ETHANOL NEUROTOXICITY IS MEDIATED BY CHANGES IN SUBUNIT COMPOSITION, SURFACE LOCALIZATION AND FUNCTIONAL PROPERTIES OF glutamate AMPA RECEPTORS

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Running title: Ethanol neurotoxicity and AMPA receptors.

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Abbreviations: AMPARs, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; CpAMPARs, Ca^{2+}-permeable AMPA receptors; EtOH, Ethanol; GluA AMPA receptor subunit; GRIP, Glutamate Receptor-Interacting Protein; NASPM, 1-naphthyl acetyl spermine; NBQX, 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide disodium salt; PI, propidium iodide; PICK1, Protein Interacting with C Kinase-1; PSD, postsynaptic density; REST, repressor element-1 (RE1) silencing transcription factor gene; SAP97, Synapse-associated protein 97; TIFs, Triton Insoluble Fraction.

Keywords: Ethanol withdrawal, AMPA Receptors, Organotypic Hippocampal Slices, CA1 injury, scaffold proteins

Abstract

Alterations in the subunit composition of the AMPA channels, with a relative decrease of the GluA2 subunit and an increase of Ca^{2+}-permeable channels, have been linked to the transition from physiological to pathological conditions in a number of contexts, including substance use disorders (SUD). Recent data from our laboratory showed that EtOH withdrawal causes CA1 pyramidal cell death in organotypic hippocampal slices through changes in glutamate homeostasis. In this paper, we studied whether Ca^{2+}-permeable AMPARs formation could be responsible for this toxicity. To verify this hypothesis, we characterized the AMPA subunit composition and localization in synaptic membranes of mature hippocampal slices exposed to EtOH withdrawal by using western blotting, surface expression assay and immunofluorescence techniques. We found that EtOH withdrawal alters AMPA subunit composition, suggesting a shift to Ca^{2+}-permeable AMPA formation. These results are also supported by the upregulation of the repressor element-1 (RE1) silencing transcription factor (REST) gene, a repressor of GluA2 AMPA receptor subunit, after EtOH withdrawal. At functional level, we found that, in EtOH-treated slices following EtOH washout, AMPA-induced current was enhanced, while AMPA-induced calcium influx was reduced, compared to non-treated control slices. Consistently, both the selective blocker of Ca^{2+}-permeable AMPARs 1-naphthyl acetyl spermine (NASPM) and the non-selective AMPA antagonist NBQX significantly attenuated EtOH-withdrawal-induced injury. Our results suggest that Ca^{2+}-permeable AMPA channels mediate the neurotoxicity induced by EtOH withdrawal in a calcium-independent manner and propose the inhibitors of Ca^{2+}-permeable AMPA channels as possible therapeutic drugs.

INTRODUCTION

Ethanol (EtOH) abuse causes persistent structural and functional alterations in the brain by mechanisms that are not yet fully understood. Many studies have shown permanent impairment in synaptic function and neuronal damage caused by EtOH exposure and withdrawal in multiple neuronal areas, including the hippocampus, with both in vivo and in vitro models of neurotoxicity (Läck et al., 2007; (Marty & Spigelman, 2012; Gerace et al., 2016; Gerace et al., 2019). AMPA receptors (AMPARs) mediate the fast component of excitatory neurotransmission, and the dynamic regulation of these receptors, both at transcriptional and posttranscriptional level, is a crucial factor in the transition from physiological to pathological conditions. The homeostasis of GluA1-2-containing AMPARs is controlled by several scaffold and adhesion complex proteins that capture, retain and promote surface expression of AMPARs, thus regulating physiological neuronal functions and, in certain conditions, cell fate. AMPARs are localised mostly in the postsynaptic density and their trafficking is tightly regulated by interactions with scaffold molecules controlling the distribution of AMPARs in the synaptic, intracellular and extrasynaptic pools (Newpher and Ehlers, 2008). Several AMPAR interacting proteins have been identified, including Glutamate Receptor-Interecting Protein (GRIP) and Protein Interacting with C Kinase-1 (PICK1), which have been shown to specifically interact with GluA2 subunit (DeSouza et al., 2002; Daw et al., 2000; Perez et al., 2001; Seidenman et al., 2003), the transmembrane protein stargazin, which interacts directly with AMPAR (Chen *et al.* 2000) and Synapse-associated protein 97 (SAP97), which specifically binds GluA1 subunit (Leonard et al., 1998; Howard *et al.* 2010).

