Pergamon

PII: S0306-4522(01)00234-2

www.elsevier.com/locate/neuroscience

Neuroscience Vol. 105, No. 4, pp. 987–997, 2001

0306-4522/01 $20.00+0.00

ALTERED EFFECTS OF ETHANOL IN NR2A$^{\Delta C/\Delta C}$ MICE EXPRESSING C-TERMINALLY TRUNCATED NR2A SUBUNIT OF NMDA RECEPTOR

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Abstract—Phosphorylation of C-termini of receptor subunits is thought to play a significant role in modulation of *N*-methyl-D-aspartic acid (NMDA) receptor function. To investigate whether the C-terminus of the NR2A subunit is involved in determining the sensitivity of NMDA receptors to ethanol we compared the effects of ethanol *in vitro* on NMDA-mediated field excitatory postsynaptic potentials (fEPSPs) in the CA1 and dentate gyrus (DG) of adult male NR2A$^{\Delta C/\Delta C}$ mice lacking the C-terminus of NR2A subunit and in their parental strain C57Bl/6. We also tested the *in vivo* effects of a hypnotic dose of ethanol in C57Bl/6 and NR2A$^{\Delta C/\Delta C}$ mice and their F2 offspring. Ifenprodil (10 μM) was used to distinguish between the NR2A and NR2B components of NMDA fEPSPs. Ethanol (100 mM) in the presence of ifenprodil inhibited the CA1 NR2A-mediated component of NMDA fEPSPs two times more in NR2A$^{\Delta C/\Delta C}$ than in C57Bl/6. Ethanol inhibition of the CA1 NR2B-mediated component was five to seven times lower in NR2A$^{\Delta C/\Delta C}$ than in C57Bl/6. In the DG ethanol had similar effects in the two strains. *In vivo* administration of ethanol (4 g/kg) induced sedation of similar duration in both strains of mice. A second administration of ethanol 7 days after the initial injection revealed an increased ethanol sensitivity of NR2A$^{\Delta C/\Delta C}$ and F2$^{\Delta C/\Delta C}$ mice including a shortened time to loss of righting reflex and an increased sleep time. The sensitization of NR2A$^{\Delta C/\Delta C}$ mice to alcohol was not accompanied by an altered ethanol sensitivity of NMDA fEPSPs recorded *in vitro*.

Our data are consistent with the inhibitory action of ethanol on NMDA receptors being mediated by a site other than the intracellular C-terminus of the NR2A subunit. The altered sensitivities to ethanol of both NR2A- and NR2B-mediated responses in the CA1 of NR2A$^{\Delta C/\Delta C}$ imply that NR2A- and NR2B subunit-containing NMDA receptors may be linked by a common target of ethanol.

Key words: field excitatory postsynaptic potential, C-terminally truncated NR2A subunit, hippocampal slice, ifenprodil, hypnotic effects of ethanol.

Ethanol inhibits neuronal *N*-methyl-D-aspartic acid (NMDA) receptors in concentrations relevant to intoxicating doses in humans and animals (10–100 mM) (Little, 1999) in a variety of preparations including brain slices (Grover et al., 1994; Lovinger et al., 1990), primary neuronal cultures (Lovinger et al., 1989; Popp et al., 1999; Wirkner et al., 1999), and anesthetized animals (Yang et al., 1996). However, in intact preparations it has been difficult to determine the NMDA receptor subtypes responsible for the ethanol effect. Studies of recombinant NMDA receptors expressed in *Xenopus* oocytes (Kuner et al., 1993; Masood et al., 1994) as well as in HEK 293 cells (Anders et al., 1999b; Blevins et al., 1995; Lovinger, 1995) have demonstrated that the inhibitory effects of ethanol may be subunit-selective.

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Abbreviations: ACSF, artificial cerebrospinal fluid; B6, C57Bl/6; CGP56433A, 3-[1-(*s*)-[[3-(cyclohexylmethyl)-hydroxyphosphinyl]-2(*s*)-hydroxypropyl]ethyl]-benzoic acid; D-APV, D-2-amino-5-phosphonovalerate; ΔC, NR2A$^{\Delta C/\Delta C}$; DG, dentate gyrus; DMSO, dimethylsulfoxide; DNQX, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione; fEPSP, field excitatory postsynaptic potential; LORR, loss of righting reflex; NMDA, *N*-methyl-D-aspartic acid; P, postnatal day; PSD, postsynaptic density.

The NMDA receptor is considered to be a heterotetramer (Laube et al., 1998), comprised of an NR1 subunit ubiquitously distributed throughout the brain (Benke et al., 1995; Petralia et al., 1994) and at least one of the four members of the NR2 subunit family (A–D) which exhibit different developmental as well as regional expression patterns (Portera-Cailliau et al., 1996; Wenzel et al., 1995, 1997). Of the four members of the NR2 family, the 2A and 2B subunits are the predominant subunits expressed in the cortex and hippocampus during development and in the adult brain (Monyer et al., 1992; Wenzel et al., 1997). In expression systems, NR1/NR2A and NR1/NR2B receptors were shown to be the most sensitive to ethanol inhibition compared to NR1/NR2C or homomeric NR1 receptors (Kuner et al., 1993; Masood et al., 1994; Mirshahi and Woodward, 1995). Phosphorylation plays a significant role in the modulation of NMDA receptor function by ethanol (Caputi et al., 1999; Kalluri and Ticku, 1999; Miyakawa et al., 1997; Yagi, 1999), and NMDA receptors can be phosphorylated on their C-termini by a variety of protein kinases. Each NMDA receptor subunit has specific protein kinases that exert their modulatory effects through the subunit's C-terminus (Gardoni et al., 1999; Kohr and Seeburg, 1996; Leonard and Hell, 1997; Leonard et al., 1999; Logan et al., 1999; Suzuki and Okumura-Noji, 1995; Tingley et al., 1997), with *fyn* tyrosine kinase

sites confined to the C-termini of NR2 subunits. *Fyn*-mediated phosphorylation of NR2A subunit was shown to reduce the sensitivity of recombinant NR1/NR2A receptors expressed in HEK 293 cells to high concentrations of ethanol (Anders et al., 1999b), while in *fyn* knockout mice the hypnotic effects of ethanol were exacerbated (Miyakawa et al., 1997). A 5 min ethanol (3.5 g/kg) administration in mice enhances the tyrosine phosphorylation of hippocampal NR2B subunits (Kalluri and Ticku, 1999). These data are consistent with an involvement of the C-termini of NR2 subunits in the effects of ethanol on NMDA receptor function.

To determine whether the C-terminus of the NR2A subunit is involved in the inhibitory effects of ethanol on NMDA receptors, we compared the effects of ethanol *in vitro* on NMDA-mediated field excitatory postsynaptic potentials (fEPSPs) in the CA1 and dentate gyrus (DG) of adult male $NR2A^{\Delta C/\Delta C}$ mice (ΔC) lacking the C-terminus of NR2A subunit (Sprengel et al., 1998) and their parental strain C57Bl/6 (B6). Furthermore, we tested the effects of a hypnotic dose of ethanol *in vivo* in B6, ΔC and in their F2 offspring. To distinguish between the NR2A and NR2B components of the total NMDA-mediated responses, we performed the experiments in the presence of the NR2B-selective NMDA receptor antagonist ifenprodil (Williams, 1993).

