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Loss of Ethanol inhibition of NMDAR-mediated currents and plasticity of cerebellar synapses in mice expressing the GluN1(F639A) subunit

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Abstract

Background—Glutamatergic N-methyl-D-aspartate receptors (NMDARs) are well known for their sensitivity to ethanol inhibition. However, the specific manner in which ethanol inhibits channel activity and how such inhibition affects neurotransmission, and ultimately behavior, remain unclear. Replacement of phenylalanine 639 with alanine (F639A) in the GluN1 subunit reduces ethanol inhibition of recombinant NMDA receptors. Mice expressing this subunit show reduced ethanol-induced anxiolysis, blunted locomotor stimulation following low dose ethanol administration and faster recovery of motor function after moderate doses of ethanol suggesting that cerebellar dysfunction may contribute to some of these behaviors. In the mature mouse cerebellum, NMDARs at the cerebellar climbing fiber (CF) to Purkinje cell (PC) synapse are inhibited by low concentrations of ethanol and the long term depression (LTD) of parallel fiber (PF) mediated currents induced by concurrent activation of PFs and CFs (PF-LTD) requires activation of ethanol-sensitive NMDA receptors. In this study, we examined cerebellar NMDA responses and NMDA-mediated synaptic plasticity in wild-type and GluN1(F639A) mice.

Methods—Patch clamp electrophysiological recordings were performed in acute cerebellar slices from adult wild type and GluN1(F639A) mice. NMDA receptor-mediated currents at the CF-PC synapse and NMDAR-dependent PF-LTD induction were compared for genotype-dependent differences.

Results—Stimulation of climbing fibers evoked robust NMDA-mediated EPSCs in PCs that were similar in amplitude and kinetics between wild-type and GluN1(F639A) mice. NMDA-mediated CF-PC excitatory postsynaptic currents (EPSCs) in wild-type mice were significantly inhibited by ethanol (50 mM) while those in mutant mice were unaffected. Concurrent stimulation of CF and PF inputs induced synaptic depression of PF-PC EPSCs in both wild-type and mutant mice and this depression was blocked by the NMDA antagonist DL-APV. The synaptic depression of PF-PC EPSCs in wild-type mice was also blocked by a low concentration of ethanol (10 mM) that had no effect on plasticity in GluN1(F639A) mice.

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Conclusions—These results demonstrate that inhibition of cerebellar NMDARs may be a key mechanism by which ethanol affects cerebellar-dependent behaviors.

Keywords

NMDA receptor; alcohol sensitivity; cerebellum; Purkinje cell; synaptic plasticity

Introduction

NMDA receptors play a fundamental role in neuronal function, synaptic transmission and are a key component of synaptic plasticity mechanisms processes such as long-term potentiation (LTP) and depression (LTD) believed to underlie learning and memory. Activation of these ion channel receptors by glutamate induces a strong neuronal depolarization and the receptor's high permeability to calcium links changes in membrane potential to downstream intracellular signaling pathways. A large number of studies have demonstrated that NMDARs are particularly sensitive to alcohol and acute inhibition of NMDAR signaling by ethanol is hypothesized to trigger compensatory changes in neuronal excitability that often develop following chronic exposure to ethanol (Chandrasekar, 2013). A key question that remains is how and to what extent ethanol inhibition of NMDARs contributes to the behaviors associated with alcohol consumption. To address this question, studies from this laboratory have identified ethanol-sensitive sites within NMDARs in order to generate animals that express ethanol-resistant NMDARs. Initial results from this approach identified a key phenylalanine residue within transmembrane domain 3 (TM3) of the GluN1 subunit that appears to regulate a significant portion of the ethanol sensitivity of recombinant NMDARs (Ronald et al., 2001, Smothers and Woodward, 2006, Smothers and Woodward, 2016). As GluN1 is an obligate subunit for functional NMDARs, all heteromeric NMDA receptors expressing GluN1(F639A) should show reduced ethanol inhibition and this was observed in oocytes and HEK293 cells expressing GluN1(F639A) and different GluN2 subunits (A–D) (Ronald et al., 2001, Smothers and Woodward, 2006, Smothers and Woodward, 2016). This finding was used to generate the first line of knock-in mice that express ethanol-resistant NMDARs (den Hartog et al., 2013) by replacing phenylalanine 639 in the TM3 domain of the GluN1 subunit with alanine (GluN1-F639A). The locomotor activating and anxiolytic effects of low-dose ethanol were blunted in these mice and they recovered faster than wild-type animals on a rotarod test of motor coordination (den Hartog et al., 2013). No genotype-dependent differences in ethanol-induced sedation, sleep time or hypothermia were observed while electrophysiological recordings demonstrated that the ethanol inhibition of synaptic NMDA responses in medial prefrontal cortex neurons was reduced in mutant mice.