Great attention has been focused on the GluA2 subunit due to its profound effects on AMPAR assembly, trafficking and ionic selectivity. A relative decrease of GluA2 subunit increases calcium permeability of AMPARs, an event that has been linked to the transition from physiological to pathological conditions in a number of contexts. For example, one of

the earliest biological manifestations of dementia in Alzheimer disease (AD) is due to a reduction of synaptic AMPARs (Shankar *et al.* 2008). Moreover, prolonged decreases in surface GluA2-containing AMPARs have been described as a causal factor for cell death of hippocampal CA1 pyramidal neurons in models of toxicity (Anzai et al., 2003; Gerace et al., 2014) and in pathological conditions including global ischemia (Pellegrini-Giampietro 1997). Interestingly, dysfunctional Q/ R editing in GluA2 occurs in the motor neurons of patients with amyotrophic lateral sclerosis (ALS, Hideyama and Kwak 2011). Calcium-permeable AMPARs (CpAMPARs) have also been demonstrated to play an essential role in substance use disorders (SUD) models, as exposure to substances lead to AMPAR molecular switch and formation of CpAMPARs (Mameli et al., 2011; Pascoli et al., 2014). For example, it was recently shown that cocaine-induced potentiation of VTA excitatory synapses is mediated by the insertion of CpAMPARs to the synaptic membranes (Mills *et al.* 2017). Moreover, cadherin adhesion complex proteins have been shown to contribute to the stabilization of AMPARs into the synaptic membranes (Saglietti et al., 2007; Tai et al., 2008) and may contribute to the susceptibility to cocaine addiction by the stabilization of GluA1-2-containing AMPARs into synaptic membranes (Mills *et al.* 2017).

A recent paper from our laboratory has demonstrated that EtOH withdrawal induces cell death in mature organotypic hippocampal slices and that glutamate receptors are mediators of EtOH withdrawal-induced toxicity (Gerace et al., 2019). In particular, we found that EtOH withdrawal increases the AMPA mediated sEPSCs as well as the expression of GluA1 but not GluA2 AMPA subunits, suggesting that the toxicity induced by EtOH withdrawal could be due to the formation of (Cp) AMPARs.

On the basis of these considerations, here we sought to investigate the role of CpAMPARs in the toxicity induced by EtOH withdrawal.

MATERIALS AND METHODS

Male and female Wistar rat pups (94 animals, 7-9 days old, weight 16±3 gr) were obtained from Charles River (MI, Italy). Animals were housed at 23±1°C under a 12 h light–dark cycle (lights on at 07:00) and were fed a standard laboratory diet with ad libitum access to water. The experimental protocols were approved by the Italian Ministry of Health (Aut. 176; 17E9C.N.VAS) and the European Communities Council Directive of 2010/63/EU.

The authors further attest that all efforts were made to minimize the number of animals used and their suffering, as reported in the Guidelines McGill Module-1. The present study was NOT pre-registered. NO subjects were excluded in the present study. No blinding for experiments was performed.

Materials

Ethanol (EtOH, CAS No: 64-17-5), 1-Naphthylacetyl spermine trihydrochloride (NASPM, catalogue number: #N193) and propidium iodide (PI, CAS No: 25535-16-4) were purchased from Sigma (St Louis, MO, USA). Tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma (St Louis, MO, USA). 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide disodium salt (NBQX, catalogue number: #1044/1), tetrodotoxin Citrate (TTX, catalogue number: #1069), DL-2-Amino-5-phosphonopentanoic acid (APV, catalogue number: #0105) and pictotoxin (catalogue number: #1128) were purchased from Tocris (Bristol, UK). (R,S)-AMPA (CAS No: #74341-63-2) was purchased from Abcam, (Cambridge, UK).

Preparation of rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as previously reported (Gerace et al., 2012; Gerace et al., 2015; Landucci et al., 2018). Briefly, after decapitation hippocampi were isolated and removed from the brains of Wistar rat pups (Harlan, MI, Italy), transverse slices (420 μm) were prepared using a McIlwain tissue chopper and then transferred onto 30 mm diameter semi-porous membranes inserts (Millicell-CM, catalogue number: #PICM03050; Millipore, Italy), which were placed in six well tissue culture plates containing 1.2 ml medium per well. Slices were maintained at 37 °C in an incubator in an atmosphere of humidified air and 5% CO_2 for 10 days. Before experiments all the slices were screened for viability by phase-contrast microscopy analysis; slices displaying signs of neurodegeneration were discarded from the study (exclusion criteria).

Ethanol exposure and Drug Treatment in organotypic hippocampal slices

The experiments were conducted as previously described in (Gerace et al., 2016; Gerace et al., 2019). Briefly, hippocampal slice cultures were exposed for 7 days to 150 mM of EtOH. The medium was changed every day adding EtOH to the fresh culture medium. For control slices the medium was changed every day by adding fresh culture medium. After 7 days of EtOH treatment, some of the slices were incubated in EtOH fresh culture medium, EtOH -free medium or in EtOH -free medium plus the non selective AMPA antagonist NBQX (10 μM) and the selective blocker of CpAMPARs NASPM (10 μM) for 24 h before they were assessed for neuronal injury using PI fluorescence.

Assessment of CA1 pyramidal cell injury

PI (5 μg/ml) was added to the medium either at the end of the 7-day EtOH incubation period or 24 h after it was removed from the medium. Thirty minutes later, fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific,

Segensworth, UK) equipped with a xenon-arc lamp, a low-power objective (4X) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1^{TM}; Intracellular Imaging Inc., Cincinnati, OH, USA) and subsequently analyzed using the Image-Pro Plus morphometric analysis software (Media Cybernetics, Silver Spring, MD, USA). In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH, Bethesda, USA) and the optical density of PI fluorescence was detected. There was a linear correlation between CA1 PI fluorescence and the number of injured CA1 pyramidal cells as detected by morphological criteria (Pellegrini-Giampietro *et al.* 1999).