EXPERIMENTAL PROCEDURES

Animals

The experiments were performed on adult male or 4–7-day-old B6 (Harlan, San Diego, CA, USA) $NR2A^{\Delta C/\Delta C}$ (Dr. Rolf Sprengel, Neuroscience Max-Planck Institute for Medical Research, Heidelberg, Germany) mice or adult F2 (wild-type and $\Delta C/\Delta C$) male progeny of the two strains. All experiments were performed in accordance with the National Institute of Health (NIH) guidelines on the ethical use of animals to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Slice preparation

Coronal or horizontal slices of the hippocampus were obtained using standard techniques. In brief, the animal was anesthetized with halothane and decapitated. The brain was cooled to 4°C, rapidly removed and whole brain slices (350 μm thick) were cut on a Leica VT100S vibroslicer (Leica Microsystems, Deerfield, IL, USA). After incubating in artificial cerebrospinal fluid (ACSF: 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose) for at least 1 h in a storage chamber at 32°C, the slices were transferred to a recording chamber, and continually perfused (2 ml/min, 31–32°C) with low (0.1 mM) Mg^{2+} ACSF in an atmosphere of humidified 95% O_2, 5% CO_2.

Extracellular recordings

Constant current stimuli (20 μs width) were delivered either to the perforant path or to the Shaffer collaterals every 15 s via bipolar stimulating electrodes. Extracellular fEPSPs were recorded in the stratum moleculare of the DG and in the stratum radiatum of the CA1. α-Amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor-mediated fEPSPs were measured in the presence of 50 μM D-2-amino-5-phosphonovalerate (D-APV). NMDA receptor-mediated responses were examined in the presence of 10 μM 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX). To exclude possible effects of $GABA_A$ receptor-mediated inhibition, 50 μM picrotoxin was always included in the ACSF.

Drugs

DNQX (Sigma, St. Louis, MO, USA), 10 mM and ifenprodil (Sigma), 100 mM stock solutions were prepared in 100% dimethylsulfoxide (DMSO); D-APV (Precision Biochemicals, Vancouver, BC, Canada), 50 mM stock was prepared in 40 mM NaOH; picrotoxin (Sigma), 50 mM stock was dissolved in ethanol (95% v/v); CGP56433A (3-[1-(*s*)-[[3-(cyclohexylmethyl)-hydroxyphosphinyl]-2(*s*)-hydroxypropyl]ethyl]-benzoic acid; a gift of Dr. W. Froestl, Ciba-Geigy, Basel, Switzerland), 50 mM stock was dissolved in water. After a stable extracellular fEPSP was obtained for each slice in low Mg^{2+} (0.1 mM) ACSF, the bath was changed to a low Mg^{2+} ACSF containing picrotoxin (50 μM) and DNQX (10 μM) to isolate NMDA receptor-mediated fEPSPs, or picrotoxin and D-APV (50 μM) to isolate AMPA- and kainate receptor-mediated fEPSPs. The final concentration of DMSO in the bath was 0.1% v/v, ethanol – 0.095% v/v). The slices were continually perfused in the recording chamber for up to 1 h prior to application of ifenprodil (10 μM final concentration) or ethanol (100 mM final concentration). DMSO alone in a concentration up to two times higher than in our experimental conditions did not alter NMDA fEPSPs. The final concentration of ifenprodil in ACSF was significantly lower than its solubility limit according to the manufacturer and thus could not be altered by the addition of 100 mM ethanol.

Data analysis

To ensure that the phosphorylation mechanisms possibly involved in the effects of ethanol on NMDA receptors do not wash out in whole-cell recordings we carried out field potential recordings that are also useful to examine the modulation of synaptic NMDA receptors (Crepel et al., 1993; Lieberman and Mody, 1999). NMDA fEPSP area rather than slope was measured in order to take into account the long time course of NMDA receptor-mediated synaptic potentials (Crepel et al., 1993; Lieberman and Mody, 1999). The fEPSP areas during the last 10 min of a control recording in low Mg^{2+}, picrotoxin and DNQX (or APV) were averaged and used as a baseline (100%). Values for all subsequent fEPSPs were expressed as a percentage of this baseline. Each experiment was repeated at least three times. The drug effects were calculated as percent of control during the last 5 min of drug application when the effect reached steady state. The figures represent the averages of several recordings ± S.E.M. Student's *t*-test for unpaired data was used for comparison of mean values, *P* values less than 0.05 were considered statistically significant. To estimate the NR2B-mediated component of the NMDA fEPSPs we perfused the slices with ifenprodil (10 μM), a specific non-competitive inhibitor of the NR2B subunit. At this concentration ifenprodil should almost fully inhibit the NR2B-mediated response (Williams, 1993). The remaining fraction of the NMDA fEPSP is thus mediated by the NR2A component. Assuming that ifenprodil does not have synergistic or allosteric interactions with ethanol on NMDA receptors that are ifenprodil-insensitive or ifenprodil-sensitive (Lovinger, 1995) we calculated the % inhibition of the NR2B-mediated component by ethanol using the following formulas:

$$A + B = 100\%, \tag{1}$$

$$T_{et} = A * A_{et} + B * B_{et}, \tag{2}$$

or

$$B_{et} = (T_{et} - A * A_{et})/B. \tag{3}$$

Where: A – fraction of the total control NMDA fEPSP not inhibited by ifenprodil (%), i.e. NR2A-mediated fEPSP; B – fraction of the total control NMDA fEPSP inhibited by ifen-

prodil (%), i.e. NR2B-mediated fEPSP; T_{et} – inhibition of the total NMDA fEPSP by ethanol (%); A_{et} – inhibition of the NR2A-mediated component of the NMDA fEPSPs by ethanol; B_{et} – inhibition of the NR2B-mediated component of the NMDA fEPSPs by ethanol.

The fEPSP decay time constants were calculated using first order exponential fitting:

$$y(t) = A * \exp(-t/\tau),$$

where: $y(t)$ – fEPSP amplitude (mV) at time t (ms); A – fEPSP amplitude at t_0 (peak of fEPSP); t – time (ms); τ – decay time constant.

Ethanol-induced sleep time

Male B6 and NR2A$^{\Delta C/\Delta C}$ (ΔC) mice or their F2 progeny were housed four per cage in standard vivarium conditions with a 12:12 h light:dark cycle. On the day of the experiment the animals were removed from the vivarium and adapted to the testing area for at least 1 h. Animals were injected i.p. with ethanol (4.0 g/kg, 15% w/v in saline). The onset of loss of righting reflex (LORR), measured in s, was used as the index of the initial sensitivity to ethanol. Righting was defined as the ability of the mouse placed in the supine position to achieve the orientation in which at least three feet were under its body and not visible from above. Sleep times were considered as the time (min) from the onset of LORR to the return of the righting reflex. The righting reflex was considered normal if an animal could right itself three times within 30 s. Ifenprodil (1 mg/kg in saline) or the vehicle was injected i.p. 5 min after the onset of LORR upon the second administration of ethanol 7 days after the initial ethanol testing. Results are given as mean ± S.E.M. Student's tests for unpaired or paired data were used for comparing mean values, P values less than 0.05 were considered statistically significant. The genotyping of the F2 (B6×ΔC) was performed after the tests and only homozygous wild-type (F2wt) and homozygous mutant (F2$^{\Delta C/\Delta C}$) littermates were used in the statistical analyses.

