Ethanol-mediated long-lasting adaptations of the NR2B-containing NMDA receptors in the dorsomedial striatum

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We recently found that ethanol-induced long-term facilitation (LTF) of NMDAR activity is mediated by NR2B-NMDARs and is observed in the dorsomedial striatum (DMS) but not in the dorsolateral striatum (DLS).9 We also showed that repeated administration of ethanol causes a long-lasting increase in NMDAR activity in the DMS, resulting from ethanol-mediated Fyn phosphorylation of NR2B subunits.9 In this addendum, we report that the different sensitivity of NMDARs to ethanol between the DMS and DLS is not attributed to the abundance of synaptic NR2B-NMDARs or differences in Fyn levels. We further show that LTF is specific for NR2B-, but not NR2A-NMDARs, and that the duration of the in vivo ethanol-mediated increase in NMDAR activity is associated with the period of ethanol exposure, but not with alteration in NR1 or NR2A protein levels. Together, these results suggest that upregulation of NR2B-NMDAR activity by ethanol is selective and that ethanol’s effect on NMDAR activity is gradual and cumulative.

Introduction

We recently found that ethanol-induced long-term facilitation (LTF) of NMDAR activity is mediated by NR2B-NMDARs and is observed in the dorsomedial striatum (DMS) but not in the dorsolateral striatum (DLS).9 We also showed that repeated administration of ethanol causes a long-lasting increase in NMDAR activity in the DMS, resulting from ethanol-mediated Fyn phosphorylation of NR2B subunits.9 In this addendum, we report that the different sensitivity of NMDARs to ethanol between the DMS and DLS is not attributed to the abundance of synaptic NR2B-NMDARs or differences in Fyn levels. We further show that LTF is specific for NR2B-, but not NR2A-NMDARs, and that the duration of the in vivo ethanol-mediated increase in NMDAR activity is associated with the period of ethanol exposure, but not with alteration in NR1 or NR2A protein levels. Together, these results suggest that upregulation of NR2B-NMDAR activity by ethanol is selective and that ethanol’s effect on NMDAR activity is gradual and cumulative.

Differential sensitivity of NMDARs to ethanol is not attributed to the abundance of synaptic NR2B-NMDARs. We previously showed that acute exposure of striatal slices to, and withdrawal from, ethanol induces LTF of NMDAR activity in the DMS but not DLS.3 We further showed that the brain region specificity is not due to differential total levels of the NR2B or NR2A subunit protein in the two brain regions.9 Heterogeneity in synaptic NR2 subunits of NMDARs in the DMS and DLS may
Differential sensitivity of NMDARs to ethanol is not attributed to differential protein levels of Fyn. We previously showed that acute ex vivo or in vivo exposure to ethanol increases Fyn activity in the hippocampus and dorsal striatum, leading to increased NR2B phosphorylation. To test whether the differential sensitivity of NMDARs to ethanol in the DMS and DLS is associated with distinct expression of Fyn, we measured protein levels of Fyn. Since the electrophysiological and behavioral experiments were performed in juvenile and adult rats respectively, we determined the level of Fyn in the DLS and DMS in both ages. We found that Fyn levels do not differ in these subregions (Fig. 2) suggesting that the protein level of Fyn is not responsible for the differential sensitivity of NMDARs to ethanol.