In addition to cortical areas, cerebellar function is particularly vulnerable to acute and chronic alcohol exposure and cerebellar-dependent functions such as postural stability, fine motion and cerebellar-dependent learning are often impaired in individuals exposed to alcohol (reviewed in (Dlugos, 2015)). Alcohol affects cerebellar neuronal networks via a plethora of mechanisms including altered synaptic transmission, synaptic plasticity and neuronal excitability, impaired energy metabolism, oxidative stress and glial abnormalities (Jaatinen and Rintala, 2008). Low concentrations of ethanol (10–25 mM) affect cerebellar

cortical circuitry at golgi, granule and unipolar brush cells synapses by enhancing GABAergic transmission via modulation of GABAA receptors (Hancher et al., 2005, Botta et al., 2007) and glycine receptor-mediated responses (Richardson and Rossi, 2017) and at synapses onto Purkinje cells (PC) via inhibition of NMDAR-mediated currents. PCs are GABAergic neurons that constitute the sole output of the cerebellar cortex and they receive excitatory glutamatergic input from two sources: granule cells of the cerebellar cortex, via the parallel fibers (PF), and inferior olivary neurons from the brainstem via the climbing fiber (CF). Plasticity at PC excitatory synapses has been shown to underlie the formation of several types of motor memory, such as adaptation of the vestibulo-ocular reflex and associative eyeblink conditioning, processes known to be sensitive to acute and chronic alcohol exposure (reviewed in (Cheng et al., 2015)). In-vivo and ex-vivo studies have shown that postsynaptic NMDARs at the CF-PC synapse contribute to the characteristic PC response to the input from the inferior olive, a unique and remarkably strong excitatory response termed the complex spike that conveys timing information and triggers synaptic plasticity (Liu et al., 2016, Piochon et al., 2007). While CF-dependent, long-term depression at PF-to-PC synapses (PF-LTD) was shown to be inhibited by 50 mM ethanol via blockade of other ethanol targets such as mGluR1 and voltage gated calcium channels (Belmeguenai et al., 2008), a recent study shows that PF-LTD is also inhibited by 10 mM ethanol likely via inhibition of CF-PC postsynaptic NMDA receptors (He et al., 2013). Given the important role of NMDARs in cerebellar function, we used wild-type and GluN1(F639A) mice to determine whether ethanol-sensitive NMDARs are required to for ethanol modulation of cerebellar glutamatergic transmission and plasticity.

Materials and Methods

Subjects

Breeding pairs of heterozygous mice expressing the GluN1(F639A) subunit were used to generate wild-type and mutant mice. As previously described, mice homozygous for the GluN1(F639A) mutation were not viable post-natally and all experiments with GluN1(F639A) mice used heterozygotes (den Hartog et al., 2013). Mice were genotyped by polymerase chain reaction from tail-derived DNA. Primers 5'-TTC ACA GAA GTG CGA TCT GG-3' and 5'-AGG GGA GGC AAC ACT GTG GAC-3' amplified a 466-base pair fragment from the wild-type allele. Primers 5'-CTT GGG TGG AGA GGC TAT TC-3' and 5'-AGG TGA GAT GAC AGG AGA TC-3' amplified a 280-base pair fragment from the knock-in allele. After weaning, mice were housed with ad libitum access to rodent chow and water with 12-h light/dark cycles (lights on at 9:00 AM). All experiments using mice were approved by the MUSC Institutional Animal Care and Use Committee and conformed to NIH guidelines for the use of animals in biomedical research.

Electrophysiology

Cerebellar slices were prepared from 9 to 30 week old wild-type (WT) and heterozygous F639A (F639A-HT). Mice were rapidly decapitated, brains were removed and placed in ice-cold carbogen-bubbled aCSF solution containing (in mM): NaCl (125), KCl (2.5), NaH₂PO₄ (1.4), CaCl₂ (2), MgCl₂ (1.3), glucose (10), ascorbic acid (0.4) and NaHCO₃ (25); osmolarity 310–320 mOsm. Parasagittal cerebellar sections were cut into 200–250 µm slices

using a Leica VT1000 vibrating microtome (Buffalo Grove, IL) with a double-walled chamber through which cooled (1–4°C) solution was circulated. Slices were collected and transferred to a chamber containing aCSF and incubated at room temperature for at least 1 hour before whole-cell patch-clamp recordings were begun.