Blood alcohol concentration

Blood samples (20–40 µl) were collected via retro-ocular venous sinus puncture with a 100 µl microcapillary pipet immediately upon induction of LORR as well as immediately after the animals regained their righting reflex after the first and the second administration of ethanol. The samples were immediately frozen at −80°C and tested at a later date by a nicotinamide adenine dinucleotide–alcohol dehydrogenase enzyme assay (Sigma) with minor modifications of the instructions.

RESULTS

Input/output (I/O) relationship of NMDA fEPSPs

We first compared the amplitudes of the NMDA receptor-mediated component of evoked field potentials in the CA1 of B6 and ΔC mice using a correlation analysis between NMDA fEPSP and fiber volley amplitudes (Fig. 1). The I/O relationship of CA1 NMDA fEPSPs was similar in B6 and ΔC mice.

Ethanol inhibition of NMDA-mediated fEPSPs

We compared the sensitivity of the NMDA fEPSPs to the inhibitory effects of ethanol. Exposure to 100 mM ethanol for a period of 30 min significantly inhibited NMDA-mediated fEPSPs in slices of both strains of mice and in both regions of the hippocampus. The inhibition was similar between the two mouse strains, but differed between the CA1 and DG. The fEPSPs recorded in the CA1 region of both strains were significantly less sensitive to ethanol than the EPSPs recorded in the DG in both strains of mice (Table 1). The inhibition of NMDA fEPSPs by ethanol in the CA1 region of young animals (postnatal day, P4–7) was similar in both strains as well.

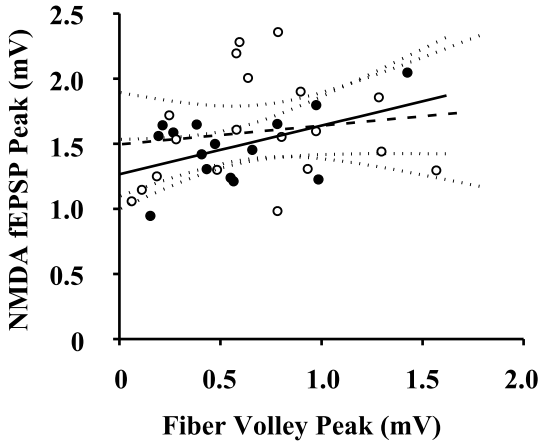

Fig. 1. Linear regression between fiber volley amplitude and the peak of NMDA fEPSPs in B6 and NR2A$^{\Delta C/\Delta C}$ mice. Dotted lines indicate 95% confidence bands. Solid line and filled circles – B6, dashed line and hollow circles – NR2A$^{\Delta C/\Delta C}$. There is no significant difference between the two slopes (0.37 ± 0.19, $n = 14$ and 0.144 ± 0.24, $n = 19$ respectively).

Inhibition of the NR2A-mediated component of NMDA fEPSPs by ethanol

To assess the individual sensitivities of the NR2A- and NR2B-mediated components of the fEPSPs to ethanol we applied 100 mM ethanol after preincubating the slices with 10 µM ifenprodil, a specific non-competitive inhibitor of NR2B subunit-containing NMDA receptors. Ifenprodil inhibited the NMDA-mediated fEPSPs in the CA1 region by $19.7 \pm 4.2\%$ ($n = 8$) in B6 and by $32.3 \pm 3.8\%$ ($n = 7$) in ΔC (Fig. 2A). In the DG the inhibition caused by 10 µM ifenprodil was significantly greater than in the CA1: $55.0 \pm 4.8\%$ ($n = 10$, $P < 0.01$) in the B6 mice and $59.6 \pm 5.6\%$ ($n = 10$, $P < 0.01$) in the ΔC (Fig. 2B). To confirm the selectivity of ifenprodil effects in our experiments we tested its effect on total NMDA-mediated fEPSPs in the CA1 region of 4–7-day-old B6 and ΔC mice. There should be a much higher inhibition of NMDA fEPSPs in young animals as until P10 the NR2B subunits are the most prominent in the brain, while NR2A subunit expression is very low (Kew et al., 1998; Laurie et al., 1997; Stocca and Vicini, 1998; Wenzel et al., 1997). The total NMDA fEPSPs were smaller than in the adult hippocampus with slower decay time constants: 200.0 ± 23.3 ms, $n = 4$ in B6 and 228.0 ± 66.8 ms, $n = 9$ in ΔC consistent with the prevalence of NR2B subunits (Monyer et al., 1994). As expected, the inhibition by ifenprodil was significantly more pronounced in the young animals than in the adults: $69.4 \pm 9.7\%$ ($n = 4$, $P < 0.01$) in the B6 and $70.9 \pm 4.9\%$ ($n = 7$, $P < 0.01$) in the ΔC mice (Fig. 2C).

Table 1. Ethanol inhibition of NMDA-mediated fEPSPs expressed as % inhibition in CA1 and DG of young (4–7 days) and adult (1–3 months) B6 and NR2A$^{\Delta C/\Delta C}$ mice

Strain	CA1	DG
B6, P4–7	23.4 ± 11.7, $n = 4$	nd
B6, adult	29.9 ± 2.4, $n = 20$	49.2 ± 3.1[a], $n = 3$
NR2A$^{\Delta C/\Delta C}$, P4–7	20.2 ± 4.0, $n = 4$	nd
NR2A$^{\Delta C/\Delta C}$, adult	31.3 ± 4.6, $n = 12$	50.6 ± 4.8[b], $n = 8$

nd – not determined.
[a]Statistically significant difference ($P < 0.01$) between the CA1 and DG (two-sample unpaired t-test).
[b]Statistically significant difference ($P < 0.05$) between CA1 and DG (two-sample unpaired t-test).

Another indicator of the involvement of NR2B and NR2A subunit-containing receptors is the difference in the receptor deactivation kinetics, which should be reflected in the decay time of fEPSPs. It has been shown that agonist-evoked responses in cells expressing NR2A subunit-containing receptors have significantly faster decay time than NR2B-containing receptors (Chen et al., 1999; Flint et al., 1997; Monyer et al., 1994). We next compared the decay time constants of the NMDA fEPSPs in the CA1 and DG of the adult animals in the presence and absence of ifenprodil (Table 2). The decay time constants of total NMDA fEPSPs in the CA1 region the ΔC mice were slightly slower than in the B6 but the difference was not statistically significant, while in the DG this interstrain difference was significant. Consistent with the kinetic behavior of the receptors, application of ifenprodil accelerated the decay time constants of NMDA fEPSPs by 23.5 ± 6.3% ($n = 12$) in the CA1 region of the ΔC mice and by 10.5 ± 8.3% ($n = 8$) in the B6 mice. In the DG the decrease in the decay time constants in the presence of ifenprodil was obvious only in the B6 mice (16.2 ± 5.4%, $n = 10$). We also observed significantly slower decay kinetics in the DG of the ΔC mice after blocking NR2B-containing receptors with ifenprodil.