Western blot analysis

The experiments were conducted as previously described in (Gerace et al., 2015; Gerace et al., 2019). Cultured slices were washed with cold 0.01 M PBS and dissolved in 1% SDS. Total protein levels were quantified using the BCA (bicinchoninic acid) Protein Assay (catalogue number: # 23225) (Pierce; Rockford, IL, USA). 40 μg of proteins were resolved by electrophoresis on SDS-polyacrylamide gel and transferred onto nitrocellulose membranes using the transblot TURBO (Bio-Rad, Hercules, CA, USA). Blots were probed overnight at 4°C with primary antibodies, all diluted 1:1000. Immunodetection was performed with secondary antibodies conjugated to horseradish peroxidase. The reactive bands were detected using chemiluminescence (ECLplus; Euroclone, Padova, Italy). Quantitative analysis was performed using the QuantityOne analysis software (Bio-Rad, Hercules, CA, USA).

Fluorescence immunohistochemistry and quantitative analysis

At the end of experiments the organotypic slices were harvested and fixed O/N in ice-cold paraformaldehyde, 4% in PBS buffer. The day after, slices were rinsed in PSB and placed for at least 2 days in 18% sucrose in PBS. Immunostaining was performed with the free-floating method reported in (Lana *et al.* 2014). Day 1: Organotypic hippocampal slices were placed in a multiwell and washed 3 times for 5 min in PBS-TX, then blocked for 60 min with BB containing 10% Normal Goat Serum. Slices were then incubated overnight at 4°C under slight agitation with a combination of two different primary antibodies (anti-NeuN, catalogue number: #ABN78, 1:400; Millipore, Billerica, MA, USA; GluA1-AMPA subunit (catalogue number: #AGC-004), 1:100 or GluA2-AMPA subunit (catalogue number: #AGC-005), 1:100; Alomone Labs, Jerusalem Israel) dissolved in BB.

Day 2: sections were incubated for 2 h at room temperature in the dark with AlexaFluor 488-conjugated donkey anti-rabbit IgG secondary antibody diluted in BB and then for 2 h at room temperature in the dark with AlexaFluor 488-conjugated donkey anti-rabbit IgG (catalogue number: #A32790) secondary antibody plus AlexaFluor 555 goat anti-mouse (catalogue number: #A-21422; Invitrogen, Thermo Fisher) both diluted 1:400 in BB. After 3 washings slices were mounted onto gelatin-coated slides using Vectashield hard set mounting medium with DAPI (catalogue number: #H-1200-10; Vector Laboratories).

Slices were observed under a LEICA TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) equipped with a 63X objective (z step of 0.3 µm). Confocal scans were acquired keeping all parameters constant. Image analyses were conducted on z-stacks projections which contained the region of interest, corresponding to the CA1 area, using Image J (National Institute of Health, http://rsb.info.nih.gov/ij). Quantification of GluA1 and GluA2 AMPA subunit immunoreactivity in CA1 was performed separately using Image J. Forty consecutive z-scans (0,3 µm each, total 12 µm) were stacked, starting at 10 µm inside the slice and an appropriate

and constant threshold level was selected. Quantitation of immunofluorescence was then obtained from the ratio between positive pixels above threshold and total pixels in each region of interest.

Real-time PCR for REST and GluA2 mRNA expression

Real time PCR was performed as previously reported in (Llorente et al., 2015; Lapucci et al., 2017). Total ribonucleic acid (RNA) was extracted using Trizol Reagent (catalogue number: #15596018, Life Technologies). Any contaminating DNA in the RNA samples was removed by incubation with DNase (Sigma Aldrich, Madrid, Spain). Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Isolated RNA was maintained at 80°C until further processing. One µg of RNA was reverse transcribed using iScript (Bio-Rad, Milan, Italy). Real-Time PCR was performed in duplicate using Rotor-Gene 3000 (Qiagen, Milan, Italy) and the Rotor-Gene TM SYBR® Green PCR Kit (Qiagen, Milan, Italy), the reactions were run at 95°C for 30 seconds, 95°C for 5 sec and 60°C for 15 sec for 45 cycles. As an internal control for normalization, PCR reactions were performed concurrently with the amplification of a reference gene, 18S ribosomal RNA (rRNA).

The following primers were used:

REST: Forward primer (5‘–3‘) GACGGAGAGCAAACCCAAGACCAG Reverse primer (5′–3′) CTTGAGCCATCTCCGAGGAGGGTTC;

GluA2: Forward primer (5‘–3‘) CCAAGGACTCGGGAAGTAAGG, Reverse primer (5′–3′) CCCCCGACAAGGATGTAGAA;

RNA 18S: Forward primer (5‘–3‘) GATTAAGTGCCCTTTGTA, Reverse primer (5′–3′) GATCCGAGGGCCTCACTAAAC.

