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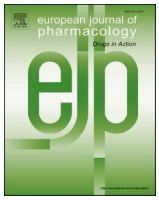

Ethanol-induced AMPA alterations are mediated by mGLU5 receptors through miRNA upregulation in hippocampal slices

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ABSTRACT

Prenatal alcohol exposure (PAE) affects neuronal networks and brain development causing a range of physical, cognitive and behavioural disorders in newborns that persist into adulthood. The array of consequences associated with PAE can be grouped under the umbrella-term 'fetal alcohol spectrum disorders' (FASD). Unfortunately, there is no cure for FASD as the molecular mechanisms underlying this pathology are still unknown. We have recently demonstrated that chronic EtOH exposure, followed by withdrawal, induces a significant decrease in AMPA receptor (AMPAR) expression and function in developing hippocampus *in vitro*. Here, we explored the EtOH-dependent pathways leading to hippocampal AMPAR suppression. Organotypic hippocampal slices (2 days in cultures) were exposed to EtOH (150 mM) for 7 days followed by 24 h EtOH withdrawal. Then, the slices were analysed by means of RT-PCR for miRNA content, western blotting for AMPA and NMDA related-synaptic proteins expression in postsynaptic compartment and electrophysiology to record electrical properties from CA1 pyramidal neurons. We observed that EtOH induces a significant downregulation of postsynaptic AMPA and NMDA subunits and relative scaffolding protein expression and, accordingly, a decrease of AMPA-mediated neurotransmission. Simultaneously, we found that chronic EtOH induced-upregulation of miRNA 137 and 501-3p and decreased AMPA-mediated neurotransmission are prevented by application of the selective mGlu5 antagonist MPEP during EtOH withdrawal. Our data indicate mGlu5 via miRNA137 and 501-3p expression as key factors in the regulation of AMPAergic neurotransmission that may contribute, at least in part, to the pathogenesis of FASD.

1. Introduction

Prenatal alcohol exposure (PAE) affects brain development causing a range of physical and cognitive abnormalities in newborns that persist into adulthood (Lange et al., 2017; Cuzon Carlson et al., 2020). During maternal ethanol (EtOH) consumption, EtOH can easily cross the placenta and reach the fetus (Lange et al., 2017) causing an array of consequences, ranging from mild cognitive impairment to neurological conditions, that are defined as fetal alcohol spectrum disorders (FASD). The etiology of FASD is very complex and little is known with respect to the exact mechanism(s) involved.

Studies in animal models have revealed various neurochemical events through which PAE impairs the complex processes underlying neurodevelopment (Dunty et al., 2001; Creeley and Olney, 2013; Tunc-Ozcan et al., 2018) and different brain structures affected by PAE. In particular, the hippocampal formation is highly susceptible to the effects of alcohol (Guerri et al., 2009). We have recently demonstrated that chronic EtOH exposure induces a significant decrease in AMPA receptor (AMPAR) expression and function and rearrangement of dendritic microtubules, suggestive of incorrect formation of neuronal circuits and

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mechanisms that may contribute to mental retardation, which may be of relevance for the alterations observed in newborns affected by FASD (Gerace et al., 2016).

Recently, it has been demonstrated that alcohol alters the relative expression of specific microRNAs (miRNAs) both in animal models (Wang et al., 2009; Mandal et al., 2018), as well as in infants (Mahnke et al., 2021). MicroRNAs are small single-stranded non-coding RNAs that regulate gene expression at post-transcriptional level, thereby influencing several physiological and pathological mechanisms (Tüfekci et al., 2014). Their dysfunction has been implicated in numerous psychiatric disorders (Issler and Chen, 2015); (Warnica et al., 2015) and also in drug addiction-related behaviors (Quinn et al., 2015). Many studies have shown that miRNAs control mechanisms involved in the development of alcohol use disorder (Wang et al., 2009; Gardiner et al., 2016; Mandal et al., 2018). For instance, the neuronal increase of miR-9 induced by alcohol exposure is associated with the modulation of ion channel functions, playing a crucial role in alcohol tolerance (Pietrzykowski et al., 2008). The assessment of the regulation of these miRNAs may provide important clues about the mechanisms underlying fetal vulnerability to alterations in the maternal-fetal environment and yields insights into the genesis of FASD. Among miRNAs, miR-137 has been linked to alcohol-related disorder in adolescent rats submitted to alcohol intermittent exposure (AIE) (Kyzar et al., 2019). Importantly, AMPA receptor subunit GluA1 is a direct target of miR-137 and miR-501-3p (Olde Loohuis et al., 2015); (Hu et al., 2015). Indeed, postsynaptic downregulation of miR-137 at CA3-CA1 hippocampal synapse selectively enhances AMPAR-mediated synaptic transmission, a mechanism mediated by metabotropic glutamate receptor 5 (mGlu5) (Olde Loohuis et al., 2015). In addition, miR-501-3p mediates the activity-dependent regulation of GluA1 expression (Hu et al., 2015). These data suggest that alcohol exposure during hippocampal development may impact the homeostasis of the glutamatergic synapse from a molecular, structural and functional point of view.

Based on the above-mentioned lines of evidence, we hypothesized a role for microRNAs 137 and 501-3p in the regulation of EtOH-induced modulation of AMPA neurotransmission, through a mechanism involving mGlu5-dependent translational repression of AMPAR subunit GluA1, in an *in vitro* model of developing hippocampal tissue chronically exposed to EtOH.

2. Material and methods

2.1. Animals

Male and female Wistar rats 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 protocol was 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.

2.2. Materials

Ethanol (EtOH, CAS No: 64-17-5) was purchased from Sigma. Tissue culture reagents were obtained from Gibco-BRL and Sigma. 2-Methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP, catalogue number: #1212/10) and 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline- 7-sulfonamide disodium salt (NBQX, #1044 catalogue number:/1) were purchased from Tocris.