Perfusion of the hippocampal slices with ethanol after preincubation with ifenprodil caused a significantly ($P < 0.01$) larger inhibition of the CA1 NMDA-mediated fEPSPs in ΔC (43.8 ± 2.9%, $n = 9$) than in B6 (19.8 ± 3.0%, $n = 12$) (Fig. 2A). The total inhibition of NMDA-mediated fEPSPs in the CA1 in the presence of both ethanol and ifenprodil was 28.2 ± 4.6% ($n = 7$) in B6 and 60.3 ± 2.9% ($n = 9$) in ΔC mice. In the DG the effect of ethanol was similar in both strains and for both strains it was significantly different from that in the CA1 region: 52.4 ± 4.8% ($n = 8$) inhibition in the B6 ($P < 0.01$) and 58.1 ± 2.2% ($n = 6$) in the ΔC ($P < 0.05$) (Fig. 2B). The total inhibition of NMDA-mediated fEPSPs in the DG in the presence of ethanol and ifenprodil was 76.3 ± 4.1% ($n = 8$) for the B6 and 82.2 ± 4.5% ($n = 6$) for the ΔC.

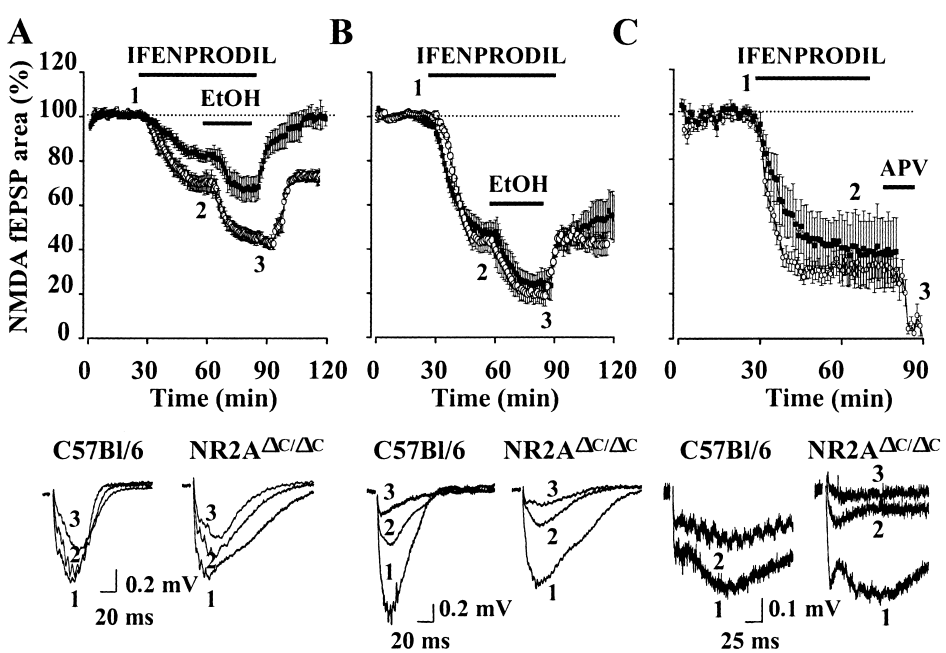

Fig. 2. (A) NMDA fEPSPs in the CA1 of the adult NR2A$^{\Delta C/\Delta C}$ mice show significantly higher sensitivity to the inhibitory effects of ifenprodil (10 μM) and ethanol (EtOH, 100 mM) than those in the adult B6 ($P < 0.01$, two-sample unpaired Student's t-test). (B) NMDA fEPSPs in the DG of the B6 and NR2A$^{\Delta C/\Delta C}$ mice are equally inhibited by ifenprodil as well as by ifenprodil+ethanol. (C) Ifenprodil (10 μM) inhibition of the NMDA fEPSPs in the CA1 region of young (P4–7) B6 and NR2A$^{\Delta C/\Delta C}$ mice is significantly higher than in the adult ($P < 0.01$, two-sample unpaired Student's t-test). Ifenprodil (10 μM) was perfused for 30 min, ethanol (100 mM) was applied after the effect of ifenprodil reached its steady state. Each graph is an average of eight to 10 independent experiments. Error bars are S.E.M. Filled squares – B6, hollow circles – NR2A$^{\Delta C/\Delta C}$. The insets represent raw traces of fEPSPs: 1, control NMDA fEPSP; 2, NMDA fEPSP upon application of ifenprodil; 3, NMDA fEPSP upon application of ifenprodil and ethanol (in C – 3, NMDA fEPSP upon application of ifenprodil and APV). The same scale bars apply to all traces in each corresponding group.

Table 2. Decay time constants of NMDA- and AMPA-mediated fEPSPs in CA1 and DG of adult (1–3 months) B6 and NR2A$^{\Delta C/\Delta C}$ mice

	CA1 (ms)	DG (ms)
NMDA B6, control	57.3 ± 14.8 (n = 8)	22.8 ± 1.67 (n = 10)
NMDA B6, IFP	44.0 ± 8.2 (n = 8)	19.4 ± 2.2 (n = 10)[a]
NMDA NR2A$^{\Delta C/\Delta C}$, control	67.8 ± 12.5 (n = 12)	42.4 ± 4.6 (n = 11)[b]
NMDA NR2A$^{\Delta C/\Delta C}$, IFP	46.2 ± 6.9 (n = 12)[a]	46.7 ± 5.3 (n = 11)[b]
AMPA B6	6.9 ± 1.0 (n = 7)	3.2 ± 0.3 (n = 3)
AMPA NR2A$^{\Delta C/\Delta C}$	9.4 ± 0.9 (n = 14)	2.8 ± 0.2 (n = 3)

The decay time constants (mean ± S.E.M.) of the NMDA fEPSPs were calculated using a first order exponential fitting (see Experimental procedures for details). IFP – ifenprodil, 10 μM.

[a]Statistically significant difference ($P < 0.05$) between the control and IFP groups (two-sample Student's paired t-test).

[b]Statistically significant difference ($P < 0.05$) between B6 and ΔC mice (two-sample Student's unpaired t-test).

Inhibition of the NR2B-mediated component of NMDA fEPSPs by ethanol

To calculate the sensitivity of the NR2B-mediated fEPSPs to ethanol we used the experimental data presented above in Eq. 3: $B_{et} = (T_{et} - A^*A_{et})/B$ (see Experimental procedures). Based on these calculations, Fig. 3 demonstrates that in the DG ethanol inhibits ~50% of the NR2B-mediated fEPSPs in both strains of mice (Fig. 3, DG NR2B). In contrast, in the CA1 the ethanol sensitivity of the NR2B component of the fEPSPs differed between the strains: 73% (range: 43–100%) of the NR2B-mediated fEPSP was inhibited in the CA1 of B6 mice but only 15% (range: 0–20%) inhibition was present in ΔC mice (Fig. 3, CA1 NR2B). We derived Eq. 3 based on the assumption that 10 μM ifenprodil is 100% selective for NR2B subunit-containing receptors. In fact, according to Williams et al. (1993), at this concentration ifenprodil leaves about 10% of NR1/NR2B receptors uninhibited, while inhibiting about 10% of NR1/NR2A receptors. This variation was well within the margin of error of our experimental data and did not significantly affect the final results.

