhibiting glutamate release (Marsicano et al., 2003). Thus, the attenuation of MSE following treatment with Bic+4-AP seems inappropriate. Perhaps MSE is just a casualty of H1a-mediated homeostatic plasticity. Viral mediated over expression of H1a *in vivo* increased resistance to status epilepticus but produced learning deficits, whereas expression of H1c enhanced cognitive performance, but had no effect on status epilepticus (Klugmann et al., 2005). Thus, Bic+4-AP induced H1a expression may be part of a compensatory mechanism to cope with aberrant patterns of synaptic activity. Alternatively, inhibition of group I mGluR-mediated 2-AG production could also inhibit eCB-mediated depression of inhibitory inputs to hippocampal pyramidal neurons (Wilson and Nicoll, 2001). Blocking eCB mediated heterosynaptic inhibitory long-term depression (Chevalleyre and Castillo, 2003) would prevent the attenuation of GABAergic input that might exacerbate excitotoxicity.

Epileptiform activity prolonged DSE. This observation is consistent with the idea that separate eCB synthetic machinery mediates MSE and DSE (Alger and Kim, 2011; Min et al., 2010). This result also indicates that the inhibition of MSE was not a result of a gross disruption of the postsynaptic cell nor a change in presynaptic sensitivity to 2-AG. In some models of epilepsy, seizures decrease CB_1 receptor levels. However, down-regulation of presynaptic CB_1 receptors could not account for the inhibition of MSE following treatment with Bic+4-AP because the same treatment failed to affect DSE, which also relies on presynaptic CB_1 receptors. Although the extent of synaptic inhibition induced by depolarization showed no change (Fig. 2), the slower decay of DSE after Bic+4-AP

treatment indicates a prolonged depression of excitatory synaptic transmission, suggesting a neuroprotective role in seizure. Indeed, the higher threshold for DSE relative to MSE is consistent with a neuroprotective role for this eCB pathway. mGluR-induced eCB signaling may play a more prominent role in synapse specific plasticity and depolarization-induced eCB signaling might play a global neuroprotective role.

4.4 Conclusions

We have shown that after sustained epileptiform activity, MSE was inhibited and DSE was prolonged. The epileptic stimulus increased the expression of H1a resulting in the subsequent inhibition of mGluR-mediated eCB signaling. The gating of the eCB production mechanism by stimuli that elevate H1a levels may explain the changing role of the eCB system in response to stimuli that increase H1a expression such as drugs of abuse, growth factors, and seizures.

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Abbreviations

eCB	endocannabinoid
DSE	depolarization-induced suppression of excitation
MSE	metabotropic-induced suppression of excitation
mGluR	metabotropic glutamate receptor
H1a	Homer isoform 1a
Bic+4-AP	Bicuculline and 4-aminopyridine
MPEP	2-Methyl-6-(phenylethynyl) pyridine hydrochloride
DHPG	dihydroxyphenylglycine
HHSS	HEPES-buffered Hanks salt solution
EVH1 domain	Ena/VASP homology domain
PLCβ	phospholipase C β
DGL	diacylglycerol lipase
IP3	inositol trisphosphate
TRPC channel	transient receptor potential cation channel
CNQX	6-cyano-2,3-dihydroxy-7-nitroquinoxaline
EPSCs	excitatory postsynaptic currents
Q-RT-PCR	quantitative real-time reverse transcription-PCR
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
NS-shRNA	non-silencing short hairpin RNA
H1-shRNA	Homer 1 short hairpin RNA

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Epileptiform activity inhibits mGluR mediated endocannabinoid production.

Epileptiform activity prolongs depolarization-induced endocannabinoid production.

An epileptiform stimulus increased homer 1a expression to uncouple mGluR signaling.

Seizures alter the mechanism of endocannabinoid-mediated synaptic plasticity.

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Fig. 1.

Bic+4-AP application induces epileptiform activity in hippocampal cultures that requires the activation of AMPA/Kainate receptors and group I mGluRs. Representative traces show experiments performed under whole-cell current-clamp mode. Hippocampal neurons were superfused with the following solutions in sequence: extracellular solution (EC), Bic+4-AP, Bic+4-AP+drug, and returned to Bic+4-AP. The indicated drugs were applied 5 min prior to and during the recordings: CNQX (10 μ M, n=4), MPEP (100 μ M, n=7) and MK801 (10 μ M, n=4).

Fig. 2.

Bic+4-AP application inhibits MSE and slows the recovery of DSE. (A–B) Plots show MSE in naïve cells (A) ($n=6$) and cells treated with Bic+4-AP ($n=7$) for 4 h (B). EPSCs were evoked by stimulating the presynaptic neuron with a bipolar platinum electrode every 2 s. MSE was induced by superfusing the neurons with DHPG (1 μ M, indicated by the horizontal bar) for 2 min. The amplitudes of EPSCs during DHPG application were normalized to those of the 30 responses before DHPG application. The insets display averaged EPSC from 30 responses before and during DHPG application. (C–D) Plots show DSE in naïve cells (C) ($n=10$) and cells treated with Bic+4-AP ($n=9$) for 4 h (D). DSE was induced by depolarizing the postsynaptic neuron (indicated by the arrow) from -70 mV to 0 mV for 15 s. The amplitudes of the responses after depolarization were normalized to those of the 30 responses before depolarization. The insets display averaged EPSCs from the 30 responses before and 2 responses immediately after depolarization. Exponential functions were fitted to plots of EPSC amplitude after depolarization using a nonlinear, least-squares

curve-fitting algorithm (Origin 8.1 software). (E) Bar graph shows the mean magnitude of MSE and DSE for naïve cells (open bars) and Bic+4-AP treated cells (solid bars). (F) Bar graph shows the mean decay time constant of DSE for naïve cells (open bar, n=7) and Bic +4-AP treated cells (solid bar, n=6). Calibration: Vertical, 50 pA; Horizontal, 50 ms. Error bars indicate SEM. * $p<0.05$, ** $p<0.01$, relative to the naïve group ANOVA with the Tukey post test.

