Ethanol affects NMDA receptor signaling at climbing fiber-Purkinje cell synapses in mice and impairs cerebellar LTD

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He Q, Titley H, Grasselli G, Piochon C, Hansel C. Ethanol affects NMDA receptor signaling at climbing fiber-Purkinje cell synapses in mice and impairs cerebellar LTD. J Neurophysiol 109: 1333–1342, 2013. First published December 5, 2012; doi:10.1152/jn.00350.2012.—Ethanol profoundly influences cerebellar circuit function and motor control. It has recently been demonstrated that functional N-methyl-D-aspartate (NMDA) receptors are postsynaptically expressed at climbing fiber (CF) to Purkinje cell synapses in the adult cerebellum. Using whole cell patch-clamp recordings from mouse cerebellar slices, we examined whether ethanol can affect NMDA receptor signaling in mature Purkinje cells. NMDA receptor-mediated currents were isolated by bath application of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[f]quinoxaline (NBQX). The remaining D-2-amino-5-phosphonovaleric acid (D-APV)-sensitive current was reduced by ethanol at concentrations as low as 10 mM. At a concentration of 50 mM ethanol, the blockade of D-APV-sensitive CF-excitatory postsynaptic currents was significantly stronger. Ethanol also altered the waveform of CF-evoked complex spikes by reducing the afterdepolarization. This effect was not seen when NMDA receptors were blocked by D-APV before ethanol wash-in. In contrast to CF synaptic transmission, parallel fiber (PF) synaptic inputs were not affected by ethanol. Finally, ethanol (10 mM) impaired long-term depression (LTD) at PF to Purkinje cell synapses as induced under control conditions by paired PF and CF activity. However, LTD induced by pairing PF stimulation with depolarizing voltage steps (substituting for CF activation) was not blocked by ethanol. These observations suggest that the sensitivity of cerebellar circuit function and plasticity to low concentrations of ethanol may be caused by an ethanol-mediated impairment of NMDA receptor signaling at CF synapses onto cerebellar Purkinje cells.

ALCOHOL IMPAIRS THE CEREBELLAR CONTRIBUTION TO MOTOR CONTROL AND THE FINE ADJUSTMENT OF MOVEMENTS (FOR REVIEW, SEE VALenzuela et al. 2010). For example, acute application of ethanol affects conditioned eyelink responses in human subjects (Hobson 1966) as well as in rabbits (Hernández et al. 1986). Chronic ethanol exposure reduces cerebellar motor coordination and causes ataxia in mice (Servais et al. 2005). Purkinje cells, which provide the sole output of the cerebellar cortex, are a main target of ethanol (Siggins and French 1979; Rogers et al. 1980; Sorensen et al. 1980). Acute application of ethanol differentially interferes with Purkinje cell activity depending on the ethanol concentration. At low doses, ethanol can increase current-evoked spiking in Purkinje cells, while high doses cause a reduction in firing rates (Chu 1983; Franklin and Gruol 1987; Urrutia and Gruol 1992; Freund et al. 1993a). This complex effect may be due to the broad spectrum of targets of ethanol, such as voltage-gated calcium channels (Walter and Messing 1999; Belmeguenai et al. 2008), GABAAergic transmission (Freund et al 1993b; Freund and Palmer 1997; Mameli et al. 2008), and type 1 metabotropic glutamate receptors (mGluR1) function (Carta et al. 2006; Belmeguenai et al. 2008). In addition, ethanol has been shown to alter cerebellar synaptic plasticity. Long-term depression (LTD) at climbing fiber (CF) synapses onto Purkinje cells (Hansel and Linden 2000) is inhibited by 50 mM ethanol (Carta et al. 2006). Similarly, parallel fiber (PF) LTD is blocked by bath application of ethanol (50 mM) in rat cerebellar slices (Belmeguenai et al. 2008; Su et al. 2010). At both types of synapses, it has been suggested that an inhibition of mGluR1 by ethanol contributes to the suppression of LTD (Carta et al. 2006; Belmeguenai et al. 2008; Su et al. 2010).