NMDAR Currents

Following incubation, slices were transferred to a recording chamber and perfused with carbogen-bubbled aCSF maintained at 32°C. For recordings of CF-NMDAR EPSCs, a concentric bipolar electrode was placed on the granule cell layer, about 150 µm from the soma of the whole-cell voltage-clamped (–70 mV) PC. CF-mediated responses were recognized by their characteristic all-or-none responses and paired pulse depression characteristics. The recording aCSF was magnesium free and was supplemented with 50 µM picrotoxin (Tocris Bioscience, Ellsville, MO) to block GABAA receptors and 25 µM glycine to facilitate NMDAR activation. NMDAR responses were isolated by addition of 10 µM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo(f)quinoxaline-7-sulfonamide (NBQX; Abcam Biochemicals, Cambridge, MA) to block AMPA receptors. 50 µM DL-2-Amino-5-phosphonopentanoic acid (DL-APV; Abcam Biochemicals, Cambridge, MA) was used to verify NMDA-mediated currents. Recording pipettes (resistance of 2–3 MΩ) were filled with internal solution containing: (in mM): 9 KCl, 10 KOH, 120 K-gluconate, 3.48 MgCl₂, 10 HEPES, 4 NaCl, 4 Na₂ATP, 0.4 Na₃GTP, and 17.5 sucrose (pH 7.25–7.35).

Synaptic plasticity

Under voltage-clamp conditions, pairs of PF-mediated excitatory postsynaptic currents (PF-EPSCs) were recorded in PCs at an inter-event interval of 60 ms, every 20s. After 5 minutes of baseline recording, the amplifier was switched to current-clamp mode and a combined PF-CF stimulation protocol was used to induce long-term depression. This consisted of a 100-Hz PF burst stimulation (9 pulses) followed, 150 ms later, by a 2 pulse CF stimulation at 20 Hz. This protocol was delivered at 0.1 Hz for 5 min (Crepel, 2009) and then the amplifier was switched back to voltage-clamp mode and PF-EPSC recordings were resumed. CF and PF inputs were stimulated using aCSF-filled glass electrodes placed in the granule cell layer and in the upper molecular layer, respectively. In this plasticity study, recordings were performed at room temperature and the aCSF contained 1.3 mM MgCl₂ and was supplemented with 50 µM picrotoxin and 25 µM glycine. Changes in synaptic strength were calculated as the percent of baseline change in the amplitude of PF-EPSCs responses following the stimulation protocol.

In all experiments, series resistance (Rs) was monitored throughout the recording and an experiment was discontinued if Rs changed more than 30%. Data were acquired using an Axon MultiClamp 700B amplifier (Molecular Devices, Union City, CA) and an ITC-18 digital interface (HEKA Instruments, Bellmore, NY) controlled by AxographX software (Axograph Scientific, Sydney, NSW, Australia). Recordings were filtered at 4 kHz, acquired at 10 kHz and analyzed offline using AxographX software. All data is shown as mean ± standard error of the mean and statistical analysis were performed using Graphpad prism 7 software.

Results

Climbing fiber-to-Purkinje cell synapses in F639A-HT mice display NMDA receptor responses similar to those in wild type mice

Evoked climbing fiber-mediated excitatory postsynaptic currents (CF-EPSCs) in Purkinje cells were first identified as described in the methods section and then 10 μ M NBQX was added to isolate the NMDAR-mediated component (CF-NMDAR EPSCs, Figure 1A). In the presence of NBQX, the NMDAR antagonist DL-APV (50 μ M) reduced CF-EPSC amplitude by 81.41 ± 4.6 % (n=5, Figure 1B). The small APV and NBQX resistant residual current has been previously reported and although it remains uncharacterized, it has been shown to be insensitive to ethanol (Piochon et al., 2007, He et al., 2013). There were no genotype specific differences in CF-NMDAR EPSC area (WT $2,182 \pm 301.5$ pA*ms; HT $2,572 \pm 285.6$ pA*ms, p=0.36; Figure 1D), amplitude (WT 78.23 ± 11.25 pA; HT 98.01 ± 15.26 pA, p=0.3; Figure 1E), decay time (WT 24.65 ± 1.4 ms; HT 24.92 ± 2.37 ms, p=0.92; Figure 1F), rise time (WT 1.44 ± 0.18 ms; HT 1.9 ± 0.3 ms, p=0.2; Figure 1G), or paired-pulse ratio, EPSC2/EPSC1 (WT 0.38 ± 0.04 ; HT 0.33 ± 0.05 , p= 0.46; Figure 1H).