Preparation of Protein Extracts and Western Blot Analyses

Proteins from hippocampal slices were extracted as previously described with minor modifications (Caffino *et al.* 2018; Fumagalli et al., 2008). Briefly, hippocampal slices were homogenized in a teflon-glass homogenizer in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 1 mM MgCl2, 1 mM NaHCO3 and 0.1 mM PMSF, in presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors and an aliquot of each homogenate was then sonicated. The remaining homogenate was centrifuged at 13000 g for 15 min obtaining a pellet. This pellet was resuspended in a buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100,000 x g for 1 h. The resulting pellet, referred as postsynaptic density (PSD) or Triton X-100 insoluble fraction (TIF), was homogenized in a glass–glass potter in 20 mM HEPES, protease and phosphatase inhibitors and stored at -20°C in presence of glycerol 30%. Total proteins have been measured in the total homogenate and in the TIF fraction according to the Bradford Protein Assay procedure (Bio-Rad Laboratories, Italy), using bovine serum albumin as calibration standard.

Equal amounts of proteins of the homogenate (6 µg) and of TIF fraction (5 µg) were run on criterion TGX precast gels (Bio-Rad Laboratories) under reducing conditions and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad Laboratories). Blots were blocked 1 h at room temperature with I-Block solution (catalogue number: # T2015, Life Technologies, Italia, Italy) in TBS + 0.1% Tween-20 buffer and incubated with antibodies against the proteins of interest.

The conditions of the primary antibodies were the following: anti N-Cadherin (catalogue number: #13116, 1:1000, Cell Signaling Technology, USA); anti mGluR5 (1:2000, Chemicon, USA); anti GRIP (catalogue number: #ABN27; 1:1000, Sigma Aldrich); anti GluA1 (catalogue number: #13185; 1:2000, Cell Signaling Technology); anti GluA2 (catalogue number: #13607; 1:1000, Cell Signaling Technology); anti SAP97 (catalogue

number: #ab2057181:1000, Abcam, Cambridge UK); anti PSD-95 (catalogue number: #2507; 1:4000, Cell Signaling Technology), anti β-catenin (catalogue number: # sc-7963; 1:1000, Santa Cruz Biotechnology, USA) and anti β-Actin (catalogue number: # A5316; 1:10000, Sigma-Aldrich). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories). Gels were run 3 times each and the results represent the average from 3 different western blots.

Electrophysiological Recordings

Whole-cell voltage-clamp recordings were performed in CA1 pyramidal neurons from organotypic hippocampal slices as previously described (Gerace et al., 2019). The slices were chronically treated with 150 mM of EtOH or under control conditions, followed by 4-6 hours of EtOH withdrawal before recordings. A single slice was removed from the culture insert, placed in a flow chamber positioned under the microscope objective and continuously perfused with warm (34-35°C) artificial Cerebrospinal Fluid (aCSF), composed of (in mM) 130 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 10 glucose, 2 $CaCl_2$ and 1 $MgSO_4$ and saturated with a 95% O_2 + 5% CO_2 gas mixture. Whole-cell pipettes were pulled from thin-walled borosilicate capillaries (Harvard Apparatus, London, UK) with a vertical puller (Narishige PP830, Narishige International Limited, London, UK) back-filled with the following intracellular solution (in mM): K^+ Methanesulfonate (120), KCl (15), HEPES (10), EGTA (0.1), $MgCl_2$ (2), Na_2PhosphoCreatine (5), Na_2GTP (0.3), MgATP (2), resulting in a bath resistance of 3-5 MΩ. For coupled recordings of electrical and fluorescent calcium signals, 0.1 mM of Fluo 4 pentapotassium salt (Molecular Probes) was added. Signals were sampled at 10 kHz and low-pass filtered at 3 kHz with an Axon Multiclamp 700B (Molecular Devices, Sunnyvale, CA, USA). After establishing a Giga-Ohm seal, a whole-cell

configuration was achieved by rupturing the neuronal membrane. Access resistance was monitored during voltage clamp recordings with brief test pulses (−10 mV, 500 ms), throughout the experiment. All data were analyzed using pCLAMP (Axon Instruments) and GraphPad Software (San Diego, CA). AMPA-mediated electrical and optical responses were studied by obtaining sequences of 30 X 30-second recording frames from individual CA1 neurons in whole-cell voltage clamp ($V_{HOLDING}$ = -65mV) configuration. After the achievement of stable baselines, a mix of inhibitors was bath-applied which included the voltage-dependent sodium channel blocker TTX (1 µM), the NMDA receptor antagonist DL-2-Amino-5-phosphonopentanoic acid (APV, 50 µM) and the $GABA_A$ receptor antagonist picrotoxin (PTX, 50 µM). After 5 minutes, 0.3 µM AMPA was applied, in presence of inhibitors, in order to record pharmacologically-isolated cellular responses.