In cerebellar cultures, ethanol alters the response of Purkinje cells to glutamate application (Franklin and Gruol 1987). In slices prepared from young adult rats, ethanol reduces mGluR1-mediated currents, whereas α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor signaling remains unaffected (Belmeguenai et al. 2008). It still is conceivable that in culture AMPA receptor signaling is impaired by ethanol as well, since in slices prepared from neonatal rats, hippocampal AMPA receptor transmission is depressed by ethanol (Mameli et al. 2005). In young adult and adult hippocampal pyramidal cells (CA1 and CA3), postsynaptic N-methyl-D-aspartate (NMDA) receptors become a main ethanol target and display a greater ethanol sensitivity than AMPA receptors (Lovingier et al. 1989, 1990; Peoples et al. 1997; Mameli et al. 2005). Until recently, it was assumed that Purkinje cells lack functional NMDA receptors (Farrant and Cull-Candy 1991; Llano et al. 1991). However, it has now been demonstrated that functional NMDA receptors, composed of NR1 and NR2-A/B subunits, are postsynaptically expressed at CF synapses onto Purkinje cells in the mature mouse cerebellum (Piochon et al. 2007; Renzi et al. 2007). NMDA receptor signaling has a late developmental onset with NMDA receptors reaching full expression levels after ~8 wk (Piochon et al. 2007). In line with these results obtained in cerebellar slices, it has been shown that Purkinje cells in old, but not young, cerebellar cultures express functional NMDA receptors (Lonchamp et al. 2012). The physiological significance of NMDA receptor signaling in Purkinje cells is supported by the observation that in slices prepared from adult mice and rats, postsynaptically expressed NMDA receptors are required for PF-LTD induction (Piochon et al. 2010).

Here, we examine whether ethanol impairs NMDA receptor signaling and synaptic plasticity in cerebellar Purkinje cells. We
show that, at concentrations as low as 10 mM, ethanol reduces \( \alpha \)-2-amino-5-phosphonovaleric acid (\( \alpha \)-APV)-sensitive synaptic responses at CF synapses and impairs PF-LTD induced by paired PF and CF activation. In contrast, LTD induced by pairing PF stimulation and application of depolarizing voltage steps is not affected by ethanol. It has previously been shown that both voltage-gated calcium channels and mGluR1 receptor signaling are affected by ethanol at concentrations of 50 mM but not 20 mM (Belmeguenai et al. 2008). Thus our data suggest that NMDA receptors are a primary ethanol target in Purkinje cells and are selectively impaired by ethanol at low concentrations.

**MATERIALS AND METHODS**

All animal procedures described were performed in accordance with a protocol approved by the Animal Care and Use Committee of the University of Chicago.

**Slice preparation.** Sagittal slices of the cerebellar vermis (190–250 \( \mu \)m) were prepared from C57BL6 mice (2- to 6-mo-old, unless specified otherwise) and were kept at room temperature in artificial cerebrospinal fluid (ACSF) containing the following (in mM): 124 NaCl, 5 KCl, 1.25 Na\(_2\)HPO\(_4\), 2 CaCl\(_2\), 26 NaHCO\(_3\), 10 HEPES, 4 NaCl, 4 Na\(_2\)ATP, and 5 KCl, 1.25 Na\(_2\)HPO\(_4\), 2 CaCl\(_2\), 26 NaHCO\(_3\), and 10 D-glucose.

**Currents were filtered at 3 kHz, digitized at 25 kHz, and acquired with EPC-10 amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany).**

Purkinje cells in acute cerebellar slices of 2- to 6-mo-old mice in the presence of 100 \( \mu \)M picrotoxin. Two CF-stimulation pulses were applied at an interval of 100 ms. CF-excitatory postsynaptic currents (EPSCs) were distinguished from PF-EPSCs by their typical paired-pulse depression and the characteristic all-or-none response. AMPA receptor-mediated currents were blocked by 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[1]quinoxaline (NBQX; 10 \( \mu \)M) in the ACSF that was supplemented with picrotoxin (100 \( \mu \)M) to block GABA\(_A\) receptors. All drugs were purchased from Sigma (St. Louis, MO).

**Whole cell patch-clamp recordings.** Patch-clamp recordings from the Purkinje cell soma were performed at room temperature using an EPC-10 amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany). Currents were filtered at 3 kHz, digitized at 25 kHz, and acquired using Patchmaster software (HEKA). Patch pipettes (2–5 M\( \Omega \)) were filled with a solution containing the following (in mM): 9 KCl, 10 KOH, 120 K-glucionate, 3.48 MgCl\(_2\), 10 HEPES, 4 NaCl, 4 Na\(_2\)ATP, 0.4 Na\(_3\)GTP, and 17.5 sucrose (pH 7.25–7.35). Purkinje cells were voltage clamped at holding potentials in the range of −65 to −78 mV. To evoke synaptic responses, PFs and CFs were activated using glass electrodes filled with ACSF. For PF stimulation, electrodes were placed in the upper molecular layer to reduce the risk of unintentional CF stimulation. For CF stimulation, electrodes were placed in the granule cell layer, just underneath the recorded Purkinje cells. In all experiments, the paired-pulse ratio was monitored using paired-pulse stimulation (100-ms interval) at a frequency of 0.05 Hz. For LTD induction, we used two different protocols. In the first protocol, 100-Hz PF burst stimulation (8–10 pulses) was followed 150 ms later by single pulse CF stimulation (current-clamp mode). This stimulation pattern was repeated at 1 Hz for 5 min. In the second protocol, depolarizing voltage steps (voltage-clamp; −75 to 0 mV; 250 ms) were paired with PF stimulation (2 pulses; 10-ms interval) at 1 Hz for 3 min. This protocol was applied for a second time after a period of 5 min. Series and input resistances were monitored throughout the experiments by applying hyperpolarizing voltage steps (−10 mV) at the end of each sweep. Recordings were excluded if series or input resistances varied by >15% over the course of the experiments. All values were averaged over time (3 successive responses) and are shown as a percentage of baseline (calculated from the last 5 min of baseline) ± SE. For statistical analysis, we used the Student’s \( t \)-test (paired/unpaired) and the Mann-Whitney \( U \) test, where appropriate.