NMDAR-mediated currents at CF-PC synapses in F639A-HT mice are less sensitive to acute ethanol

To assess the ethanol sensitivity of PC NMDARs, pairs of CF-NMDAR EPSCs were monitored under control conditions and then the bath solution was switched to one containing ethanol (50 mM) for 5 minutes followed by a wash out period. In PCs from wild type mice, ethanol significantly reduced the amplitude of CF-NMDAR EPSCs (70.93 ± 3.52 % of baseline, p < 0.0001, n=10) while those from F639A-HT mice were unaffected (99.46 ± 4.14 % of baseline, p = 0.8985, n=10; Figure 2A). Similarly, the total charge transfer of CF-EPSCs in wild-type mice was reduced by ethanol in WT mice (83.33 ± 5.18 % of baseline, p = 0.0105) but not in F639A-HT mice (HT: 111.0 ± 8.195 % of baseline, p = 0.2116). Ethanol had no effect on the paired-pulse ratio of CF-NMDAR EPSC amplitudes (EPSC2/EPSC1) in neurons from WT (0.39 ± 0.02 for baseline and 0.3936 ± 0.03 for ethanol, p = 0.98) or F639A-HT mice (0.42 ± 0.03 for baseline and 0.47 ± 0.03 for ethanol, p = 0.21; Figure 2B)

NMDA-dependent PF-EPSC long-term depression is blocked by ethanol in WT but not F639A-HT mice

As previously reported (Piochon et al., 2010), concomitant stimulation of CF and PF inputs induced a long-term depression of PF-mediated synaptic responses (Figure 3A). PF-LTD was blocked by the NMDAR antagonist DL-APV (126.9 ± 14.66 % of baseline, p=0.13; Figure 3A). In the presence of 10 mM ethanol, PF-LTD in WT mice was also blocked and instead PF-EPSCs were now significantly potentiated (control: 84.83 ± 4.2 % of baseline, p= 0.0033; 10 mM ethanol: 133.8 ± 12.7 % of baseline, p= 0.028; Figure 3A) confirming previously reported findings (He et al., 2013). The change in PF-EPSC amplitude after the induction protocol was significantly different between control and ethanol (p= 0.008) and control and DL-APV (p=0.019), but not between ethanol and DL-APV groups (>0.999). As with WT mice, the PF-LTD induction protocol significantly reduced the amplitude of PF-EPSCs in slices from F639A-HT mice (84.33 ± 4.9 % of baseline, p= 0.0069; Figure 3B)

and the LTD was prevented by APV (114.3 ± 6.45 % of baseline, $p = 0.05$; Figure 3B). However, unlike WT mice, bath application of 10 mM ethanol had no effect on PF-LTD in F639A-HT mice (85.64 ± 5.95 % of baseline, $p = 0.036$; Figure 3B). Statistical analysis of this data revealed that, in F639-HT mice, the change in PF-EPSC amplitude of control vs in the presence of DL-APV ($p = 0.0022$) and ethanol ($p = 0.0057$) differed significantly while there was not a significant difference in PF-LTD induction between control and ethanol groups (>0.999).