Ifenprodil inhibition of NMDA-mediated fEPSPs in the presence of ethanol

The results presented in Fig. 3 should be valid regard-

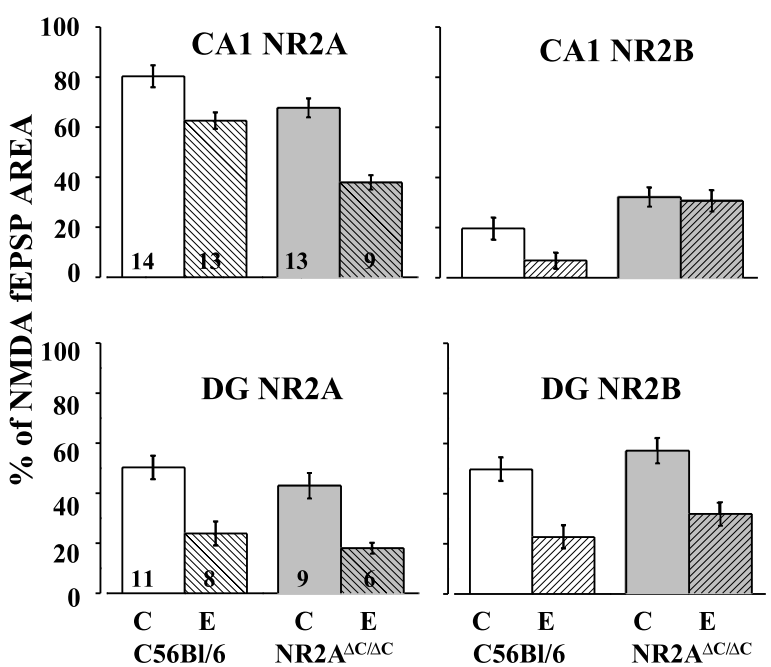

Fig. 3. Relative contributions of the NR2A- and NR2B-mediated components to the total NMDA fEPSPs and their inhibition by ethanol recorded in the CA1 and the DG of hippocampal slices of B6 (white bars) and NR2A$^{\Delta C/\Delta C}$ (shaded bars) mice. CA1 NR2A, the ethanol inhibition of the NR2A-mediated fraction of the NMDA fEPSPs in the NR2A$^{\Delta C/\Delta C}$ is significantly higher than in the B6 ($P < 0.01$, two-sample unpaired Student's t-test). CA1 NR2B, the NR2B-mediated fraction of the NMDA fEPSPs in NR2A$^{\Delta C/\Delta C}$ is five to seven times less sensitive to ethanol inhibition than in B6. DG NR2A, the sensitivity of NR2A-mediated component of NMDA fEPSPs to ethanol in DG is significantly higher than in the CA1 ($P < 0.01$, two-sample unpaired Student's t-test). DG NR2B, the ratio of NR2A/NR2B components of NMDA fEPSPs as well as their sensitivity to ethanol inhibition in DG are comparable in both strains. C and E refer to the ratios of NR2A- to NR2B-mediated fractions of NMDA fEPSPs in control conditions and in the presence of 100 mM ethanol respectively. B6 – white bars, NR2A$^{\Delta C/\Delta C}$ – shaded bars. Same legends apply to all graphs. See text for details on the statistical significance of the values. Numbers on the bars refer to number of experiments. Each bar is a mean ± S.E.M.

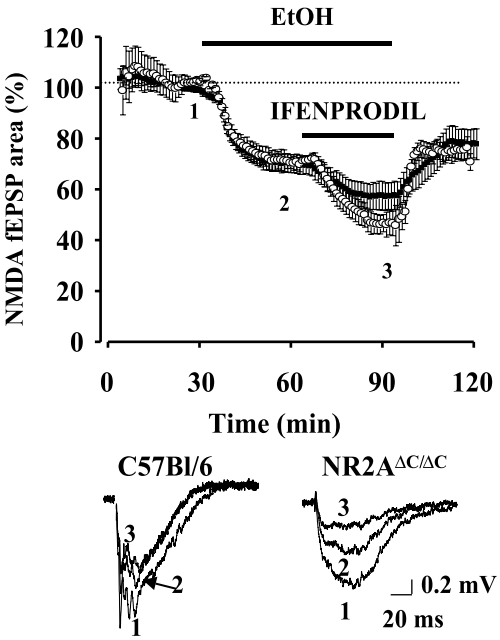

Fig. 4. Ifenprodil inhibition of NMDA fEPSP in the CA1 of NR2A$^{\Delta C/\Delta C}$ mice following preincubation with ethanol (EtOH) was significantly higher than in the B6 mice ($P<0.01$, two-sample unpaired Student's t-test). Ifenprodil (10 μM) was perfused after the inhibitory effect of ethanol (100 mM) reached the steady state. Each graph is an average of eight independent experiments. Error bars are S.E.M. Filled squares – B6, hollow circles – NR2A$^{\Delta C/\Delta C}$. The insets represent raw traces of fEPSPs: 1, control NMDA fEPSP; 2, NMDA fEPSP upon application of ethanol; 3, NMDA fEPSP upon application of ethanol+ifenprodil. The same scale bars apply to all traces.

less of the experimental sequence of drug application. Therefore we reversed the sequence of applying ifenprodil (10 μM) and ethanol (100 mM) to the slices. Figure 4 demonstrates the effect of 10 μM ifenprodil on NMDA-mediated fEPSPs in the CA1 of the adult B6 and ΔC mice after preincubation of the slices with 100 mM ethanol for at least 30 min. Ifenprodil inhibited an additional $10.9\pm1.6\%$ ($n=8$) of the CA1 fEPSPs in B6, and $30.4\pm1.8\%$ ($n=8$) in the ΔC. The total inhibition of NMDA fEPSPs by ethanol and ifenprodil in the CA1 was $41.0\pm4.8\%$ ($n=9$) in B6 and $64.2\pm4.3\%$ ($n=10$) in ΔC. In these experiments the fraction of NR2B-mediated fEPSP remaining after ethanol treatment that was still available for inhibition by ifenprodil (dB) can be calculated as follows:

$$dB = B - B * B_{et}, \quad (4)$$

or

$$B_{et} = 1 - dB/B. \quad (5)$$

Where: B – fraction of the total control NMDA fEPSP inhibited by ifenprodil (%), i.e. NR2B-mediated component of the fEPSP; B_{et} – inhibition of the NR2B-mediated component of the NMDA fEPSPs by ethanol.

Incorporating the experimental data into this equation yields that ethanol inhibition of the NR2B-mediated component of fEPSPs is 45% (range: 35–100%) in the CA1 of B6, but only 6% (range: 0–25%) in the CA1 of the ΔC mice. These values are within the range of those obtained with the inverse sequence of drug perfusion, and the relative differences between the effects in the two strains are similar. Thus, using either method, the sensitivity of the NR2B-mediated component of NMDA fEPSPs to ethanol in the CA1 of ΔC mice is 4.9–7.5 times lower than that in B6 mice.