**RESULTS**

Whole cell voltage-clamp recordings were performed from Purkinje cells in acute cerebellar slices of 2- to 6-mo-old mice in the presence of 100 \( \mu \)M picrotoxin. Two CF-stimulation pulses were applied at an interval of 100 ms. CF-excitatory postsynaptic currents (EPSCs) were distinguished from PF-EPSCs by their typical paired-pulse depression and the characteristic all-or-none response. AMPA receptor-mediated currents were blocked by bath application of 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[1]quinoxaline (NBQX; 10 \( \mu \)M), unveiling a residual current of 150.8 ± 13.8 pA (EPSC\(_1\), \( n = 5 \); Fig. 1A; see Piochon et al. 2007). This residual current was significantly reduced by subsequent bath application of the NMDA receptor antagonist \( \alpha \)-APV (50 \( \mu \)M; 37.2 ± 9.3 pA; 24.7 ± 3.5%; \( n = 5 \); \( P < 0.05 \); Fig. 1B), suggesting that this current is largely NMDA receptor dependent. Upon washout of \( \alpha \)-APV, the residual current recovered to 126.5 ± 31.1 pA (83.9 ± 16.3%; \( n = 5 \); \( P < 0.05 \); Fig. 1C). The paired-pulse ratio of the residual CF-EPSC did not change with \( \alpha \)-APV bath application (0.55 ± 0.04 under control conditions; 0.53 ± 0.01 under control conditions; 0.54 ± 0.02 under \( \alpha \)-APV application).

Fig. 1. \( N \)-methyl-\( \alpha \)-aspartate (NMDA) receptor-mediated currents at cerebellar climbing fiber-Purkinje cell synapses. A: typical traces. Climbing fiber (CF)-excitatory postsynaptic currents (EPSCs) recorded in the presence of 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[1]quinoxaline (NBQX; 10 \( \mu \)M; top); the residual current was further inhibited by \( \alpha \)-2-amino-5-phosphonovaleric acid (\( \alpha \)-APV; 50 \( \mu \)M; middle). After washout of \( \alpha \)-APV, the current recovered (bottom). B: time graph of \( \alpha \)-APV inhibition of NBQX-resistant CF-EPSCs (EPSC\(_1\); \( n = 5 \)). Black bar represents the presence of NBQX (10 \( \mu \)M) in the bath. White bar indicates the presence of \( \alpha \)-APV (50 \( \mu \)M) in the bath. C: bar graph showing the reduction of NBQX-resistant currents by \( \alpha \)-APV and the recovery during washout. Washout was performed at \( n = 5 \); the data were obtained during a 5-min baseline period, during 5 min of maximal reduction by \( \alpha \)-APV, and during 5 min of steady-state recovery; \( * P < 0.05 \); paired Student’s \( t \)-test. Error bars are means ± SE.

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0.58 ± 0.05 in the presence of d-APV; n = 5; P > 0.05; see Fig. 3), suggesting that the NMDA receptors blocked by d-APV were of postsynaptic origin.