2.3. Preparation of rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as previously reported (Gerace et al., 2012). Briefly, after decapitation hippocampi were isolated and removed from the brains of male and female Wistar rat pups (PND 7), 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; 4 slices per insert), which were placed in six well tissue culture plates containing 1.2-ml medium per well. Typically, slices were maintained at 37 °C in an incubator in an atmosphere of humidified air and 5% CO 2 for 10 days to reach maturity *in vitro*. For these experiments, organotypic hippocampal slices were used after 2 days *in vitro,* in order to study the effects of EtOH in developing hippocampus. Before experiments all the slices were screened for viability by phase-contrast microscopy analysis; slices displaying signs of neurodegeneration were discarded from the study.

2.4. Ethanol exposure and Drug Treatment in immature organotypic hippocampal slices

The experiments were conducted as previously described in (Gerace et al., 2016). Briefly, hippocampal slice cultures were exposed for 7 days to 150 mM of EtOH after 2 days (immature) of culture *in vitro*. To reproduce a chronic assumption of EtOH, the medium was changed every day adding EtOH to the fresh culture medium (chronic EtOH). For control slices, the medium was changed every day by adding fresh culture medium. After 7 days of EtOH treatment, to reproduce EtOH withdrawal *in vitro*, some of the slices were incubated in EtOH fresh culture medium (EtOH withdrawal). To test the effect of mGlu5 inhibition, the slices were exposed to the selective antagonist of metabotropic glutamate receptor-5 MPEP (30 μM) EtOH-free medium during the 24 h of EtOH withdrawal. As reported (Gerace et al., 2016), following exposure to 150 mM (6.9 g/l) EtOH the concentration of EtOH in the medium was reduced by 15% 1 h later and by 79% 24 h later, resulting in an average daily concentration in the range 127 to 32 mM (5.8–1.5 g/l) determined by gas chromatography–mass spectrometry (GC/MS). Importantly, the maximal EtOH concentration detected in hippocampal slices was 2 mM (0.1 g/l) 1 h after EtOH exposure. As discussed (Gerace et al., 2012, 2016), the concentrations of drugs used in organotypic hippocampal slice experiments are generally higher than those expected from their Kd values and those used in cell cultures and need to be further increased because only a fraction of the drug diffuses across the membrane and reaches the tissue. At the end of the experiments, the slices were analysed for miRNA and electrophysiological investigations. All experiments were performed in primary slice cultures obtained from different litters.

2.5. Preparation of protein extracts and western blot analyses

Proteins from hippocampal slices were extracted as previously described with minor modifications (Gerace et al., 2021). 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) and phosphatase (Sigma-Aldrich) inhibitors and an aliquot of each homogenate was then sonicated. The remaining homogenate was centrifuged at 13,000 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 g for 1 h. The resulting pellet, referred as post-synaptic 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 GluN1 (RRID: AB_1904067; 1:1000 iblock buffer O/N 4 °C; Cell Signaling Technology), anti GluN2A (RRID: AB_2112295; 1:1000 iblock buffer O/N 4 °C; Cell Signaling Technology), anti GluN2B (RRID: AB_2798506; 1:1000 iblock buffer O/N 4 °C; Cell Signaling Technology); anti GRIP (RRID: AB_887728; 1:2000 iblock buffer O/N 4 °C; Synaptic System); anti GluA1 (RRID: AB_641040; 1:2000 iblock buffer O/N 4 °C; Cell Signaling Technology); anti GluA2 (RRID: AB_10622024; 1:1000 iblock buffer O/N 4 °C; Cell Signaling Technology); anti SAP102 (RRID: AB_2799325; 1:1000 iblock buffer O/N 4 °C; Cell Signaling Technology); anti SAP97 (RRID: AB_2091910; 1:1000 iblock buffer O/N 4 °C; Cell Signaling Technology); anti PSD95 (RRID: AB_561221; 1:4000 iblock buffer O/N 4 °C, Cell Signaling Technology), and anti β-Actin (RRID: AB_476697; 1:10,000 iblock buffer O/N 4 °C; 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 (BioRad Laboratories). Gels were run three times each and the results represent the average from three different runs (the full-size cropped WB bands are presented in Supplementary Figs. S1 and S2). We used a correction factor to average the different gels: correction factor gel B = average of (OD protein of interest/OD β-actin for each sample loaded in gel A)/(OD protein of interest/OD β-actin for the same sample loaded in gel B) (Caffino et al., 2020).

2.6. Real time PCR (qPCR) for microRNA measurement

Total RNA was isolated from organotypic hippocampal slices using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA was quantified by using the NanoPhotometer® (Implen, Munich, Germany) measuring the absorbance at 260 nm. cDNA was synthesized using the miRCURY LNA RT kit (Qiagen, Hilden, Germany). cDNA samples were stored at −20 °C. RT-quantitative PCR (qPCR) was performed using miRCURY LNA SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. The following primers from miRCURY LNA miRNA PCR Assays were used: (mir-137-3p: GeneGlobe ID - YP00206062, Qiagen; mir-501-3p: GeneGlobe ID - YP00205037, Quiagen; RNU6b: GeneGlobe ID - YP00203907, Qiagen). The analyses were performed using the Rotor-Gene Q thermal cycler (Qiagen). Each sample was tested in triplicate. RNU6b was selected as reference due to its stability across samples. The calculation of relative expressions was performed using the $2^{-\Delta\Delta Ct}$ formula.

2.7. Electrophysiological recordings

Whole-cell voltage-clamp recordings were performed in CA1 pyramidal neurons from immature organotypic hippocampal slices as previously described (Gerace et al., 2021). The slices were chronically treated with 150 mM of EtOH or under control conditions, followed by 24 of EtOH withdrawal or EtOH withdrawal plus 30 μM MPEP before recordings. Moreover, as experimental controls, the slices were also exposed to 30 μM MPEP alone for 24 h before recording or to acute application of EtOH (150 mM, 10 min) during 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 NaH2PO4, 24 NaHCO3, 10 glucose, 2 CaCl2 and 1 MgSO4 and saturated with a 95% O2 + 5% CO2 gas mixture. Whole-cell pipettes were pulled from thin-walled borosilicate capillaries (Harvard Apparatus) with a vertical puller (Narishige PP830, Narishige International Limited) back-filled with the following intracellular solution (in mM):K + Gluconate, KCl (15), HEPES (10), EGTA (0.1), MgCl2 (2), Na2PhosphoCreatine (5), Na 2GTP (0.3) and MgATP (2), resulting in a bath resistance of 3–5 MΩ. 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. All data were analysed using pCLAMP (Axon Instruments) and GraphPad Software (San Diego, CA). The AMPA receptor blocker NBQX (10 μM) was added at the end of the experiments to verify that the sEPSCs were AMPA receptor mediated, as previously reported in [11]. Moreover, passive properties of CA1 neurons were evaluated in the five experimental conditions (control, ethanol withdrawal, ethanol withdrawal + MPEP, control + MPEP and control plus acute EtOH) by measuring membrane capacitance, membrane resistance and resting membrane potential. Finally, to study the effects on the whole intrinsic excitability, increasing steps of depolarizing current (20 pA) were imposed in current-clamp configuration to CA1 neurons to measure: number and threshold of evoked action potentials (APs).