Ethanol-mediated LTF is observed in the DMS of NR2A knockout (KO) mice. We previously showed that the NR2B subunit is required for ethanol-mediated LTF of NMDAR activity in the dorsal striatum. To determine whether the NMDAR-containing NR2B subunit (NR2A-NMDAR), another major NR2 subunit in the dorsal striatum, also contributes to LTF, we compared the level of LTF upon acute ex vivo ethanol exposure and withdrawal in the DMS of NR2A KO and wild-type (WT) littermate mice. We reasoned that if NR2A-NMDARs are required for LTF, we should observe less or no LTF in brain slices from NR2A KO mice compared to WT littermate mice. We found that LTF of NMDAR-EPSCs following acute ethanol withdrawal was not smaller but actually greater in DMS neurons from NR2A KO mice compared to WT mice. This suggests that NR2A-NMDARs do not contribute to ethanol-mediated LTF in the DLS and DMS.
Figure 4. Duration of the ethanol-mediated increase in NMDAR activity is associated with the period of ethanol exposure. (A) Single administration of ethanol did not lead to a detectable change in NMDA-induced current. Rats were systemically administered with 2 g/kg of ethanol or saline and striatal slices were prepared 16 hrs after the administration to measure NMDA-induced currents. Left, Changes in holding currents were measured after NMDA (10 μM, 30 s) was applied to the slices. n = 16 (saline) and 16 (EtOH). Right, Bar graph summarizing the peak amplitudes of NMDA-induced currents in DMS neurons from ethanol- and saline-treated rats. p > 0.05 by t-test. (B) Repeated ethanol administration does not alter presynaptic release of neurotransmitter. The frequency of miniature EPSCs (mEPSCs) were compared in DMS neurons from saline- and ethanol-treated animals. n = 25 (saline) and 29 (ethanol) slices. p > 0.05, Mann-Whitney Rank Sum test. (C) Seven administrations of ethanol did not alter NMDA-induced currents 40 hrs later after the last treatment. Rats were systemically administered with 2 g/kg of ethanol or saline daily for seven days and striatal slices were prepared 40 hrs after the last administration to measure NMDA-induced currents. Left, Changes in holding currents were measured after NMDA (10 μM, 30 s) was applied to the slices. n = 14 (saline) and 14 (EtOH). Right, Bar graph summarizing the peak amplitudes of NMDA-induced currents in DMS neurons from ethanol- and saline-treated rats. p > 0.05 by t-test.

These results suggest that NR2A subunits are not required for LTF in response to acute ethanol exposure, and strongly suggest a specific contribution of NR2B-NMDARs to ethanol-mediated LTF in the DMS.

The duration of ethanol-mediated increases in NMDAR activity is associated with the period of ethanol exposure. Next, we examined whether in vivo ethanol exposure and withdrawal cause an increase in NMDAR activity in the DMS. We found that a single systemic administration of ethanol did not alter NMDAR activity in the DMS when measured 16 hrs after the ethanol treatment (Fig. 4A). These findings lead to the hypothesis that repeated ethanol exposure and withdrawal is required to obtain a gradual and cumulative increase in NMDAR activity. As predicted, we observed that once-daily administration of ethanol for seven days increases NMDA-elicited currents and NMDAR-EPSC9 but not the frequency of miniature EPSCs (mEPSCs) in DMS neurons (Fig. 4B), suggesting that repeated ethanol administration increases postsynaptic NMDAR function. The increases were observed 16 hrs,9 but not 40 hrs after the last of seven ethanol administrations (Fig. 4C). However, prolonged 14 daily administrations of ethanol caused similar increases in NMDAR function 40 hrs after the last ethanol administration.9 These results suggest that repeated ethanol exposure and withdrawal results in a cumulative long-lasting increase in NMDAR activity in the DMS, supporting the model described by Wang et al.9

Repeated administration of ethanol or excessive ethanol consumption does not alter protein levels of NR1 or NR2A subunits in the DMS. We previously reported that repeated systemic administration of ethanol or high levels of voluntary ethanol drinking results in a long-lasting increase in NMDAR activity.9 Moreover, we found that the NR2B-NMDAR contribution to the overall channel activity was associated with increased phosphorylation and membrane localization of NR2B subunits in the DMS.9 Here, we report that repeated daily administration of ethanol did not
KO mice and their corresponding WT littermates were generated by in-house breeding of male and female NR2A heterozygous mice. Mice genotype was determined by polymerase chain reaction (PCR) analysis of products derived from tail DNA, and were used for experiments at three to five weeks of age. For electrophysiological recordings, NMDAR-mediated NR2B-NMDARs in the DMS resulting in an increase in channel function.