To examine the sensitivity of NMDA receptor-mediated currents in Purkinje cells to ethanol, we repeated the same type of experiment but subsequently added ethanol to the bath. In the presence of NBQX (10 μM), the CF-EPSC amplitude was reduced to 180.9 ± 23.6 pA (n = 6). Subsequent application of d-APV (50 μM) reduced these currents to 26.4 ± 2.5 pA (14.6 ± 3.6%; n = 6; P < 0.05; Fig. 2, A–C). After d-APV washout the current recovered to 87.9 ± 19.6 pA (48.6 ± 6.9%; n = 6; P < 0.05; Fig. 2, A–C). We then applied 10 mM ethanol to the recovered NMDA receptor-mediated current, which reduced this current to 40.2 ± 6.9 pA (45.7 ± 6.8% of the recovered current after d-APV washout; n = 6; P < 0.05; Fig. 2, A–C). Next, we performed this experiment using 50 mM ethanol. In the presence of NBQX (10 μM), the residual current amounted to 195.8 ± 19.3 pA and was inhibited to 24.3 ± 4.5 pA by d-APV (50 μM) bath application (12.4 ± 6.9%; n = 9, P < 0.05; Fig. 2, D–F). Washout of d-APV led to a recovery of this current to 136.1 ± 20.3 pA (69.5 ± 6.8%; n = 9; P < 0.05; Fig. 2, D–F). Application of 50 mM ethanol reduced this current to 18.2 ± 6.9 pA (13.4 ± 5.6% of the recovered current after d-APV washout; n = 9; P < 0.05; Fig. 2, D–F). The reduction of the recovered NMDA current by ethanol was significantly more pronounced at a concentration of 50 mM than at 10 mM (P < 0.05; Fig. 2). These data show that ethanol inhibits NMDA receptor signaling in Purkinje cells in a dose-dependent manner. However, a significant reduction of NMDA current amplitudes was already observed when 10 mM ethanol was bath applied. Ethanol bath application did not significantly alter the rise time (10–90%) or decay time constants of d-APV-sensitive EPSCs (10 mM EtOH: control: rise time, 3.85 ± 0.5 ms; decay time constant, 20.3 ± 0.6 ms; EtOH: rise time, 4.53 ± 0.4 ms; decay time constant, 17.7 ± 1.1 ms; n = 6; P > 0.05; and 50 mM EtOH: control: rise time, 3.61 ± 0.6 ms; decay time constant, 17.9 ± 1.4 ms; EtOH: rise time, 5.07 ± 0.5 ms; decay time constant, 19.2 ± 0.8 ms; n = 9; P > 0.05), which suggests that the residual current recorded in the presence of 50 mM ethanol is either NMDA receptor mediated as well or that a residual non-NMDA receptor current had similar kinetics. Ethanol bath application did not affect the paired-pulse ratio (10 mM EtOH: baseline, 0.61 ± 0.10; EtOH, 0.59 ± 0.08; n = 6; P > 0.05; 50 mM EtOH: baseline, 0.52 ± 0.11; EtOH, 0.53 ± 0.10; n = 9; P > 0.05; Fig. 3), suggesting that EtOH acted postsynaptically.

To specifically address the question whether the residual current that can be recorded in the presence of NBQX and d-APV (see Figs. 1, 2, 5) is ethanol sensitive, we measured CF-EPSCs in the presence of NBQX (10 μM) and d-APV (50 μM). Under

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**Fig. 2.** Ethanol inhibits d-APV-sensitive currents. **A–C:** effects of 10 mM EtOH. **A:** typical traces. **First trace:** CF-EPSCs recorded in the presence of NBQX. **Second trace:** d-APV inhibition of the NBQX-resistant currents. **Third trace:** recovery from d-APV inhibition. **Fourth trace:** inhibition of the recovered NMDA receptor-mediated current by 10 mM EtOH. **B:** time graph showing EtOH inhibition of NMDA receptor-mediated CF-EPSCs (EPSC1; n = 6). Bars indicate periods of drug application. After a stable baseline was established in the presence of NBQX (10 μM; black bar), d-APV (50 μM) was applied until steady inhibition was achieved (white bar). Subsequently, d-APV was washed out and the NMDA receptor-mediated current was allowed to recover before the application of EtOH (10 mM; grey bar). **C:** bar graph showing the reduction of NBQX-resistant currents by d-APV (50 μM), d-APV washout, and suppression by 10 mM EtOH (n = 6). Amplitude of recovered EPSCs after d-APV washout was normalized to baseline values before calculating the EPSC reduction in the presence of EtOH. **D–F:** bars indicate the suppression of d-APV-sensitive currents by 50 mM EtOH. **D:** typical traces show the suppression of d-APV-sensitive currents by 50 mM EtOH. **E:** time graph showing EPSC blockade by d-APV, recovery after washout, and subsequent suppression by 50 mM EtOH (n = 9). **F:** bar graph showing the reduction of NBQX-resistant currents by d-APV (50 μM), d-APV washout, and suppression by 50 mM EtOH (n = 9; *P < 0.05; paired Student’s t-test). Error bars are means ± SE.
induction protocol resulted in long-term potentiation (LTP) instead (128.1 ± 12.7%; n = 6; P < 0.05; Fig. 6, A and B). These alterations were not accompanied by changes in the paired-pulse facilitation (PPF) ratio (data not shown). Under naïve conditions (without prior μ-APV application/washout), 10 mM EtOH reduces CF-EPSCs recorded in the presence of NBQX (10 μM) to 88.6% (Fig. 5, A–C). We next asked whether LTD was similarly impaired in the presence of a low concentration of μ-APV that would reduce CF-EPSCs to a comparably low degree. We determined that μ-APV at a concentration of 0.2 μM reduces CF-EPSCs recorded in the presence of NBQX (10 μM) to 81.3 ± 5.7% (n = 3; Fig. 6C). In the presence of μ-APV at 0.2 μM, PF-LTD induction was impaired and a potentiation was observed instead (PPSC1: 121.9 ± 19.8%; n = 6; t = 44–48 min; P < 0.05; Fig. 6, D and E). This change in EPSC amplitudes was significantly different from that observed under control conditions (see above; P < 0.01; Mann-Whitney U-test). The alterations seen in the presence of 0.2 μM μ-APV were not accompanied by changes in the PPF ratio (data not shown). These results show that comparably moderate reductions in CF-EPSC amplitude by either ethanol (10 mM) or μ-APV (0.2 μM) cause a similar impairment of PF-LTD, supporting the notion that at low concentrations EtOH may affect the LTD/LTP balance by modulating NMDA receptor signaling.