2.8. Statistical analysis

Data are presented as means ± SEM of n experiments from independent cell preparations. Each experimental point consisted of 40–72 hippocampal slices for RT-PCR and western blotting experiments and of 12–20 hippocampal slices for electrophysiological experiments. Statistical significance of differences between Western blot optical densities and electrophysiological experiments was evaluated by performing one-way ANOVA followed by Tukey's w test for multiple comparisons for unpaired samples or by Student's *t*-test for unpaired samples, as appropriate. Statistical significance of differences between miRNA levels was evaluated by performing one-way ANOVA followed by Dunnet's w test and two-way ANOVA followed by Tukey's w test for multiple comparisons for unpaired samples. Differences were considered significant for $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. All statistical calculations were performed using GRAPH-PAD PRISM v.8 for Windows (GraphPad Software).

3. Results

To determine whether EtOH alters the glutamatergic synapses, we examined the expression of the main AMPA and NMDA subunits and their specific scaffolding proteins in the total homogenate and in the post-synaptic density (PSD) of immature hippocampal slices immediately after chronic exposure to EtOH (150 mM, 7 days) and following 24h of withdrawal (150 mM for 7 days followed by 24h EtOH washout). As previously observed, the expression levels of both AMPA receptor subunits GluA1 and GluA2 in total homogenate are significantly decreased both after chronic EtOH and EtOH-withdrawal treatment (Table 1 and Gerace et al., 2016). We obtained similar results analysing GluA1 and GluA2 in PSD fraction, suggesting that AMPARs are significantly reduced in the postsynaptic compartment (Fig. 2 panel A). Consistently, a significant reduction has been also observed in their specific scaffolding proteins SAP97 and GRIP in the same experimental conditions and both in the total homogenate (Table 1) and in the PSD fraction (Fig. 2 panel C). Chronic EtOH and EtOH-withdrawal reduced the expression of GluN1, the obligatory subunit of NMDA receptor, and GluN2A and GluN2B, accessory subunits of NMDA receptor, in both whole homogenate (Table 1) and in the PSD fraction (Fig. 2 panel B). Accordingly, the expression levels of SAP102 and PSD95, the specific scaffolding proteins of the NMDA receptor, are significantly reduced in the same experimental conditions (Homogenate: Table 1; PSD: Fig. 2 patel D).

We focused on EtOH induced AMPAR modifications and to define one possible mechanistic explanation, we hypothesized that EtOH may induce increased levels of both miR-137 and miR-501-3p leading to a translational repression of GluA1 subunit. The levels of miR-137 (Fig. 3, left panel) and miR-501-3p (Fig. 3, right panel) were assessed by means of RT-PCR after acute EtOH application (150 mM, 30 min) or chronic

Table 1
Effect of EtOH on the expression of glutamatergic makers in the homogenate of immature hippocampal slices, expressed as mean % ± SEM. Detailed F values, degrees of freedom and p values relative to one-way ANOVA analyses of protein levels are presented. One-way ANOVA followed by Tukey's multiple comparisons test: *p < 0,05; **p < 0,01; ***p < 0,001 vs CTRL.

	CTRL	Chronic EtOH	EtOH withdrawal	One-way ANOVA	
				F value	P value
GluA1	100 ± 15	51 ± 10 *	50 ± 5 *	F (2, 12) = 6862	P = 0,0103
GluA2	100 ± 17	53 ± 9 *	53 ± 6 *	F (2, 12) = 5161	P = 0,0241
GRIP	100 ± 13	45 ± 9 **	42 ± 7 **	F (2, 12) = 10,91	P = 0,0020
SAP97	100 ± 6	66 ± 5 ***	71 ± 3 **	F (2, 12) = 15,22	P = 0,0005
SAP102	100 ± 9	69 ± 11*	68 ± 7 *	F (2, 11) = 7368	P = 0,0093
GluN2A	100 ± 7	64 ± 8 **	61 ± 6 **	F (2, 12) = 9485	P = 0,0034
GluN2B	100 ± 17	37 ± 12 *	49 ± 7 *	F (2, 11) = 6665	P = 0,0127
GluN1	100 ± 15	58 ± 9 *	56 ± 5 *	F (2, 11) = 5637	P = 0,0206
PSD95	100 ± 7	76 ± 6	76 ± 6	F (2, 10) = 4885	P = 0,0331

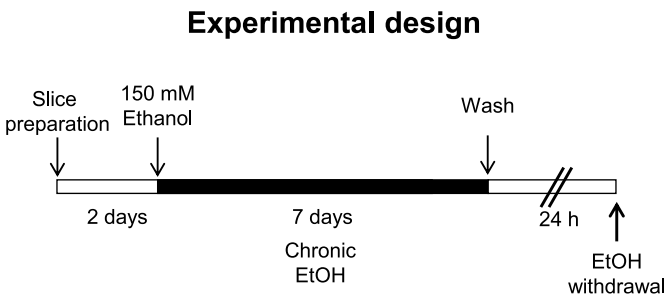

Fig. 1. *Experimental protocol* showing organotypic hippocampal slices after 2 days *in vitro* (immature) exposed to EtOH (150 mM) for 7 days (chronic EtOH) followed by 24 h of EtOH withdrawal.