Microfluorometric Determination of Calcium Responses

AMPA-induced calcium responses were studied as described in Carbone et al. (2017) with some modifications. During electrophysiological recordings, fluorescence signal was collected from a square-shaped window comprising the cell body of the neuron under investigation loaded with 100 µM of the high-affinity, non-ratiometric calcium dye Fluo 4 pentapotassium salt (Molecular Probes). Fluorescence was elicited with a 488 nm LED and collected with a photomultiplier tube (PMT; Cairns Research) with a 10 kHz sampling rate. LED excitation was triggered with the electrophysiological protocol and consisted of 1 sec of LED ON at the end of each 30 second gap free recording. Somatic calcium responses (SCRs) are reported as $\Delta F/F_0$, where F_0 signal was the baseline emission of the loaded neuron at rest, and ΔF was defined as $F_{AMPA} - F_0$ where F_{AMPA} was taken at the end of AMPA application. Background fluorescence was obtained by measuring the emission of a Fluo 4-free area of the

slice and subtracting the obtained value from F_0. Off-line analysis was performed with Clampfit 10 (Molecular Devices) and Origin 9.1.

Statistical analysis

Data are presented as means ± SEM of n experiments from independent cell preparations. Statistical significance of differences between PI fluorescence intensities, immunostaining or Western blot optical densities was evaluated by performing one-way ANOVA followed by Tukey's w test for multiple comparisons. For electrophysiological experiments and determination of calcium response, statistical significance was evaluated by performing a Student's t test for unpaired samples. Differences were considered significant for *p < 0.05, **p < 0.01, ***p < 0.001. All statistical calculations were performed using GRAPH-PAD PRISM v.5 for Windows (GraphPad Software, San Diego, CA, USA). No sample calculation was performed to predetermine the sample size. Data were not assessed for normality. NO test for outliers was conducted.

RESULTS

We used rat organotypic hippocampal slice cultures exposed to 150 mM EtOH for 7 days (chronic) to mimic chronic EtOH consumption and then we incubated the slices in EtOH-free medium for the subsequent 24 h to mimic EtOH withdrawal *in vitro*. In these experimental conditions, EtOH withdrawal led to a dose-dependent CA1 pyramidal cell injury examined using propidium iodide fluorescence as previously demonstrated and published (Gerace et al., 2016; Gerace et al., 2019). With the aim to study the mechanism of EtOH withdrawal toxicity, we firstly performed western blotting experiments to characterize the composition of AMPA receptor tetramers after EtOH withdrawal. We used specific antibodies

directed against GluA1, GluA2 and GluA3 AMPAR subunits (Fig. 1, A, B and C). We found that EtOH withdrawal induced a significant increase in the expression of GluA1 (Fig 1, panel A), no change was observed for GluA3 (Fig 1, panel C), while a relative trend in a reduction was observed in the expression of GluA2 (Fig 1, panel B) subunit. To this matter, the analysis of GluA1/GluA2 was significant after EtOH withdrawal as compared to control and to chronic EtOH and GluA3/GluA2 ratio expression was significant only versus control after EtOH withdrawal, while no changes was noticed for GluA1/GluA3 ratio. These data suggest that EtOH withdrawal induces relevant modification of AMPAR subunit composition leading to formation of GluA2-lacking, CpAMPARs (Fig. 1, D, E and F). We confirmed the data obtained by western blot analysis with immunofluorescence technique. Figure 2A shows that GluA1 expression is significantly increased while GluA2 appears to be decreased (Fig. 2B) in CA1 neurons after 24h of EtOH withdrawal compared to control slices. In order to elucidate the mechanisms of GluA2 lacking AMPA formation, we considered another type of evidence resulting from the analysis of mRNA expression levels. In particular, we used real time PCR to study the transcriptional repressor element-1 (RE1) silencing transcription factor (REST) gene, which was demonstrated to repress the transcription of GluA2 AMPAR subunit (Noh *et al.* 2012). The results showed that EtOH withdrawal induces the upregulation of REST gene, conversely GluA2 mRNA gene was downregulated (Fig. 3), indicating that GluA2 lacking AMPARs formation could be due to a transcriptional process. Furthermore, in order to study the trafficking of AMPARs, we measured the expression levels of GluA1 and GluA2 in enriched postsynaptic membranes (PSD, SI, figure 1) under control, chronic EtOH or EtOH withdrawal (Figure 4). In contrast with the previously shown increase of GluA1 subunit in total homogenates, these data showed that EtOH downregulates the expression of both GluA1 and GluA2 AMPA subunits in PSD, suggesting that AMPARs should be relocated in the intracellular and/or extrasynaptic membrane compartments after EtOH treatment. Since