To ensure that the observed differences were not due to a differential modulation of NMDA fEPSPs by $GABA_B$ receptors (Steffensen et al., 2000) we included a potent $GABA_B$ receptor antagonist CGP56433A (5 μM final concentration) in the bath prior to application of ethanol or ifenprodil. While the NMDA fEPSPs were increased by $10.2\pm0.2\%$ ($n=10$, $P<0.01$) upon the superfusion of the slices with CGP56433A, the inhibitory effects of ethanol on the total NMDA-mediated field potentials in the CA1 of ΔC mice were not significantly altered compared with NMDA fEPSPs recorded from the slices not treated with the $GABA_B$ receptor antagonist ($38.6\pm2.6\%$, $n=10$ with CGP56433A vs. $31.3\pm4.6\%$, $n=12$ without CGP56433A, $P>0.05$). The presence of CGP56433A in the bath did not affect the inhibition of the NR2A-mediated component of the NMDA fEPSPs by ethanol as well ($50.3\pm4.6\%$, $n=5$ with CGP56433A vs. $43.8\pm2.8\%$, $n=9$ without CGP56433A, $P>0.05$).

Ethanol inhibition of non-NMDA receptor-mediated fEPSPs

To establish if the difference in ethanol sensitivity pertains exclusively to the NMDA fEPSPs and to assess any potential presynaptic effects of ethanol, we tested the effects of ethanol on AMPA receptor-mediated fEPSPs. Figure 5 shows the inhibitory effect of 100 mM ethanol on the area of non-NMDA-mediated fEPSPs in the CA1. The inhibition was similar between the B6 and ΔC mice: $31.0\pm2.1\%$ ($n=3$) and $34.2\pm16.7\%$ ($n=3$) respectively in the DG, and $27.8\pm4.3\%$ ($n=7$) and $21.8\pm4.8\%$ ($n=10$) respectively in the CA1. There were no significant differences in the decay time constants of the AMPA fEPSPs between the B6 and the ΔC mice in both regions (Table 2).

Hypnotic effects of ethanol

To test the general sensitivities of B6 and ΔC mice to the effects of high doses of ethanol that correspond to its *in vitro* concentration of 100 mM (Little, 1999) we measured the hypnotic effects of ethanol (4 g/kg, i.p.) in the two strains. Regardless of the strain, injection of ethanol in naive mice caused loss of the righting reflex within 4 min. The ethanol-induced sleeping times were not significantly different between the B6 and the ΔC mice upon the first injection of ethanol when both strains were tested on the same day to reduce the possibility of 'seasonal' variations (Markel et al., 1995). When the same mice were given a second dose of ethanol 7 days after the first, we observed a significant increase in the ethanol-induced sleep times in ΔC mice (Fig. 6) compared to B6. To ensure that the observed differences were not due to a difference in the general excitability between the two

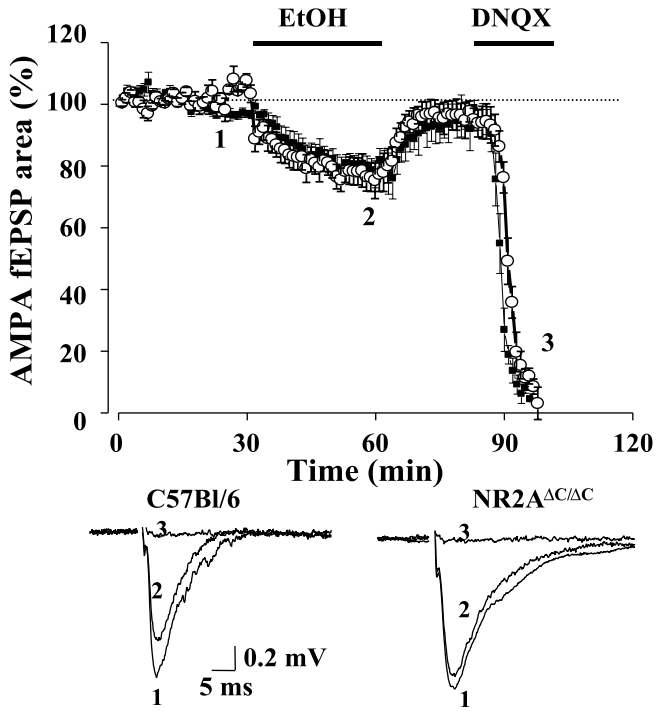

Fig. 5. The sensitivity of the AMPA-mediated fEPSPs to the inhibitory effects of 100 mM ethanol (EtOH) in the CA1 area of the hippocampus was similar between NR2A$^{\Delta C/\Delta C}$ and B6 mice. Each graph is an average of seven to 10 independent experiments. Error bars are S.E.M. Filled squares – B6, hollow circles – NR2A$^{\Delta C/\Delta C}$. The insets represent raw traces of fEPSPs: 1, control AMPA fEPSP; 2, AMPA fEPSP upon application of 100 mM ethanol; 3, AMPA fEPSP upon application of 10 μM DNQX. The same scale bars apply to all traces.

strains, we compared the hypnotic effects of pentobarbital (50 mg/kg) in the B6 and ΔC mice. No significant differences were found in the duration of sleep induced by pentobarbital (Fig. 6).

To establish that the observed differences in effects of alcohol were not due to interstrain differences unrelated to the mutation we repeated the experiments on the F2 progeny of the B6 and ΔC mice. We compared the wild-

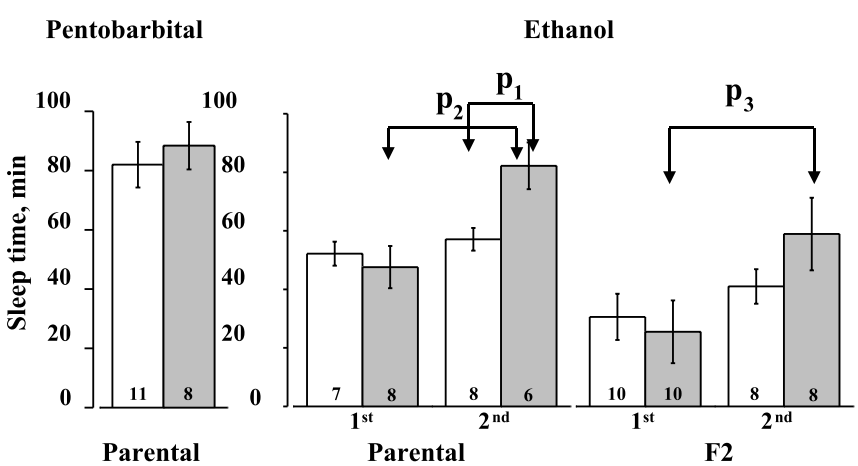

Fig. 6. NR2A$^{\Delta C/\Delta C}$ and F2$^{\Delta C/\Delta C}$ mice show significant increase in sensitivity to the hypnotic effects of the second administration (7 day after the first) of ethanol (4 g/kg, i.p.). The hypnotic effect was measured as sleep time induced by pentobarbital (50 mg/kg, i.p.), or ethanol (4 g/kg, i.p.). 1st and 2nd – respectively first and second administration of ethanol. Parental – effects of administration of ethanol to B6 (white bars) and NR2A$^{\Delta C/\Delta C}$ mice (shaded bars). F2 (C67Bl/6×NR2A$^{\Delta C/\Delta C}$) – effects of administration of ethanol to F2 wild-type (white bar) and F2$^{\Delta C/\Delta C}$ mice (shaded bar). Student's *t*-tests for unpaired (p_1 and p_2) or paired (p_3) data were used to compare mean values. p_1 ($P < 0.05$) – after ethanol preexposure the sedation of NR2A$^{\Delta C/\Delta C}$ mice was significantly longer than of B6 mice. p_2 ($P < 0.05$) – after ethanol preexposure the sedation of NR2A$^{\Delta C/\Delta C}$ mice was significantly longer than of naive animals. p_3 ($P < 0.05$) – after ethanol preexposure the sedation of F2$^{\Delta C/\Delta C}$ mice was significantly longer than of naive animals. Numbers in the bars represent number of animals tested in each group.