EtOH exposure. Fig. 3 shows that immature hippocampal cultures exhibit increased levels of both miRNA 137 and 501-3p after chronic but not acute exposure to EtOH. Since, the link between mGlu5and EtOH has been largely described (Stevenson et al., 2019), we theorized that this process is mediated by mGlu5. Incubation with the mGlu5 negative allosteric modulator (NAM) MPEP in control conditions did not alter miRNA 137 and miR-501-3p levels, indicating that mGlu5 antagonism does not modify the physiological conditions (Fig. 4, grey column). Then, in order to reproduce *in vitro* the clinical situation according to which newborns exposed to alcohol during pregnancy are born in abstinence conditions, we measured miR-137 and miR-501 content after EtOH withdrawal with or without application of MPEP (30 μM). Interestingly, the increase of both miR-137 and miR-501-3p induced by chronic EtOH is preserved also after 24-h EtOH withdrawal and the application of MPEP significantly reduced miR-137 and miR-501-3p expression, proving that mGlu5s are mediators of this finely tuned mechanism activated by EtOH (Fig. 4).

To evaluate a more direct correlation between miRNA alterations induced by EtOH and the influence of MPEP on AMPAR dysregulation, we performed functional investigations on CA1 pyramidal neurons by using patch clamp technique. With whole-cell patch clamp recordings, we measured the AMPAR-mediated sEPSCs from CA1 pyramidal cells in control, after 24h MPEP application alone or after 24h EtOH withdrawal and EtOH withdrawal plus MPEP application. Our results show that EtOH withdrawal significantly decreased both frequency and amplitude of spontaneous AMPA-mediated EPSCs and MPEP application alone did not alter the electrical properties or the AMPA-mediated EPSCs in CA1 neurons (Supplementary data, Fig. 3), but prevented in part the decrease in amplitude (but not in frequency), suggesting that mGlu5 antagonism may induce modulation at postsynaptic level (Fig. 5). Furthermore, in the same experimental set, we measured the intrinsic somatic excitability of CA1 neurons. We found that there are no differences in the basal parameters such as membrane capacitance, membrane resistance and resting membrane potentials, suggesting that our protocol of EtOH exposure does not induce broad physicochemical effects (Fig. 6, panel A). Conversely, EtOH withdrawal neurons (Fig. 6, panel C, black circles) show a decreased number of the total action potential (AP) compared to control slices (Fig. 6, panel C, white circles), that is restored by MPEP (Fig. 6, panel C, grey circles), without affecting the threshold (Fig. 6, panel D). Finally, acute EtOH application (150 mM, 10 min) induced an increased number of AP compared to control slices and led to enhancement of frequency but not of amplitude of AMPA-mediated EPSCs (Supplementary data, Fig. 4).

4. Discussion

We have previously demonstrated that tissues at different time of maturation show differential sentitivity to EtOH exposure. Indeed, EtOH withdrawal elicited a selective CA1 pyramidal cell injury in mature (10 days *in vitro*), but not in immature (2 days *in vitro*), slices. The differential age-dependent effects of EtOH were highlighted also by electron microscopy, which revealed, despite the lack of toxicity observed with PI, a clear disorganization of microtubuli in neural processes after chronic EtOH exposure in immature slices in concomitance with alterations in the expression of pre- and postsynaptic proteins and impairment of excitatory synaptic transmission (Gerace et al., 2016). In particular we observed that chronic EtOH exposure followed by withdrawal elicits a significant decrease in the expression of AMPARs in developing hippocampal slices (Gerace et al., 2016). Here, we established a role for mGlu5 in EtOH-induced AMPA dysregulation via changes in microRNA 137 and 501-3p levels, hypothesizing that EtOH may promote miR-137 and miR-501 overexpression, causing epigenetic reprinting and consequently reduction of AMPAR function. To validate our hypothesis, we measured miR-137 and miR-501-3p content in immature hippocampal slices after acute or chronic EtOH exposure and we observed a significant increase of both miRNAs induced by chronic but not acute application of EtOH. This increase is maintained after 24h EtOH withdrawal and is abolished by the application of mGlu5 antagonist MPEP during EtOH withdrawal period, indicating that miRNA-induced overexpression is mediated by mGlu5 activation. The decision to apply MPEP during EtOH withdrawal was a technical choice and may be rationalized by the fact that the experimental protocol was standardized (EtOH is usually removed for 24h) and the neuronal network is more stabilized. In addition, it should be considered a useful therapeutical window for translational studies, since mGlu5 can be seen as a target for therapeutic interventions.

Numerous papers have defined miRNA and related mechanisms involved in regulating AMPAR expression at synapses (Hanley, 2021). For example, the regulation of AMPARs in physiological synaptic plasticity is dependent on miR-92a, miR-124, miR-186–5p, miR-218 and miR-134 (Lagos-Quintana et al., 2002); (Letellier et al., 2014); (Silva et al., 2019); (Rocchi et al., 2019). In addition, the regulation of AMPA in disorders such as Alzheimer's disease, dementia, ischemia or schizophrenia, is dependent on MiR-181a, mir-30b, miR-124 and miR-223 (Ansai et al., 2019); (Hsu et al., 2019); (Gascon et al., 2014); (Harraz et al., 2014); (Amoah et al., 2020). Recently, it was reported that AMPAR subunit GluA1 is specifically targeted by miR-137 and miR-501-3p (Olde Loohuis et al., 2015); (Hu et al., 2015). Olde-Loouis and colleagues have shown that experimental up- or down-regulation of miR-137 parallels changes in GluA1 expression and that its postsynaptic downregulation selectively enhances AMPAR-mediated