The experiments described above were performed in 0 mM external Mg2+. To examine whether ethanol impairs LTD under more physiological conditions, we repeated these experiments when 2 mM MgSO4 were added to the ACSF. Under control conditions, LTD amounted to 65.5 ± 6.4% (n = 8; P < 0.05; Fig. 7). In the presence of 10 mM ethanol, LTD was blocked (99.2 ± 9.8%; n = 9; P > 0.05; Fig. 7). However, LTD was not reversed to LTP as observed in Mg2+ free solution. These results show that low-dosage ethanol application impairs LTD induction in Mg2+ free solution (facilitating NMDA currents) but also under more physiological conditions (2 mM MgSO4).

We next reversed the application sequence of μ-APV and ethanol, respectively. Bath application of 10 mM ethanol caused a reduction in the amplitude of NMDA-mediated currents to 88.6 ± 4.0% (NBQX alone: 248.0 ± 97.8 pA; NBQX + EtOH: 209.1 ± 79.3 pA; t = 8–12 min; n = 4; P < 0.01; Fig. 5, A–C). After washout of ethanol, the current recovered to 98.7 ± 6.0% (228.3 ± 80.9 pA). Subsequent application of μ-APV (50 μM) reduced the amplitude to 22.3 ± 5.6% of the recovered current (52.9 ± 36.0 pA; t = 49–53 min; n = 3; P < 0.01; Fig. 5, A–C). Bath application of 50 mM ethanol resulted in a more pronounced reduction in the amplitude of NMDA-mediated currents to 49.2 ± 2.8% (NBQX alone: 159.3 ± 17.7 pA; NBQX + EtOH: 78.3 ± 13.2 pA; n = 5; P < 0.05; Fig. 5, D–F). After washout of ethanol, the current recovered to 71.8 ± 10.6% (114.4 ± 2.3 pA). Subsequent application of μ-APV (50 μM) caused a decrease of the recovered current to 25.9 ± 12.6% (29.6 ± 3.8 pA; n = 5; P < 0.05; Fig. 5, D–F), confirming that the ethanol-sensitive current was mediated by NMDA receptors.

It has recently been shown that activation of Purkinje cell NMDA receptors is required for PF-LTD induction in the mature mouse cerebellum (Piochon et al. 2010). Thus we examined whether LTD induced with the modified protocol described by Piochon et al. (2010) (8–10 PF pulses at 100 Hz, followed after a delay of 150 ms by a single CF pulse; repeated at 1 Hz for 5 min) is affected by ethanol in cerebellar slices prepared from 2- to 6-month-old mice. PF-EPSCs were evoked by paired-pulse stimulation (100-ms interval) delivered to the outer molecular layer using a glass pipette filled with ACSF. Application of the pairing protocol induced PF-LTD (EPSC1: 75.0 ± 12.8%; n = 12; t = 44–48 min; P < 0.01; Fig. 6, A and B). In the presence of 10 mM ethanol, application of the same

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**Fig. 3.** Paired-pulse ratio of CF-EPSCs remains unaffected in the presence of μ-APV and ethanol, respectively. Top: typical traces under control conditions and after treatment with μ-APV (left), 10 mM EtOH (middle), and 50 mM EthOH (right), respectively. Bottom: bar graphs showing paired-pulse ratio values before and during μ-APV application (n = 5), as well as during the application of EthOH at concentrations of 10 mM (n = 6) and 50 mM (n = 9), respectively.

**Fig. 4.** NBQX/μ-APV-insensitive residual current is not blocked by ethanol. A: typical traces show CF-EPSCs in the presence of NBQX (10 μM) and μ-APV (50 μM; left) and after ethanol (50 mM) wash-in (middle). Right: overlay of traces. B: time graph showing CF-EPSC amplitude changes upon wash-in of ethanol (50 mM; n = 4). Bars represent the presence of drugs in the bath. Error bars are means ± SE.
We next asked the question whether 10 mM ethanol also impairs LTD induced by a protocol in which CF activation is replaced by somatic depolarization, thus bypassing the activation of NMDA receptors at CF synapses. LTD was triggered by pairing depolarizing voltage steps [-75 to 0 mV; 250 ms; age: postnatal day (P)24–90] with PF activation (2 pulses at an interval of 10 ms) at 1 Hz for 3 min (2×, 5-min interval; 2 mM MgSO4). Under control conditions, this pairing protocol resulted in a depression of PF response amplitudes (69.7 ± 12.3%; n = 7; t = 36–40 min; P < 0.01; Fig. 8). In the presence of 10 mM ethanol in the bath, LTD could still be induced (71.9 ± 7.2%; n = 8; P < 0.01; Fig. 8). The difference in LTD amplitudes between the two experimental conditions was not significant (P > 0.05; Mann-Whitney U-test). These results show that a form of LTD that does not involve NMDA receptor signaling at CF synapses is not sensitive to 10 mM ethanol.