Discussion

The major findings of this study show that GluN1(F639A) mice are resistant to ethanol-mediated inhibition of NMDAR-mediated currents at cerebellar CF-PC synapses and LTD induction at PF-to-PC synapses. These findings are consistent with those from our previous studies showing that the GluN1(F639A) subunit dramatically reduces the ethanol sensitivity of NMDARs in heterologous cells (Ronald et al., 2001, Smothers and Woodward, 2006, Smothers and Woodward, 2016) and neurons (den Hartog et al., 2013). The inability of ethanol to block PF synaptic depression in GluN1(F639A) mice highlights the role of ethanol-sensitive NMDARs in mediating effects of sub-intoxicating concentrations of ethanol on cerebellar function (10 mM is equivalent to $\sim 0.05\%$ blood ethanol). In addition, these data provide a potential cellular correlate to alterations in some of the ethanol-induced motor behaviors displayed by F639A-HT mice (den Hartog et al., 2013). In particular, these mice showed a faster recovery in rotarod performance than wild-type mice after an acute injection of ethanol. While ethanol-insensitive NMDARs in the cerebellum of these mice could underlie this effect, it should be noted that this shift in motor recovery was only observed at the higher dose of ethanol (2.5 g/kg; i.p.) as no genotype-dependent difference in rotarod performance was observed in mice receiving a 2 g/kg dose of ethanol. These findings highlight the awareness that ethanol's actions on behavior likely involve multiple cellular targets and circuits that show differential sensitivity to ethanol (Lovinger and Roberto, 2013).

Of the many molecular targets of alcohol, NMDARs are among the most sensitive and are often inhibited by alcohol at concentrations below those that affect other molecular targets, including those implicated in alcohol-induced cerebellar dysfunction (for review see (Valenzuela et al., 2010)). In juvenile rats, PF-PC LTD has been shown to be prevented by bath application of 50 mM ethanol (Belmeguenai et al., 2008). The authors of that study suggested that this occurred via inhibition of known facilitators of LTD induction, mGluR1 receptors and voltage-gated calcium channels, as these processes are sensitive to 50 and 80 mM ethanol but not 20 mM ethanol (Belmeguenai et al., 2008, Carta et al., 2006). In contrast, NMDAR-mediated currents have been reported to be inhibited by ethanol at concentrations as low as 5–10 mM (Lovinger et al., 1989, Smothers et al., 2016). In addition, even though lower concentrations of ethanol induce sub-maximal inhibition of NMDAR-mediated currents at the channel level, these effects may be amplified in circuits and processes such as cortical up-states that are NMDA-dependent (Tu et al., 2007).

In the cerebellar cortex, NMDA receptors are expressed in various cell types throughout life and are involved in processes such as developing granule cell migration (Gerber and

Vallano, 2006) synapse refinement onto Purkinje cells (Rabacchi et al., 1992), and modulation of neurotransmission and plasticity processes at various synapses within the juvenile cerebellar cortex circuitry (Bidoret et al., 2015, Armano et al., 2000, Bouvier et al., 2016, Klintsova et al., 2002, Mapelli et al., 2016). However, while the involvement of NMDARs in glutamatergic signaling and plasticity in other areas of the mature brain is well accepted, their role in PC function has been somewhat controversial. This is likely due to a developmentally delayed onset of functional synaptic NMDA receptors at CF-to-PC synapses and the use of young animals in many cerebellar slice electrophysiology studies. NMDAR currents are detected at 4 weeks of age and only reach maximum strength at approximately 8 weeks of age (Piochon et al., 2007). We confirmed this and observed robust and reproducible NMDA-mediated currents and synaptic depression in the 9–30 week old animals used in this study. The induction of PF-to-PC synaptic depression starts with a rapid increase in intracellular Ca^{2+} which, via activation of protein kinase C signaling, results in phosphorylation and subsequent internalization of AMPA receptors. Historically, the increase in intracellular PC Ca^{2+} has been attributed to the release of calcium from the endoplasmic reticulum upon activation of perisynaptic mGlu1R-Gq protein-coupled receptors located on PC dendrites that are innervated by the stimulated PF, and the nearly coincident Ca^{2+} entry through voltage-gated channels at the CF synapse. However, in adult animals, activation of postsynaptic NMDARs at the CF-to-PC synapse mediates Ca^{2+} entrance to PCs and unlike in juvenile cerebellum, NMDARs are required for PF-LTD induction in older animals (Piochon et al., 2007, Piochon et al., 2010). In contrast with other brain regions, PF-PC LTD requires higher calcium concentrations than LTP does and activation of NMDARs at the CF-PC synapse during LTD induction significantly enhances and prolongs levels of intracellular calcium, thereby mediating the induction of LTD (Piochon et al., 2010). PF-LTP requires lower calcium concentrations and can be induced by repetitive activation of PFs without concomitant CF activation. Results from the current study show that when NMDA receptors are inhibited with APV or a low concentration of ethanol, concomitant activation of PFs and CF could result in PF synapse potentiation instead of depression, suggesting that reducing the calcium contribution from NMDARs at the CF-PC synapse can invert the sign of synaptic plasticity.