**Materials and Methods**

All materials and methods used in the current study, except for the information regarding the NR2A KO mice, are described in Wang et al. Briefly, NR2A KO mice and their corresponding WT littermates were generated by in-house breeding of male and female NR2A heterozygous mice. Mice genotype was determined by polymerase chain reaction (PCR) analysis of products derived from tail DNA, and were used for experiments at three to five weeks of age. For electrophysiological recordings, NMDAR-mediated NR2B-NMDARs in the DMS resulting in an increase in channel function.

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**Figure 5.** Repeated ethanol administration did not alter the protein levels of NR1 or NR2A subunits in the DMS. (A and B) Sprague-Dawley rats were treated with saline or ethanol (2 g/kg, i.p.) once a day for seven days. DMS was dissected 16 hrs after the last injection. (A) Repeated ethanol administration did not alter the protein levels of NR1 or NR2A subunits in total DMS homogenates. Left, image is representative of n = 3 for NR1 (top) and n = 9 for NR2A (bottom). p > 0.05 vs. saline-treated rats (two-tailed t-test). (B) Repeated ethanol administration did not alter the protein levels of synaptosomal NR1 or NR2A subunits in the DMS. Left, image is representative of n = 6 (saline) and n = 6 (ethanol) for NR1 (top). n = 6 (saline) and n = 5 (ethanol) for NR2A (bottom). (A and B) Middle and right, bar graphs summarizing the averaged changes in protein levels of NR1 (middle) and NR2A subunits (right). Western blot data was normalized to GAPDH and plotted as percentage of saline treatment. p > 0.05 vs. saline-treated rats (two-tailed t-test). (C) Intermittent access to ethanol did not alter the protein levels of NR1 or NR2A in total DMS homogenates. Long Evans rats were exposed to 18 ethanol-drinking sessions under an intermittent access to 20% ethanol in a two-bottle-choice paradigm. One day after the last drinking session, DMS tissue was dissected out and protein levels of NR1 and NR2A subunits in homogenates were measured. Left, image is representative of n = 7 (water), n = 8 (ethanol). Middle and right, bar graphs summarizing the averaged changes in protein levels of NR1 (middle) and NR2A subunits (right). Western blot data was normalized to GAPDH and plotted as percentage of water treatment. p > 0.05 vs. control (two-tailed t-test).
EPSCs were recorded at -70 mV and in the presence of 0.05 mM Mg²⁺, 0.01 mM NBQX and 0.1 mM picrotoxin in the bath solution. To measure NMDA-induced currents, NMDA (10 μM) was bath applied for 30 s and holding currents were measured every 5 s.

**Conclusions**

We show that neither the abundance of synaptic NR2B-NMDARs nor Fyn protein level accounts for the difference in NMDAR sensitivity to ethanol between the DMS and DLS. We also report that NR2A-NMDARs are not required for LTF of NMDAR activity. Finally, we found that the duration of ethanol-mediated increase in NMDAR activity is determined by the period of ethanol exposure and is associated with neither protein levels nor membrane localization of NR1 and NR2A subunits. Together, these results suggest that ethanol exposure causes a selective long-lasting alteration in NR2B-NMDARs in the DMS resulting in an increase in channel function.

We recently demonstrated that inhibition of NR2B-NMDAR/Fyn pathway in the DMS reduces operant ethanol self-administration and reinstatement of ethanol seeking. These data, together with the data presented herein, strongly suggest that the specific long-term activation of the Fyn/NR2B-NMDA pathway specifically within the DMS is an important player in the mechanism underlying excessive ethanol-drinking behavior. Facilitation of NMDAR activity following repeated cycles of ethanol bouts and withdrawal, may lead to alternation of AMPAR-LTP in the DMS, which is NMDAR-dependent. Such aberrant plasticity may be part of the mechanism involved in the transition from social drinking to excessive and compulsive ethanol intake and relapse.

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