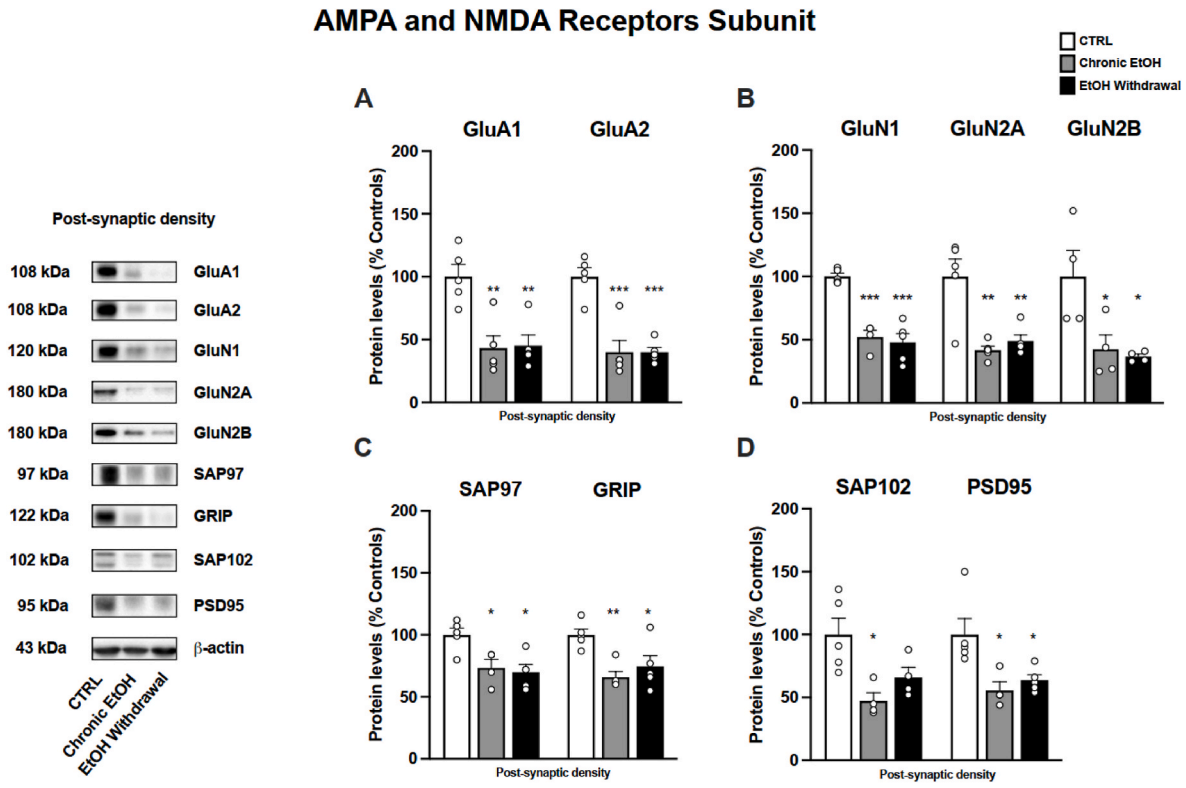

Fig. 2. *EtOH reduces the expression of AMPA and NMDA receptor subunits and related scaffolding proteins in the Post Synaptic Densities (PSD) of immature slices exposed to EtOH.* The experiments were conducted as described in Fig. 1. The main AMPA receptor subunits GluA1 and GluA2 (panel **A**), the NMDA receptor subunits GluN1, GluN2A and GluN2B (panel **B**), the AMPA specific scaffold proteins synapse-associated protein 97 (SAP97) and glutamate receptor-interacting protein (GRIP) (panel **C**) and the NMDA specific scaffold proteins synapse-associated protein 102 (SAP102) and post-synaptic density protein 95 (PSD95) (panel **D**) are significantly decreased after chronic EtOH and EtOH-withdrawal treatment. Representative immunoblots of the proteins of interest are shown in the left panel. Data are expressed as percentage of control protein levels (white column). Bar represent the mean ± SEM of six experiments from independent cell preparations (about ≥72 slices for each experimental point). ***p < 0.001, **p < 0.01 and *p < 0.05 versus. CRL (one-way ANOVA + Tukey's w test).

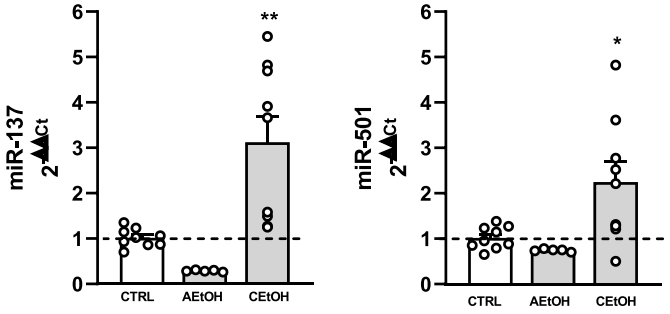

Fig. 3. *Chronic EtOH-induced upregulation of miR-137 and miR-501.* Real-time PCR was performed to assess miR-137 (left panel) and miR-501-3p (right panel) expression after acute EtOH (AEtOH; 150 mM for 30 min) and Chronic EtOH (CEtOH; 150 mM EtOH for 7 days). Data are expressed as $2^{-\Delta\Delta Ct}$. Bars represent the mean ± SEM of 5 experiments from independent cell preparations (about ≥40 slices for each experimental point). ***p < 0.01 versus. miR-137 CRL, *p < 0.00.001 versus. miR-501-3p CRL, **p < 0.01 EtOHw versus miR-501-3p CRL (one-way ANOVA + Dunnet's w test).

synaptic transmission, with a mechanism, that involves mGluR5Rs at CA3-CA1 hippocampal synapse (Olde Loohuis et al., 2015). Furthermore, miR-501–3p is important in regulating GluA1 levels through development; its expression is developmentally regulated and it was shown that miR-501-3p mediates the activity-dependent regulation of the expression of GluA1 (Hu et al., 2015). We focussed on GluA1 subunit considering that the majority of AMPARs in the brain are GluA1/2 heteromers, followed by GluA2/3 (Lu et al., 2009; Wenthold et al., 1996).

In our hippocampal slice model, the upregulation of miR-137 and 501-3p induced by EtOH may be linked to the decreased translation and retention in the active zone of the synapse of GluA1 and GluA2 AMPAR subunits, as demonstrated by their measure in total homogenate and in PSD. Interestingly, this reduction is maintained after 24h of EtOH withdrawal. We can speculate that the overexpressed miR-137 and miR-501-3p are responsible for GluA1 downregulation, by correlating miRNA and protein results. Thus, AMPA receptors are reduced and less stably retained at the membrane leading to an unstable AMPA-mediated glutamate synapse and, thereby, an incorrect formation of neuronal circuits.