cadherin adhesion complex proteins have been recently shown to contribute to the stabilization of GluA1/2-containing AMPARs into the synaptic membranes (Mameli et al., 2011; Mills et al., 2017), we analyzed the levels of the scaffold proteins SAP97, GRIP and of N-cadherins in total homogenate and PSD preparations of organotypic slices under control, chronic EtOH or EtOH withdrawal conditions (Fig. 5). Our results showed that in total homogenate there is no modification of the expression level of the proteins examined in any experimental conditions. On the contrary, a clear reduction of their expression was present in PSD. Collectively, these results indicate that chronic EtOH induces profound alterations in the molecular composition and surface expression of AMPARs. We then sought evidence that EtOH-induced molecular rearrangements result in increased calcium conductance through CpAMPARs. To directly test this hypothesis, we simultaneously measured electrophysiological and fluorescence calcium responses induced by AMPA stimulation in organotypic hippocampal slices during EtOH withdrawal and in control conditions. These experiments were performed during early withdrawal (4-6 hours), when neurons are still viable and amenable to functional investigation. CA1 pyramidal neurons were individually loaded with the non-ratiometric calcium dye Fluo4 and then AMPA-mediated electrical and fluorescence responses were elicited by agonist application. Our results show that AMPA reliably induces an inward cationic current in both controls and EtOH-treated slices. Moreover, both peak value and area under the curve (AUC) of AMPA-induced inward currents are significantly increased in CA1 neuron after EtOH withdrawal compared to control slices (Figure 6). These findings confirm that the EtOH-dependent rearrangements in expression and localization of AMPARs that we observed lead to functional changes. Of note, the increase in AMPA current is reflective of an actual increase in current density, since no differences in whole-cell capacitance (a proxy for soma size) were observed between the two conditions (SI, figure 5). AMPA application also induced detectable elevations in intracellular

calcium in both experimental groups but, quite unexpectedly, the magnitude of AMPA-induced calcium transients was significantly smaller in CA1 neurons from EtOH-treated slices compared to controls (Figure 6D). Our data suggest that CpAMPARs may lead to excitotoxicity and neuronal death by driving network hyperexcitability rather than by a direct contribution of CpAMPAR-mediated calcium influx. To selectively assess the contribution of CpAMPARs in EtOH-induced hyperexcitation, we tested the neuroprotective efficacy of 1-naphthyl acetyl spermine (NASPM), a selective blocker of CpAMPARs, to protect CA1 neurons from EtOH withdrawal toxicity, compared to NBQX (a non-selective AMPA antagonist). Our data showed that incubation of NASPM (10 μM) during the 24h of EtOH withdrawal significantly attenuated EtOH-withdrawal-induced injury as well as NBQX, suggesting that the neurotoxicity induced by EtOH withdrawal is largely mediated by CpAMPARs (Figure 7).

DISCUSSION

We previously demonstrated that EtOH withdrawal induces cell death in organotypic hippocampal slices. Cell death was largely prevented by the non selective AMPAR blocker NBQX and preceded by an increase in the amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) and in GluA1/GluA2 expression ratio, thus pointing to CpAMPARs, and to CpAMPAR-mediated calcium currents, as a possible death mechanism involved in EtOH toxicity (Gerace et al., 2019). Building on this hypothesis, we investigated the implication of AMPARs, and CpAMPARs in particular, in EtOH toxicity, by using biochemistry, molecular biology, microscopy and physiology. We first examined the expression levels of the main AMPAR subunits (GluA1, GluA2, GluA3) in the total homogenate of hippocampal slices chronically exposed to EtOH for 7 days followed by 24 hours withdrawal. We found that EtOH withdrawal modifies (alters) AMPA subunit composition leading to formation of

GluA2-lacking CpAMPARs (Fig. 1), in line with previous published manuscripts (Acosta et al., 2012; Jin et al., 2014; Li et al., 2017). Remarkably, the switch to CpAMPARs has been implicated in the alterations associated to EtOH consumption, such as behavioral reinforcement (Mameli et al., 2011; Marty et al., 2012). In addition, it has been reported that repeated systemic administration of EtOH causes facilitation of LTP that is mediated by the insertion of AMPAR into the synaptic membrane and in a long-lasting increase of the GluA1 and GluA2 AMPAR subunits expression in the dorsomedial striatum of rats (Wang *et al.* 2012).

In an attempt to reveal the mechanism underlying the formation of GluA2-lacking and CpAMPARs as a consequence of EtOH withdrawal, we examined the mRNA levels of REST gene, one of the repressors involved in the regulation of the AMPAR subunit GluA2 (Noh *et al.* 2012). The activation of REST is clinically relevant in a model of ischemic stroke *in vivo* where it was reported to bind a subset of transcriptionally responsive genes, including GluA2 (Noh *et al.* 2012). Consistently, we found that REST gene expression is significantly upregulated as a consequence of withdrawal and, at the same time, GluA2 gene expression is significantly reduced.