type F2 mice to their homozygous mutant littermates ($F2^{\Delta C/\Delta C}$) with respect to their responses to the initial as well as to the second ethanol injection. As in their parental strains there was no difference in sleep times or in LORR between the F2 littermates upon the first injection of ethanol. The second application of alcohol revealed a significant augmentation of ethanol sensitivity judged by a reduced time to lose their righting reflex (209 ± 18.9 s – first ethanol exposure vs. 134 ± 7.3 s – second ethanol exposure, $n = 15$, $P < 0.01$, paired Student's *t*-test) and increased sleep time only in the homozygous mutants (25.5 ± 10.7 min – first ethanol exposure vs. 58.9 ± 12.4 min – second ethanol exposure, $n = 8$, $P < 0.05$, paired Student's *t*-test) but not in their wild-type littermates (first LORR: 192 ± 18.9 s, second LORR: 167.8 ± 21 s, $n = 15$; first sleep: 30.6 ± 7.0 min, second sleep: 41.0 ± 12.4 min, $n = 10$). Analysis of the blood samples taken at the time of recovery from LORR revealed no significant differences in blood alcohol concentrations between the tested strains of mice after the first (75.8 ± 2.3 and 70.0 ± 1.2 mmol/l respectively) and second administration of alcohol (74.2 ± 3.2 and 74.0 ± 2.0 mmol/l respectively). Therefore, it is unlikely that the sensitization to alcohol in mice with terminally truncated NR2A subunit of NMDA receptor is due to changes in pharmacokinetic or metabolic factors or to genetic differences between the strains unrelated to the alteration of the NR2A subunit. Our attempt to distinguish between the contribution of NR2A and NR2B subunits to the hypnotic effect of ethanol by injecting ifenprodil (0.5–2 mg/kg, i.p.) (Malinowska et al., 1999) 5 min after the second ethanol injection yielded no additional changes in behavior in any of the genotypes tested (data not shown).

Ethanol inhibition of NMDA fEPSPs in CA1 of mice preexposed to the hypnotic dose of alcohol

After observing a significant change in the alcohol sensitivity of ΔC mice 7 days after their exposure to a single hypnotic dose of ethanol we tested the effects of 100 mM ethanol on NMDA fEPSPs in the CA1 of ΔC mice preexposed to a hypnotic dose of alcohol (4 g/kg, i.p.) 7 days prior to the *in vitro* experiment. We found no differences in the inhibitory effect of ethanol (100 mM) on the total NMDA fEPSPs compared to the naive animals ($38.9 \pm 3.8\%$, $n = 11$ and $31.3 \pm 4.6\%$, $n = 12$ respectively). Moreover, there were no changes in the sensitivities to 10 μM ifenprodil or to ethanol in the presence of ifenprodil (data not shown).

DISCUSSION

We examined the effects of ethanol on NMDA fEPSPs in the CA1 and DG of $NR2A^{\Delta C/\Delta C}$ mice that lack the intracellular C-terminus of the NR2A subunit of NMDA receptor and compared them to those recorded in its parental strain B6. The NR2A-mediated fEPSP was isolated by the use of a non-competitive NMDA receptor inhibitor ifenprodil with high affinity for the NR2B subunit (Williams, 1993). Our findings can be summarized as follows:

1. There is a prevalence of NR2A- over NR2B-mediated component in the NMDA fEPSPs in the CA1 area of both B6 and $NR2A^{\Delta C/\Delta C}$ mice, while in the DG of both strains NR2A and NR2B contribute equally to the NMDA fEPSPs.
2. In the CA1 of adult $NR2A^{\Delta C/\Delta C}$ mice, the NR2A-mediated component of NMDA fEPSPs was 20% smaller than that in B6.
3. The ethanol inhibition of the truncated NR2A-mediated component of CA1 NMDA fEPSPs was increased two-fold, but remained unchanged in the DG.
4. The ethanol inhibition of the calculated NR2B-mediated component of NMDA fEPSPs in the CA1 of $NR2A^{\Delta C/\Delta C}$ was reduced 5–7-fold.

Steigerwald et al. (2000) have previously reported that at CA1 pyramidal cell synapses but not on cell somata the C-terminally truncated NR2A subunits were less concentrated than the full-length subunits, and that the fractional NMDA component of evoked EPSCs was reduced in the mutants. In our preparation the I/O relationship of CA1 NMDA fEPSPs was similar in B6 and ΔC mice. However, the significant reduction of the NR2A-mediated component of the NMDA EPSPs in the CA1 of adult $NR2A^{\Delta C/\Delta C}$ mice is in agreement with a reduced NR2A protein staining in hippocampal dendrites as well as a reduced enrichment of NR2AΔC subunits in the postsynaptic densities (PSDs) of $NR2A^{\Delta C/\Delta C}$ mice (Steigerwald et al., 2000). Agonist-evoked responses decay faster in cells expressing NR2A subunit-containing receptors than in cells with NR2B subunit-containing receptors (Chen et al., 1999; Flint et al., 1997; Monyer et al., 1994). Accordingly, in the presence of the NR2B-specific NMDA receptor inhibitor ifenprodil the NMDA fEPSPs decayed faster in the CA1 of the $NR2A^{\Delta C/\Delta C}$, but not in the B6 mice, which is consistent with the increased relative contribution of the NR2B-mediated component of NMDA fEPSPs in the CA1 of the $NR2A^{\Delta C/\Delta C}$ mice compared to B6. Steigerwald et al. (2000) reported similar accelerating effects of the ifenprodil derivative CP-101,606 on the deactivation kinetics of stimulus-evoked NMDA EPSCs in the CA1 of $NR2A^{\Delta C/\Delta C}$ mice, as well as a lack of this acceleration in the presence of the NR2B subunit-specific inhibitor in the CA1 of their adult wild-type littermates. In contrast to Steigerwald and coworkers we did not observe slower decay rates of CA1 NMDA fEPSPs in the $NR2A^{\Delta C/\Delta C}$ mice compared to B6 in the presence of ifenprodil. Differences in the experimental designs (whole-cell currents vs. fEPSPs) probably account for the discrepancy. However, our findings are in agreement with Kohr and Seeburg (1996) who reported that the amplitudes of the whole-cell currents and their deactivation time courses were comparable between the wild-type NR1/NR2A and the mutant NR1/NR2AΔC channels expressed in HEK 293 cells. It also remains to be determined why in the DG of both $NR2A^{\Delta C/\Delta C}$ and B6 mice the

NMDA fEPSPs decayed significantly faster than in the CA1 region.