The effects of EtOH on AMPA responses has been previously shown in other experimental models (Li et al., 2017); (Varodayan et al., 2018); (Gerace et al., 2018, 2021). In particular, in the early stage of development, such as in the fetal period, EtOH exposure leads to postnatal attenuation of AMPA receptor-mediated synaptic transmission in CA1 pyramidal cells (Bellinger et al., 2002); (Wijayawardhane et al., 2007); (Puglia and Valenzuela, 2009) and depression of inward currents evoked by application of AMPA (Mameli et al., 2005). In support, there are some papers demonstrating that Aniracetam, an allosteric modulator of AMPARs, improved AMPAR-mediated synaptic transmission dysregulation and reversed learning and memory deficits induced by PAE in adolescent rats (Wijayawardhane et al., 2007); (Vaglenova et al., 2008); (Wijayawardhane et al., 2008).

Moreover, our findings show that following chronic EtOH incubation and its withdrawal there is a decrease also in the expression of NMDAR subunits (Fig. 2), in agreement with numerous reports in literature. Indeed, the role of NMDARs in EtOH dependence and withdrawal has

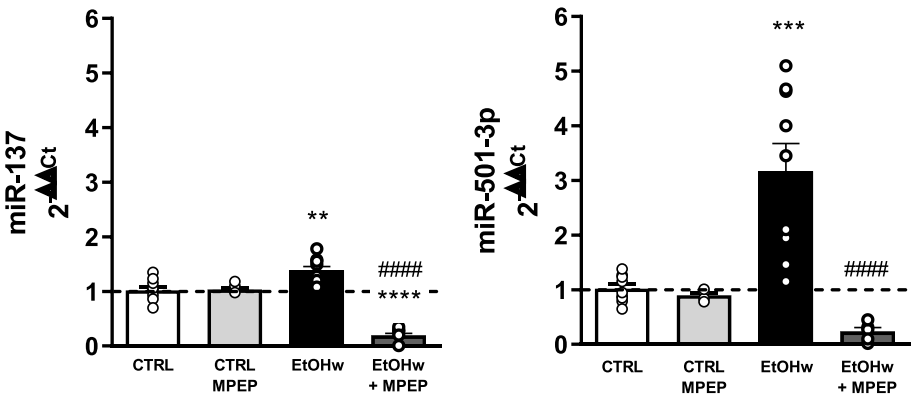

Fig. 4. *EtOH withdrawal-induced upregulation of miR-137 and miR-501 is mediated by mGlu5 receptors.* Real-time PCR was performed to assess miR-137 (left panel) and miR-501-3p (right panel) expression after EtOH withdrawal (EtOHw) or EtOH withdrawal + the mGlu5 antagonist MPEP (EtOHw + MPEP). Data are expressed as $2^{-\Delta\Delta Ct}$. Bars represent the mean ± SEM of 5 experiments from independent cell preparations (about ≥40 slices for each experimental point). **p < 0.01 versus. miR-137 CRL, ****p < 0.00.001 versus. miR-137 CRL, $^{\#\#\#\#}$p < 0.01 EtOHw + MPEP versus. miR-137 EtOHw, ***p < 0.01 versus. miR-501-3p CRL, $^{\#\#\#\#}$p < 0.001 EtOHw vs EtOHw + MPEP (miR-501-3p) (two-way ANOVA + Tukey's w test).

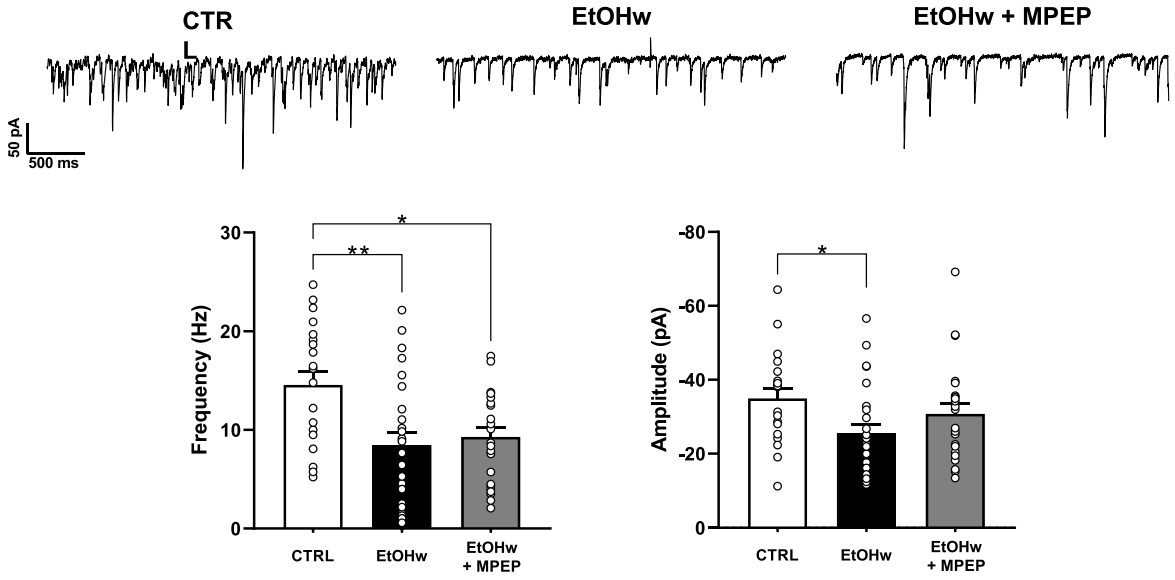

Fig. 5. *Excitatory neurotransmission alterations induced by Ethanol withdrawal and effects of MPEP in immature organotypic hippocampal slices.* Spontaneous excitatory postsynaptic currents (sEPSCs) AMPA-mediated recorded in whole-cell voltage-clamp configuration from CA1 pyramidal cells in CTRL, EtOHw and EtOHw + MPEP. Upper panel: traces are representative of results obtained in independent neurons for each condition (CTRL = 20, EtOHw = 25, EtOHw + MPEP = 24). Lower panel: bar chart of quantitative data expressed as control frequency (Hz) and amplitude (pA) showing that EtOHw significantly decreases both frequency and amplitude of sEPSCs and MPEP application prevent in part the reduced amplitude. Bars represent the mean ± SEM, **p < 0.01; *p < 0.1 vs CRL (one-way ANOVA + Tukey's w test).