In order to get further insights into the modulation of AMPAR expression and function, we investigated the proteins regulating the trafficking and localization of these receptors in synaptic membranes. To this end, we examined the levels of GluA1 and GluA2, as well as scaffold proteins SAP97, GRIP, N-cadherin and beta catenin in total homogenate and PSD-enriched fractions from organotypic slices in control, chronic EtOH or EtOH withdrawal. In contrast with the previously shown increase of GluA1 subunit in total homogenates, we observed a clear reduction of the expression of all the proteins analyzed (GRIP, SAP97, N-cadherin) in the PSD preparation. To explain our results, we can speculate that EtOH withdrawal may induce the insertion in PSD of CpAMPARs in the early phase of EtOH

withdrawal. This is consistent with the results of our previous work showing an increase of AMPA-mediated sEPSCs immediately after EtOH withdrawal, (Gerace et al., 2019) and with the neuroprotective effects of NASPM from EtOH withdrawal toxicity. AMPARs may be then trafficked by lateral diffusion in the extra-synaptic and intracellular pools, triggering calcium signaling pathways leading to excitotoxicity and CA1 neuronal death *in vitro*. Anyway, the extra-synaptic distribution of AMPARs is critical for synapse function and the lateral distribution of AMPARs within the PSD provides an effective mechanism for modulating synaptic strength (Newpher & Ehlers, 2008; MacGillavry et al., 2011). For example, hippocampal neurons exposed to TNFα treatment display an increased surface level of GluA2-lacking CpAMPARs after 15 min, the majority of which are relocalized to extrasynaptic sites. This mechanism has been proposed to contribute to excitotoxic neuron death and is prevented by CpAMPAR antagonist NASPM (Leonoudakis *et al.* 2008). Moreover, it was largely described how the extra-synaptic NMDA receptor signaling promoted mitochondrial dysfunction, loss of integrity of neuronal structures and connectivity triggering cell death pathways (Hardingham et al., 2002; Hardingham & Bading, 2010). Functional determination of AMPA-mediated currents and calcium influx during early EtOH withdrawal confirms that remodeling of AMPARs results in functional changes, as we observe a significant increase of AMPA-induced currents. This is consistent with the increase in EPSC amplitude we previously reported (Gerace et al., 2019) and with pioneering single channel recordings reporting larger unitary conductance in GluA2-lacking CpAMPARs (Swanson et al., 1997). At the same time, our experiments seem to exclude a direct involvement of CpAMPAR-mediated calcium in neurotoxicity. The molecular mechanism underlying paradoxical reduction of AMPA-induced calcium influx in presence of increased CpAMPAR levels requires specific investigation, which goes beyond the scope of the present study.

In light (view) of these results, we propose that increased network excitability, promoted by simultaneous increase of AMPAR function and removal of EtOH-mediated potentiation of inhibition, is the main mechanism of EtOH excitotoxicity, thus explaining the protective effect exerted by AMPAR blockers. In this regard, the evidence that the non-selective blocker NBQX and the selective CpAMPAR blocker NASPM show equal neuroprotective action suggests that, at molecular level, CpAMPARs are the main molecular entities accounting for the increased AMPA-induced current and thereby represent a potential disease-specific target for the development of neuroprotectants against the neurotoxic effects of EtOH withdrawal.

Acknowledgments and conflict of interest disclosure

This work was supported by grants from Ente Cassa di Risparmio di Firenze, University of Florence, Zardi-Gori foundation and MIUR Progetto Eccellenza. The authors have no conflict of interest to declare.

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LEGENDS TO FIGURES

Figure 1

Ethanol withdrawal induces changes in AMPA receptors subunit composition. **Top**: experimental protocol (**A**). **Middle:** representative Western blots using antibodies directed against the AMPA subunit GluA1, GluA2 and GluA3 (**B, C, D**) and quantitative analysis of immunoreactive bands (**E, F, G**), showing that EtOH withdrawal increases the expression of the AMPA subunit GluA1. Tubulin was used as loading control. **Bottom**: analysis of the ratio between GluA1/GluA2, GluA1/GluA3 and GluA3/GluA2 AMPA subunits expression in mature slices after chronic EtOH and EtOH withdrawal suggesting that EtOH withdrawal modifies AMPA subunit composition leading to GluA2-lacking and Ca^{2+}−permeable AMPARs. Data are expressed as a percentage of control. Bars represent the mean ± SEM of at least 5 experiments from independent cell preparations (about ≥8 slices for each experimental point). *P<0.05 vs. CRL and #P<0.05 vs. Chronic EtOH (ANOVA + Tuckey's test).

Figure 2

Figure 2. Ethanol withdrawal increases GluA1 AMPA subunit expression in CA1 Stratum pyramidale of organotypic hippocampal slices. A-B2: Representative confocal microscopy images showing CA1 immunostaining of neurons (NeuN, red, A1,B1), and GluA1- AMPA subunit (GluA1, green A2,B2) in CA1 pyramidal neurons of organotypic hippocampal slices in control (**A-A2**) or after 24h of EtOH withdrawal (**B-B2**). Scale bar: 10 μm. **C**: quantitative analysis of GluA1 immunoreactivity in CA1 Stratum pyramidale of control (CRL) (white column, n= 6), and EtOH withdrawal slices (black column, n = 6) (**P<0.05 EtOH withdrawal vs CTR, Student's t test). **Figure 2: C-D2:** Representative confocal microscopy images showing CA1 immunostaining of neurons (NeuN, red, C1,D1), and GluA2- AMPA subunit (GluA2, green, C2,D2) in CA1 Stratum pyramidale of

organotypic hippocampal slices in control (**C-C2**) or after 24h of EtOH withdrawal (**D-D2)**. Scale bar: 10 μm. **E**: quantitative analysis of GluA2 immunoreactivity in CA1 Stratum pyramidale of control (CRL) (white column, n= 4), and EtOH withdrawal slices (black column, n = 3). The decrease was not statistically significant (Student's t test).