Although results in the literature and from the present study suggest that post-synaptic NMDARs on PCs mediate PF-LTD, NMDARs are also found on axon terminals at PF-PC synapses. These presynaptic NMDARs have been linked to PF-PC LTD induced by pairing of PF stimulation with PC depolarization, in the place of CF stimulation (Casado et al., 2002); and are involved in the PF-PC LTP observed in transverse but not sagittal slice preparations (Bouvier et al., 2016). Future studies with wild type and ethanol-insensitive NMDAR mutant mice are needed to determine the role of presynaptic NMDARs in mediating plasticity at PF-PC synapses.

Interestingly, PCs in younger animals express NMDA receptors containing the GluN2D subunit that generates receptors with lower single channel conductance, slower kinetic properties and less sensitivity to magnesium block (Cull-Candy et al., 1998). GluN2D subunits are later replaced by high-conductance, magnesium-sensitive NMDARs containing GluN2A/B subunits (Piochon et al., 2010, Renzi et al., 2007, Piochon et al., 2007) that are more suitable for fast synaptic transmission. Previous work from this laboratory has

demonstrated that both GluN2A and GluN2D containing receptors are sensitive to ethanol with some differences noted depending on the GluN1 splice variant expressed (Jin and Woodward, 2006). This could result in developmental-dependent differences in the ethanol inhibition of NMDAR-mediated currents and plasticity in cerebellar PCs. To address this issue, we have recently developed two new mouse lines expressing ethanol-insensitive GluN2A or GluN2D containing NMDARs and studies using these mice are underway to assess the role of different NMDAR subunits in the ethanol sensitivity of PF-synaptic depression.

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Figure 1. NMDAR- mediated currents at climbing fiber synapses in WT and F639A-HT mice
 (A) CF-EPSC sample traces from a WT mouse in the presence of the AMPA receptor antagonist NBQX (black trace) and after application of the NMDA receptor antagonist APV (blue trace); scale: 100 pA/200 ms. (B) Average percent inhibition of CF-EPSCs by APV. Data are mean $\pm$ SEM. (C) Superimposed CF-EPSCs from WT (black trace) and F639A-HT mouse (red trace); traces normalized to amplitude. Scale: 50 ms. (D–H) CF-EPSCs show no genotype specific differences (by unpaired t-test) in area (D), amplitude (E), decay time (F), rise time (G) or paired pulse ratio (H). Data are mean $\pm$ SEM.

Figure 2. NMDAR-mediated CF-EPSCs in F639A-HT mice are less sensitive to acute ethanol
 (A) Top panel: Sample traces of CF-NMDA EPSCs from WT and F639A-HT mice; scale: 100 pA/100 ms (left). Bottom panel: Time course (left) and summary (right) of effects of 50 mM ethanol on CF-NMDA EPSC1 amplitude. Data are percent of baseline (mean $\pm$ SEM). Symbol (*): value significantly different from baseline amplitude, $p < 0.001$; one sample t-test; (**) value significantly different from WT, $p < 0.001$; unpaired t-test. (B) Paired-pulse ratio of CF-EPSCs before and during ethanol bath application for WT (left) and F639A-HT mice (right).

Figure 3. NMDA-dependent PF-EPSC long-term depression is blocked by ethanol in WT but not F639A-HT mice

(A) Top panel: Representative traces showing NMDA EPSCs in Purkinje neuron from WT mouse before (baseline) and after (post-stimulation) plasticity protocol (see insert in Figure A) under control, ethanol (10 mM) or APV (20 μ M) conditions. Sample traces scale: WT, 200 pA/200 ms. Bottom panel: Time course and summary plot of effect of ethanol or APV on PF-CF LTD in WT mice. (B) Top panel: Representative traces showing NMDA EPSCs in Purkinje neuron from F639A-HT mouse before (baseline) and after (post-stimulation) LTD stimulation protocol under control, ethanol (10 mM) or APV (20 μ M) conditions. Scale bars: 100 pA/50 ms. Bottom panel: Time course and summary plot of effect of ethanol or APV on PF-CF LTD in F639A-HT mice. Data are mean $\pm$ SEM expressed as percent of baseline. Data are mean $\pm$ SEM expressed as percent of baseline. Symbol (**) $p < 0.05$, One-way

Anova, Benferroni's multiple comparison test ;(*) $p < 0.05$ value significantly different than baseline; unpaired t-test.

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