been established and the differential age-dependent effects of EtOH have been highlighted in vivo, as well as *in vitro* studies. In particular, adult offspring prenatally exposed to EtOH showed reduction of plasticity and alterations of NMDA receptor subunit composition and function (Hughes et al., 1998; Samudio-Ruiz et al., 2009; Brady et al., 2013). Conversely, in adult animals after chronic EtOH exposure, both NMDAR-mediated glutamatergic synaptic transmission and NMDAR expression were increased (Smothers et al., 1997; Cebere et al., 1999; Läck et al., 2007).

At functional level, we observed that EtOH represses AMPA-receptor-mediated synaptic transmission by decreasing both frequency and amplitude of sEPSCs. Interestingly, 24h MPEP application in control condition did not alter the electrical properties or the AMPA-mediated EPSCs in CA1 neurons (Supplementary Fig. 3). However, MPEP application during 24h EtOH withdrawal was able to restore the decreased amplitude induced by EtOH, but has no effect on sEPSCs frequency (Fig. 5), suggesting a postsynaptic modulation of AMPARs. Surprisingly, we observed an important effect on intrinsic excitability, resulting in a significant reduction of APs in CA1 neurons after 24h EtOH withdrawal. This effect was opposite to the one observed after acute EtOH application (that provoked an evident increase in APs and also in sEPSCs frequency; Supplementary Fig. 4) but is strictly (tightly) mediated by mGlu5 receptors, given that MPEP application restored APs to control levels and the mechanisms underlying this effect has to be explored and need further investigations (Fig. 6).

The connection between mGlu5 and EtOH is essentially established. mGlu5 activity is required for EtOH-related behavioral pathologies (Stevenson et al., 2019). A significant increase of mGlu5 gene expression was observed in the amygdala and in the NAc shell of EtOH-withdrawn rats (Kumar et al., 2016); (Lee et al., 2018). Moreover, it was suggested the important role of this receptor subtype in EtOH drinking (Cozzoli et al., 2009, 2012); (Besheer et al., 2010); (Sinclair et al., 2012) and in memory formation, which appears to be responsible for the chronic relapsing nature of EtOH abuse (Obara et al., 2009). In fact, the systemic administration of mGlu5 antagonists attenuate cue-evoked reinstatement of ethanol-seeking behavior in (Bäckström et al., 2004), (Adams et al., 2008); (Sinclair et al., 2012). Similarly, studies with mGlu5 negative allosteric modulators performed in animal models of EtOH consumption have shown a reduction of the reinforcing effects of EtOH, as well as decreased motivation during self-administration (Mihov and