The data shown above indicate that ethanol at low doses (10 mM) impairs cerebellar LTD induced by paired PF and CF activation and identify NMDA receptors at CF synapses as a primary ethanol target at low concentrations. Since the paired synaptic stimulation protocol was applied in current-clamp mode, we determined the effects of 10 mM ethanol on CF-evoked complex spikes recorded in current clamp. It has previously been shown that complex spikes are affected by ethanol: at a concentration of 50 mM, ethanol reduces the late phase of the complex spike waveform. It has been suggested that this effect results from an inhibition of mGluR1 signaling by ethanol (Carta et al. 2006). However, at lower doses (20 mM), ethanol does not have a significant effect on mGluR1 receptors (Belmeguenai et al. 2008). To examine whether ethanol can also affect the complex spike waveform at low doses, we recorded complex spikes (in 0 mM Mg2+) and bath-applied ethanol (10 mM). In the presence of ethanol, the amplitude of the first spike and the early spikelets did not change (Fig. 9A), suggesting that ethanol did not reduce voltage-gated sodium currents (Camacho-Nasi and Treistman 1986; Wu and Kendig 1998). In contrast, the afterdepolarization (ADP) was reduced when ethanol was bath applied (area under the curve: 100 ms period after last spikelet: 93.3 ± 2.7%; t = 20–24 min; n = 8; P < 0.01; Fig. 9A). The ADP recovered after ethanol washout (100.4 ± 0.9%; t = 105–109 min; n = 3; P < 0.01; Fig. 9A). In the presence of d-APV (50 μM), bath application of ethanol (10 mM) did not cause a reduction in the ADP amplitude (99.4 ± 0.8%; t = 10–14 min; n = 12; P > 0.05; Fig. 9B). These data show that the complex spike ADP is reduced by 10 mM ethanol and that at this low concentration this ethanol effect is specifically related to a reduction in NMDA receptor-mediated response components. In some recordings, the ADP reduction observed after ethanol wash-in was accompanied by a reduction in the spikelet number (data not shown). At a concentration of 10 mM, ethanol does not affect mGluR1 receptor signaling or voltage-gated calcium channels (Belmeguenai et al. 2008). The results presented here show that 1) 10 mM ethanol reduces the complex spike ADP and 2) this effect is not observed when NMDA receptors are blocked by d-APV thus suggest that at low concentrations NMDA receptors are the main ethanol target and that NMDA receptor blockade may cause the impairment of LTD.
We have previously shown that ethanol (50 mM) does not affect PF synaptic transmission in P18–30 rats (Belmeguenai et al. 2008). To exclude the possibility that in the more mature animals tested here LTD could be impaired by ethanol effects on PF responses, we monitored PF synaptic transmission in 2-mo-old mice. Ethanol bath application (10 mM) had no effect on the PF responses, we monitored PF synaptic transmission in 2-mo-old mice. Ethanol bath application (10 mM) had no effect on the PF-excitatory postsynaptic potential (EPSP) amplitude (EPSP1: 97.5 ± 3.9%; t = 15–19 min; n = 6; P > 0.05; Fig. 10, A and B) and did not affect the paired-pulse facilitation ratio (103.1 ± 2.6%; P > 0.05; Fig. 10, A and C). Thus LTD induced by paired PF and CF activation was not blocked by 10 mM ethanol because of ethanol affects at PF synapses but rather because of its reduction of NMDA receptor signaling at CF synapses.