A higher ratio of the NR2A- to NR2B-containing NMDA receptors in the DG is inconsistent with our finding that the inhibitory effect of ifenprodil on NMDA EPSPs was significantly higher in this region than in the CA1. Based on the faster decay time constants of the AMPA EPSPs recorded in the DG compared to the CA1 of both strains the different membrane properties of the cells or region-specific expression of different protein kinases in the two regions may explain our findings.

NMDA receptors are subject to modulation by phosphorylation of the C-termini of its subunits by a variety of protein kinases. Protein kinase C positively modulates NMDA receptor currents through the phosphorylation site on the C-terminal of NR1 subunit (Logan et al., 1999), while CaM-KII was shown to exert its modulatory effect through the C-terminus of the NR2B subunit (Gardoni et al., 1999; Leonard et al., 1999). Protein kinase A phosphorylates both NR1 and NR2 (A and B) subunits (Leonard and Hell, 1997; Tingley et al., 1997), while tyrosine kinases phosphorylate NR2A and NR2B subunits but not NR1 subunits (Kohr and Seeburg, 1996; Suzuki and Okumura-Noji, 1995). Anders et al. (1999a,b) reported that the ethanol inhibition of NR1/NR2A but not of NR1/NR2B was specifically reduced upon *fyn* tyrosine phosphorylation, while other members of the tyrosine kinase family had no such effect, consistent with a *fyn*-mediated tyrosine phosphorylation of NR2A subunits being involved in the reduction of sensitivity of NR1/NR2A receptors to high (sedative) concentrations of ethanol. Our finding that truncation of the C-terminus of the NR2A subunit increased the inhibitory effect of ethanol on NMDA-mediated responses in the CA1 region of the hippocampus indicates that the site of ethanol action is not the C-terminus of the NR2A subunit. Peoples and Stewart (1999, 2000) reported similar findings in their study of recombinant NMDA receptors in CHO-K1 expressing NR1/NR2B receptors with truncated C-termini for both subunits. The authors observed a decreased IC_{50} for ethanol in the mutant receptors. Taken together with our present findings, the site of ethanol effect on NMDA receptors is not confined to the C-termini of NR2 subunits but rather to a site located in a domain exposed to, or only accessible from, the extracellular environment (Peoples and Stewart, 2000) or some associated targeting, anchoring, signaling, or modulatory protein (Zheng et al., 1999).

NMDA receptors at synapses are clustered with a complex of proteins including protein kinases and phosphatases linked to scaffolding proteins in the PSD (Kennedy, 2000). Each of the kinases and phosphatases interacts with a different scaffolding protein. The yotiao protein anchors protein kinase A to NR1 subunit-containing C1 exon (Westphal et al., 1999). Spectrin is involved in anchoring NR1 as well as NR2A and B subunits, and this function depends on the presence of *fyn* tyrosine kinase in case of NR2 subunits or on the presence of CaM, protein kinase A and C in case of NR1 (Wechsler and Teichberg, 1998). PSD-95 serves as another molecular scaffold for anchoring protein tyrosine kinases to NR2A and NR2B subunits (Tezuka et al., 1999), while α-actinin (actin-binding protein) binds to NR1 and NR2B but not NR2A subunits (Wyszynski et al., 1997). Each of the scaffolding proteins displays differential localization in the brain. We found that truncation of the NR2A C-terminus has a different effect on the ethanol sensitivity of the NMDA-mediated responses depending on the hippocampal subfield. This might indicate that NR2A-containing NMDA receptors may be regulated and/or anchored by different mechanisms depending on the brain region.

Previous studies on recombinant NMDA receptors expressed in *Xenopus* oocytes (Kuner et al., 1993; Masood et al., 1994) or in HEK 293 cells (Anders et al., 1999b) demonstrated subunit-selective inhibitory effects of ethanol. Thus, Kuner et al. reported that in Ca^{2+}-containing medium the recombinant NR1/NR2B receptors expressed in *Xenopus* oocytes were the most sensitive to inhibition by 50–100 mM ethanol. Concurrently, Masood et al. found that recombinant NR1/NR2B receptors in *Xenopus* oocytes were maximally inhibited (28%) by 25 mM ethanol while NR1/NR2A receptors were inhibited by elevated concentrations of ethanol, with 100 mM causing 44% inhibition (Masood et al., 1994). Similarly, in HEK 293 cells expressing recombinant NMDA receptors ethanol (50–100 mM) inhibition of NR1/NR2A receptors was less than that of NR1/NR2B (Lovinger, 1995). On the contrary, Anders et al. reported that the NR1/NR2B receptors expressed in HEK 293 cells were inhibited by 100 mM ethanol to a lesser degree than the NR1/NR2A receptors (Anders et al., 1999b), while Blevins et al. (1997) did not find any statistically significant differences in ethanol's potency or maximal inhibition between any of NMDA receptor subunit combinations tested in HEK 293 cells. We found that NR2A and NR2B subunit sensitivity to ethanol inhibition may be region-specific, as in the CA1 the NR2A-mediated fEPSPs were less sensitive to ethanol inhibition than the NR2B, while in the DG both subtypes were equally inhibited by ethanol.

We also found a change in ethanol sensitivity of NR2B-mediated NMDA responses in the $NR2A^{\Delta C/\Delta C}$ mice specific to the CA1 region. Thus there may be regional differences in the regulation and/or anchoring of the NR2B-containing receptors in the hippocampus. In the CA1 region of $NR2A^{\Delta C/\Delta C}$ mice both subunits display altered sensitivity to ethanol when only the NR2A subunit is truncated. Since there is no reported compensatory increase in the expression of NR2B subunits (Steigerwald et al., 2000), and a significant compensatory increase in the expression of NR2C or NR2D subunits is unlikely (Laurie et al., 1997; Monyer et al., 1994; Wenzel et al., 1995), it is possible that in the CA1 but not in the DG NR2A and NR2B subunits are linked by a common regulatory protein targeted by ethanol. As shown by Wyszynski et al. (1998) α-actinin is expressed at much higher levels in the DG than in the CA1 thus pointing to a strong possibility that other NMDA receptor regulatory and anchoring proteins may underlie region-specific control of NMDA receptor function.

We observed a significant increase in the sensitivity to a hypnotic dose of alcohol after preexposure of animals to alcohol only in mice expressing the C-terminally truncated version of the NR2A subunit. This finding implies the importance of the C-terminus of the NR2A subunit in the sensitization to the *in vivo* effects of alcohol. Yet, we did not find any corresponding changes in the ethanol effects on NMDA receptor-mediated field potentials recorded from hippocampal slices of animals preexposed to hypnotic doses of alcohol. These findings are consistent with a lack of involvement of hippocampal NMDA receptor ethanol sensitivity in the *in vivo* sensitization to the hypnotic effects of ethanol. Future studies will have to address the mechanism underlying ethanol sensitization in ΔC mutants as it may be due to various factors including alterations in the CNS of the mutant mice due to the presence of truncated NR2A subunits during development.

Acknowledgements—This work was supported by NIH Grant NS-27528 (I.M.). We thank Dr. Rolf Sprengel of the Max-Planck Institute for Medical Research for the generous gift of the $NR2A^{\Delta C/\Delta C}$ mice.

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(*Accepted* 22 *May* 2001)