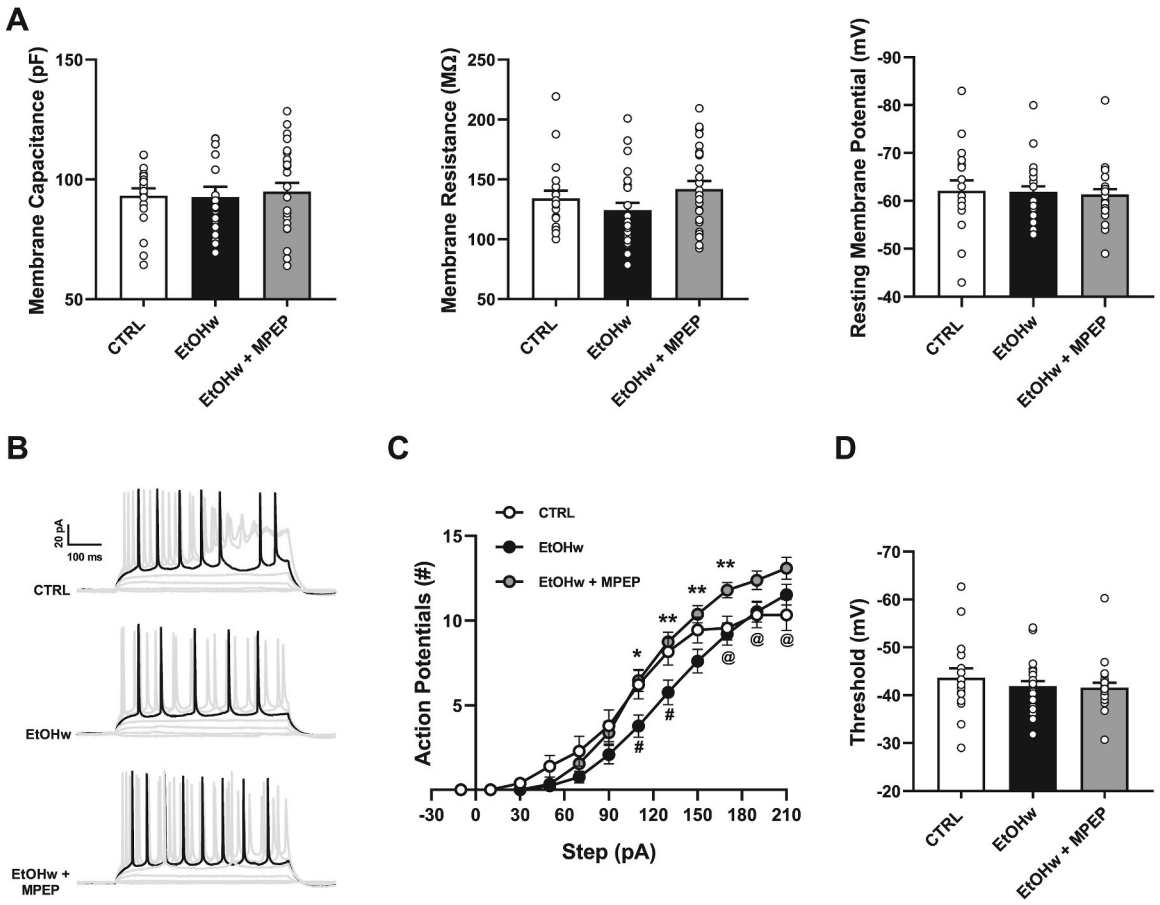

Fig. 6. *Intrinsic somatic excitability alterations induced by Ethanol withdrawal and effects of MPEP in immature organotypic hippocampal slices.* **A)** Bar graphs showing no differences in the passive properties of CA1 pyramidal neurons (membrane capacitance, membrane resistance and resting membrane potential) between control (CTRL; white column), after EtOH withdrawal (EtOHw, 24h; black column) and after mGlu5 antagonist MPEP (30 μM) application during 24h of EtOH withdrawal. (EtOHw + MPEP; grey column) (one-way ANOVA + Tukey's w test**). B)** Representative traces of whole-cell current clamp action potentials (APs) recorded from CA1 pyramidal cells evoked by a depolarizing single step (20 pA), in CTRL, EtOHw and EtOHw + MPEP. Representative black traces evidentiate AP at 130 pA. **C**) Bar chart showing the decrease of the total AP number in EtOHw treated neurons (black circles) compared to CTRL (white circles) and the effects of MPEP application (grey circles). Bars represent the mean ± SEM, *$p < 0.05$, **$p < 0.01$ vs CTRL, $^{\#}p < 0.05$ vs CTRL and $^{@}p < 0.05$ vs EtOHw + MPEP (one-way ANOVA + Tukey's w test**). (D)** Bar chart showing no differences in the threshold values (mV) between the three experimental groups.

Hasler, 2016), (Stevenson et al., 2019) and EtOH withdrawal-induced anxiety-like syndrome in rats (Kumar et al., 2013).

While we here propose for the first time a previously unknown molecular mechanism underlying EtOH-induced AMPA dysregulation in immature hippocampus, we are aware that this study has some limitations, the major relying on the fact that we do not directly link the effect on miRNAs with AMPA receptor downregulation. However, other studies have shown very elegantly that AMPA receptor subunit GluA1 is a direct target of miR-137 and miR-501-3p (Olde Loohuis et al., 2015); (Hu et al., 2015), thus allowing us to hypothesize a causality, albeit indirect, between the two events. Moreover, we did not provide evidence for how mGlu5 become activated in the presence of EtOH. The direct interaction between EtOH and mGluR5Rs has never been shown yet. All the evidence reported in the literature refer to studies in which mGlu5 antagonists and/or mGlu5 negative allosteric modulators have been used to correlate EtOH effects together with behavioral parameters. In the present study, we demonstrated that mGlu5 are mediators of decreased AMPA-mediated neurotransmission induced by chronic EtOH application (the implication of mGluR5 is demonstrated pharmacologically by the effect of MPEP on miRNA expression) and we consider that this is one of the numerous mechanisms by which EtOH affects neurotransmission in immature tissue.

5. Conclusions

In the present study, we explored the EtOH-dependent pathways leading to hippocampal AMPAR suppression. This mechanism suggests that EtOH exposure in developing brain tissue leads to increased miR-137 and 501-3p expression that downregulates AMPA synaptic transmission, a process mediated by mGlu5 in immature organotypic hippocampal slices (Fig. 7). Our results rely on a previously unknown molecular mechanism underlying EtOH-induced AMPA dysregulation in immature hippocampus, which is essential for brain function and plasticity, as well as development of cognition. To conclude, we propose that mGlu5 may be used as therapeutic targets for EtOH-related pathologies in infants or adolescence and, most importantly, our data point to miR-137 and miR-501-3p as potential, novel diagnostic tools for PAE and FASD.

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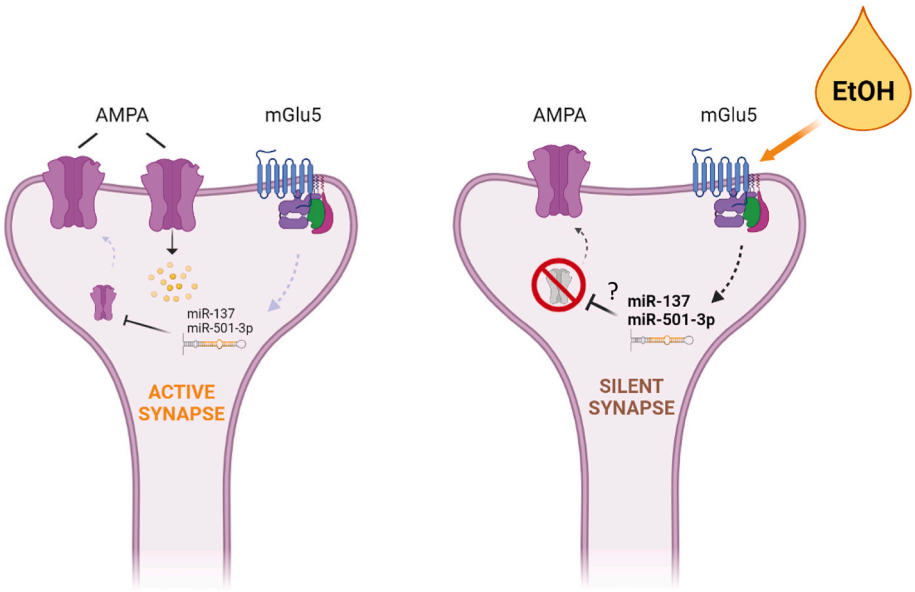

Fig. 7. Hypothetical model to explain mechanisms underlying ethanol effects on immature organotypic hippocampal slices. Ethanol induces the overexpression of miR-137 and 501-3p that lead to dysregulation of AMPA synaptic transmission through mGluR5Rs.

Author contribution

EG designed the study, performed experiments and data analysis and wrote the first draft of the manuscript. LC, EB, FM, FCD and AI performed experiments and data analysis. LC, CL, CD, FF, AM and GM contributed intellectually to the interpretation of the data. LC, FF, AM and GM revised the manuscript. All authors have contributed to and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2023.175878.

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