**DISCUSSION**

The results presented here show that ethanol at low doses (10 mM) impairs cerebellar synaptic plasticity and that at this concentration NMDA receptors are a main ethanol target. It has previously been demonstrated that in Purkinje cells voltage-gated calcium channels and mGluR1 receptors are affected by ethanol. Like NMDA receptors, both provide sources for calcium influx that are likely involved in LTD induction. However, voltage-gated calcium channels and mGluR1 receptors are characterized by a lower ethanol sensitivity than NMDA receptors and are affected by ethanol at a concentration of 50 mM but not at 20 mM (Carta et al. 2006; Belmeguenai et al. 2008). Slow currents triggered by mGluR1 receptor activation can be suppressed by 10 mM ethanol, but only when ethanol is applied internally (added to the pipette saline). A reduction in mGluR1 signaling by externally applied ethanol was only observed with higher doses of ethanol (50 mM; Su et al. 2010). The high ethanol sensitivity of NMDA receptors reported here falls into the same range as that observed in the hippocampus, where a significant reduction in the amplitude of NMDA receptor-mediated field EPSPs was observed when ethanol was applied at a concentration of 25 mM, and a reduction that did not reach statistical significance was observed at 1 mM (Lovinger et al. 1990). At 50 mM, ethanol blocks the d-APV-sensitive current in Purkinje cells almost entirely (Fig. 2, D–F). This pronounced reduction might be due to the fact that, in addition to its effects on the phosphorylation state of NMDA receptors and potential consequences for their biophysical properties (Miyakawa et al. 1997; Alvestad et al. 2003; Yaka et al. 2003; Hicklin et al. 2011), ethanolic can induce internalization of NMDA receptors, thus lowering the receptor density in the membrane (Suvanara et al. 2005). This possibility is further strengthened by the observation that the effects of ethanol (50 mM) were only partially reversible. At 10 mM ethanol, CF responses returned to baseline following ethanol washout (Fig. 5). The reduction of CF response amplitudes was more pronounced when ethanol was applied after previous d-APV application/washout (Fig. 2) compared with the reduction that was seen when ethanol was applied first (Fig. 5). This unexpected result was obtained when using ethanol at concentrations of 10 and 50 mM, respectively. Future studies will have to address whether this effect may be due to changes in the subunit composition or surface expression of NMDA receptors or any other factor that might regulate ethanol sensitivity.
revisited the question of whether ethanol can impair PF-LTD to mM (Carta et al. 2006; Belmeguenai et al. 2008). Here, we not PF-LTP, are prevented by ethanol at a concentration of 50 range as those reached by mild alcohol consumption in hu-
ing and LTD are impaired at low doses that are in the same
living organisms, it can be noted that NMDA receptor signal-
relevance of alcohol concentrations used in vitro to those in
mM. While we acknowledge that it is difficult to compare the
old is 0.08 g/dl, which is equivalent to a concentration of 17
exposure. In the United States, the legal blood alcohol thresh-
Morisset et al. 1991). In our hands, 10 mM ethanol blocked
it is induced by a protocol in which depolarizing voltage steps
substitute for CF stimulation, thus bypassing NMDA receptor
activation at CF synapses. In previous studies, it was suggested
that the antagonistic effect of ethanol on NMDA currents is
enhanced in the presence of Mg$^{2+}$ ions (Martin et al. 1991; 
Morisset et al. 1991). In our hands, 10 mM ethanol blocked
LTD in 2 mM MgSO$_4$, but we even observed a reversal toward
LTP in Mg$^{2+}$ free solution (Fig. 6). Under control conditions,
the Mg$^{2+}$ concentration did not affect the ability of PF syn-
apses to express LTD. It seems that during application of the
LTD induction protocol sufficient depolarization is reached to
remove the Mg$^{2+}$ block from NMDA channels. However,
ethanol seems to unveil a potentiation mechanism that is
suppressed when Mg$^{2+}$ ions are present in the ACSF.

Throughout our recordings, we observed a small excitatory
current that was resistant to bath application of NBQX and d-
APV. This residual current was not affected by ethanol (50 mM).
We did not attempt to further characterize or identify this current.

Taken together, our data show that in Purkinje cells NMDA
receptors are the primary ethanol target at low-dose ethanol
exposure. In the United States, the legal blood alcohol thresh-
hold is 0.08 g/dl, which is equivalent to a concentration of 17
mM. While we acknowledge that it is difficult to compare the
relevance of alcohol concentrations used in vitro to those in
living organisms, it can be noted that NMDA receptor signal-
ning and LTD are impaired at low doses that are in the same
range as those reached by mild alcohol consumption in hu-