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Fig. 3.

Bic+4-AP induces H1a expression by activating of group I mGluRs. RNA isolation, Q-RT-PCR and primers are described in Methods. (A) Plot displays H1a mRNA induction in hippocampal cultures treated with Bic+4-AP for the times indicated. (B) Bar graph displays induction of the indicated mRNAs following 4 h treatment with Bic+4-AP. * $p < 0.05$, paired t test, $2^{-\Delta\text{CT}}$ before compared to after Bic+4-AP (Schmittgen and Livak, 2008). Long Homer transcripts were not significantly changed by treatment with Bic+4-AP. (C) Hippocampal cultures were incubated with the indicated drugs for 4 h and Q-RT-PCR performed. DHPG and MPEP were applied a concentration of 100 μM . **, $p < 0.01$ relative to Bic+4-AP; *, $p < 0.05$ relative to DHPG, ANOVA with the Tukey post test. Error bars indicate SEM (n = 5). (D) The scheme shows the proposed interactions that underlie Bic+4-AP-induced epileptiform activity and H1a expression.

Fig. 4. MPEP blocks Bic+4-AP-induced inhibition of MSE. (A–B) Plots show MSE in cells pretreated with Bic+4-AP (A) ($n=7$) or Bic+4-AP+100 μ M MPEP (B) ($n=5$). The insets display averaged EPSCs from the 30 responses before and during DHPG application. Calibration: Vertical, 50 pA; Horizontal, 50 ms. Error bars indicate SEM.

Fig. 5.

H1a expression mimics and knockdown of H1a prevents Bic+4-AP-induced uncoupling of mGluR-mediated Ca^{2+} mobilization. Hippocampal neurons were loaded with fura-2 by single cell electroporation and $[\text{Ca}^{2+}]_i$ recorded using digital imaging as described in Methods. (A) Representative image shows fura-2 loaded cell. (B) Plot displays $[\text{Ca}^{2+}]_i$ recorded from the proximal dendrite of the cell shown in A. The cell was superfused with 50 mM K^+ and 10 μM DHPG at the times indicated by the horizontal bars. (C) Time lapse images display $[\text{Ca}^{2+}]_i$ recorded from the boxed region in (A). Pseudocolor images were scaled to the color bar in (B). Images were acquired at the times indicated by the lower case letters in B (a–n). (D) Plots show DHPG-evoked $[\text{Ca}^{2+}]_i$ responses from the proximal

dendrites of cells treated according to the protocol shown in (B). Experiments were performed on cells expressing RFP, H1a or H1aW24A as indicated (n=4). (E) Bar graph summarizes DHPG-evoked $[Ca^{2+}]_i$ increases from the cells indicated. **, $p<0.01$ relative to RFP expressing cells; †, $p<0.05$ relative to H1a expressing cells ANOVA with the Tukey post test. (F) Plots show DHPG-evoked $[Ca^{2+}]_i$ responses from the proximal dendrites of cells treated according to the protocol shown in (B). Experiments were performed on cells expressing NS-shRNA (*a–b*) or H1-shRNA (*c–d*) and were either untreated (*a and c*) or treated with Bic+4-AP for 4 h prior to recording (*b and d*). (G) Bar graph summarizes DHPG-evoked $[Ca^{2+}]_i$ increases from the cells indicated. Error bars indicate SEM (n = 4). **, $p<0.01$ relative to corresponding untreated cells; ††, $p<0.01$ relative to Bic+4-AP-treated NS-shRNA cells ANOVA with the Tukey post test.

Fig. 6. H1a but not H1aW24A attenuates MSE. (A–B) Plots show MSE in cells expressing H1aW24A (A) ($n=6$) or H1a (B) ($n=6$). The insets display averaged EPSCs from the 30 responses before and during DHPG application. Calibration: Vertical, 50 pA; Horizontal, 50 ms. (C) Bar graph shows the magnitude of MSE in cells expressing the indicated constructs. Error bars indicate SEM. *** $p<0.001$; relative to RFP, ANOVA with the Tukey post test.

Fig. 7.

Knock down of H1a blocks Bic+4-AP-induced MSE inhibition. (A) Plots show MSE in cells expressing NS-shRNA (n=6). (B–C) Plots show MSE in cells expressing NS-shRNA (B) (n=7) or shRNA for Homer1 (C) (n=6) after treating with Bic+4-AP for 4 h. 1 μ M DHPG was applied at the time indicated by the horizontal line. The insets display averaged EPSCs from 30 responses before and during DHPG application. Calibration: Vertical, 50 pA; Horizontal, 50 ms. (D) Bar graph shows the magnitude of MSE following the indicated treatments. Error bars indicate SEM. ** $p < 0.01$; relative to the indicated group ANOVA with the Tukey post test.