Figure 3

Ethanol withdrawal induces the upregulation of the transcriptional repressor element-1 (RE1) silencing transcription factor (REST). Real time PCR assessed mRNA expression levels of REST and of the AMPA subunit GluA2 gene after 24h of EtOH withdrawal. Data are expressed as percentage of control protein levels (white column). Bars represent the mean ± SEM of 4 experiments from independent cell preparations (about ≥4 slices for each experimental point). *p <0.05 vs. CRL (ANOVA + Tukey's w test).

Figure 4

EtOH induces a significant reduction of GluA1 and GluA2 AMPA receptor subunit expression in the postsynaptic densities (PSD). The experiments were conducted as described in Figure 1. Representative immunoblots showing that the main AMPA receptor subunits GluA1 (left panel) and GluA2 (right panel) are significantly decreased after chronic EtOH and EtOH withdrawal treatment in the PSD of organotypic hippocampal slices. Data are expressed as percentage of control protein levels (white column). Bar represent the mean ± SEM of 6 experiments from independent cell preparations (about ≥12 slices for each experimental point). **p <0.01 and *p <0.05 vs. CRL (ANOVA + Tukey's w test).

Figure 5

Effects of EtOH withdrawal on GRIP, SAP97 and N-cadherin expression in the whole homogenate and postsynaptic densities (PSD). (**A**) Representative immunoblots showing that GRIP, SAP97 and N-cadherin proteins expression are significantly decreased after chronic EtOH and EtOH withdrawal treatment in the PSD, but not in the homogenate of organotypic hippocampal slices. Panel (**B**) shows the protein levels of GRIP1 (upper panels), SAP97 (middle panels) and N-cadherin (lower panel). Data are expressed as percentage of control protein levels (white column). Bar represent the mean ± SEM of 6 experiments from independent cell preparations (about ≥12 slices for each experimental point). **p <0.01 and *p <0.05 vs. CRL (ANOVA + Tukey's w test).

Figure 6

Electrophysiological and fluorescence calcium responses induced by AMPA stimulation in organotypic hippocampal slices. (**A**) CA1 pyramidal cell filled with Fluo-4 excited with blue LED light. (**B**) Experimental paradigm. *Top*: Electrophysiological protocol consisted of 30X, 30-second gap free recordings. *Middle and bottom*: Optical protocol consisted of 1 second LED pulse and fluorescence collection with PMT at the end of each 30 second gap free recording. (**C**) *Top*: time course of isolated AMPA current reported as average of "n" superimposed recordings ± SEM (gray shading) from CRL (left, n = 11) and EtOH withdrawal group (right, n = 16). *Bottom*: quantification of AMPA-mediated current expressed as peak amplitude (*left*; CRL: -53.11 ± 6.38 pA, n = 11; EtOH withdrawal: -81.82 ± 8.58 pA, n = 16) and AUC (*right*; CRL: -1.51 ± 0.20 107pA·ms, n = 11; EtOH withdrawal: -2.20 ± 0.22 107pA·ms, n = 16). (**D**) *Top*: averaged fluorescence responses to pulsed LED excitation before (F0) and after (F_{AMPA}) agonist application, from CRL (left, n = 10) and EtOH withdrawal group (*right*, n = 14). Scale bar, x = 500 ms, y = 0.2 ΔF/F0. *Bottom*: quantification of absolute ΔF/F0 (*left*, CRL: 0.11 ± 0.02, n = 11; EtOH withdrawal: 0.05 ±

0.01, n = 16) and $\Delta F/F_0$ normalized to total AMPA current (*right*, CRL: -0.83 ± 0.19, n = 11; EtOH withdrawal: -0.23 ± 0.04, n = 16). All histograms report data as mean values ± SEM vs CRL group. *p<0.05 and **p<0.01 vs CRL group (Student's t-test).

Figure 7

Neuroprotective effects of the selective Ca^{2+}-permeable AMPARs antagonist NASPM on EtOH withdrawal toxicity. A) Experimental protocol. **B)** Hippocampal slices displaying background levels of PI fluorescence under control conditions, an intense PI labeling in the CA1 subregion 24 h after EtOH withdrawal and a reduction of CA1 PI fluorescence when incubated with non-selective AMPA antagonist NBQX and the selective blocker of Ca^{2+}-permeable AMPARs NASPM. **C)** Quantitative analysis of CA1 region expressed as percentage of CRL PI fluorescence. Values represent the mean ± SEM of at least 5 experiments from independent cell preparations (about ≥8 slices for each experimental point). ***P < 0.001 vs. CRL and # < 0.05 vs. EtOH withdrawal alone (ANOVA þ Tukey's w test).