It has previously been shown that CF-LTD and PF-LTD, but
not PF-LTP, are prevented by ethanol at a concentration of 50
mM (Carta et al. 2006; Belmeguenai et al. 2008). Here, we
revisited the question of whether ethanol can impair PF-LTD to
specifically examine whether ethanol can have this effect at
low doses, at which NMDA receptors, but not voltage-gated
calcium channels or mGluR1 receptors are affected. Our re-
results show that 10 mM ethanol blocks LTD. It remains possible
that relevant factors other than NMDA receptors are affected by
10 mM ethanol. However, the recent demonstration that
Purkinje cell NMDA receptors are required for LTD induction
(Piochon et al. 2010) suggests that the blockade of NMDA
receptor-mediated currents at this ethanol concentration con-
tribute to the suppression of LTD. This notion is supported by
two additional observations. First, LTD was similarly impaired
in the presence of 0.2 μM d-APV, which reduces NMDA
receptor-mediated CF-EPSCs to a comparable degree as 10
mM ethanol does, suggesting that at this concentration ethanol
indeed may act primarily through the reduction of NMDA
receptor signaling. Second, LTD is not ethanol sensitive when
it is induced by a protocol in which depolarizing voltage steps
substitute for CF stimulation, thus bypassing NMDA receptor
activation at CF synapses. In previous studies, it was suggested
that the antagonistic effect of ethanol on NMDA currents is
enhanced in the presence of Mg$^{2+}$ ions (Martin et al. 1991; 
Morisset et al. 1991). In our hands, 10 mM ethanol blocked
LTD in 2 mM MgSO$_4$, but we even observed a reversal toward
LTP in Mg$^{2+}$ free solution (Fig. 6). Under control conditions,
the Mg$^{2+}$ concentration did not affect the ability of PF syn-
apses to express LTD. It seems that during application of the
LTD induction protocol sufficient depolarization is reached to
remove the Mg$^{2+}$ block from NMDA channels. However,
ethanol seems to unveil a potentiation mechanism that is
suppressed when Mg$^{2+}$ ions are present in the ACSF.

In young rats (P15–26), it has been found that LTD induc-
tion requires the activation of NMDA receptors located at PF
terminals (Casado et al. 2002). However, this finding has been
controversial as later studies reported that NMDA receptor
blockade neither affects calcium transients in PF terminals
(Shin and Linden 2005; Qiu and Knöpfel 2007) nor the frequency of miniature EPSCs (Shin and Linden 2005). In our
previous study using 2- to 6-mo-old mice, d-APV bath appli-
cation neither changed the PPF ratio at PF synapses nor the
paired-pulse depression ratio at CF synapses, suggesting that in
the mature mouse cerebellum presynaptic NMDA receptors are
not activated at these synapses (Piochon et al. 2010). In the
present study, in which we again used 2- to 6-mo-old mice, we
confirmed that d-APV bath application reduces CF-EPSC am-
plitudes (Fig. 1) but does not affect the paired-pulse depression
ratio at CF synapses (Fig. 3). These data suggest that the NMDA receptors studied here are postsynaptically expressed. Moreover, we demonstrate in mature Purkinje cells that ethanol (10 mM) does not affect PF synaptic responses (Fig. 10) but reduces CF responses (Figs. 2, 5, and 9). The latter effect was not seen when NMDA receptors were blocked by D-APV (50 µM; Fig. 9). Together, these data show that at this concentration ethanol selectively reduces NMDA receptor signaling at CF synapses.

At high doses, ethanol has relatively unspecific effects on signaling mechanisms affecting, among others, voltage-gated calcium channels (Walter and Messing 1999; Belmeguenai et al. 2008), mGluR1 receptors (Carta et al. 2006; Belmeguenai et al. 2008), and NMDA receptors (Lovinger et al. 1989, 1990;
Schummers and Browning 2001; this study). It is therefore surprising that ethanol selectively suppresses cerebellar LTD, but not LTP (Belmeguenai et al. 2008). A possible explanation is that at cerebellar PF synapses onto Purkinje cells, larger calcium transients are required for LTD than for LTP induction (Coesmans et al. 2004). In contrast to LTD, LTP requires neither mGluR1 (Belmeguenai et al. 2008) nor NMDA receptor activation for induction (Picchioni et al. 2010). It is thus likely that ethanol does not block LTP (at concentrations as high as 50 mM), because it suppresses signaling pathways that are specifically involved in LTD induction and because LTP is less sensitive to a reduction in the amplitude of calcium transients. At low doses, ethanol effects become more specific. At a concentration of 10 mM, ethanol reduces NMDA receptor-mediated currents (shown here) but does not affect voltage-gated calcium currents or mGluR1 receptors (Belmeguenai et al. 2008). These data support the notion that NMDA receptors are a primary target of ethanol and emphasize the key role of NMDA receptors in cerebellar LTD. Recent data suggest that LTD alone cannot account for cerebellar motor learning but that other plasticity mechanisms, such as LTP, might play a critical role as well (Schonewille et al. 2010, 2011). It is thus conceivable that motor coordination deficits resulting from alcohol consumption (for review, see Valenzuela et al. 2010) are partially due to ethanol-mediated alterations in the LTD / LTP balance and the resulting deficits in the fast adjustment of PF synaptic strength.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: Q.H., H.T., G.G., C.P., and C.H. conception and design of research; Q.H., H.T., and G.G. performed experiments; Q.H., H.T., and G.G. analyzed data; Q.H., H.T., G.G., C.P., and C.H. interpreted results of experiments; Q.H. and H.T. prepared figures; Q.H., H.T., G.G., C.P., and C.H. approved final version of manuscript; C.H. drafted manuscript; C.H. edited and revised manuscript.

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