day 5 or 6 with an HIV-1 p24 ELISA (Organon Teknika, Durham, NC). The suppressive activity of each supernatant was calculated as the percentage of inhibition of HIV-1 p24 antigen production compared with controls. HIV-1_{IIIB} virus stock was prepared from chronically infected Molt3–HIV-1_{IIIB} cell lines, whereas the previously described primary NSI and SI isolates (*8*) [R. I. Connor *et al.*, *J. Virol.* **67**, 1772 (1993)] were propagated in primary PBMCs. All isolates were titrated to determine $TCID_{50}$ in PHA-stimulated normal PBMCs.

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12. Culture supernatant (1200 ml) from F3b clone 19, grown to high cell density in serum-free medium supplemented with rIL-2 (*11*), was subjected to centrifugation at 100,000*g* for 60 min at 4°C, and the resulting soluble fraction was applied to a 5-ml Hi-Trap heparin affinity FPLC (fast protein liquid chromatography) column (Pharmacia) that had been equilibrated with 10 mM tris-HCl (pH 7.6) containing 0.1 M NaCl (column buffer). The column was then washed extensively with column buffer, after which the bound proteins were eluted with 10 mM tris-HCl (pH 7.6) containing 2.0 M NaCl at a flow rate of 1 ml/min. The column eluate was adjusted to pH 2.0 by addition of trifluoroacetic acid (TFA) and subjected to reversed-phase HPLC on a PEEK C_{18} column (Waters Instruments, Millford, MA) that had been equilibrated with H_2O containing 0.1% TFA. Proteins bound to the column were eluted with a 5-min linear gradient of aqueous acetonitrile (0 to 35%) containing 0.1% TFA. After 10 min at 35% acetonitrile, the column was further subjected to a 60-min linear gradient of 35 to 70% aqueous acetonitrile containing 0.1% TFA. The flow rate was maintained at 0.5 to 1 ml/min. The resulting fractions were tested for suppressor activity in the infectivity assay with HIV-1_{IIIB}. Active fractions were pooled, diluted twofold in H_2O containing 0.1% TFA, and reapplied to the column. Proteins were eluted with a 30-min linear gradient of aqueous acetonitrile (0 to 60%) containing 0.1% TFA at a flow rate of 0.5 to 1 ml/min. The fractions obtained were assayed as above. Active fractions were pooled, diluted with H_2O containing 0.1% TFA, and fractionated under the same conditions to obtain a single protein peak. The fraction corresponding to the peak and flanking fractions were tested in the infectivity assay to verify that suppressor activity co-fractionated with the protein.
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15. Amino acid analysis of the purified protein was performed with a Beckman 6300 amino acid analyzer. Samples were hydrolyzed for 24 hours at 110°C in the presence of 6 M HCl and then reconstituted in loading buffer. Amino acids were analyzed by post-column derivatization with ninhydrin. NH_2-terminal microsequencing was performed by automated Edman degradation with a Hewlett-Packard G1005A protein sequencing system, which was operated with standard reagents, solvents, and programs (routine 3.0) supplied by the manufacturer. Abbreviations for the amino acid residues are A, Ala; D, Asp; E, Glu; G, Gly; L, Leu; M, Met; N, Asn; P, Pro; R, Arg; S, Ser; V, Val; and Y, Tyr.
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21. PBMCs (American Red Cross) from a healthy donor were purified by centrifugation in Histopaque (Sigma) and harvested either immediately or after activation for 48 hours with PHA (5 μg/ml) and rIL-2 (10 ng/ml). The HUT 78 human T cell line was cultured in the presence (50 U/ml) or absence of IL-2 (Boehringer, Mannheim, Germany). RNA was isolated by the RNAzol procedure (Tel-Test, Friendswood, TX), and 10 μg of total cellular RNA was separated by electrophoresis on a denaturing formaldehyde-agarose gel and then transferred to a nylon membrane. The membrane was subjected to hybridization with an MDC-specific probe and washed under stringent conditions as described [A. Garzino-Demo, R. C. Gallo, S. K. Arya, *Hum. Gene Ther.* **6**, 177 (1995)]. The probe for Northern hybridizations was generated by reverse transcription and polymerase chain reaction with MDC-specific primers.
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Fyn-Kinase as a Determinant of Ethanol Sensitivity: Relation to NMDA-Receptor Function

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Animals vary in their sensitivity to ethanol, a trait at least partly determined by genetic factors. In order to identify possible responsible genes, mice lacking Fyn, a non–receptor type tyrosine kinase, were investigated. These mice were hypersensitive to the hypnotic effect of ethanol. The administration of ethanol enhanced tyrosine phosphorylation of the *N*-methyl-D-aspartate receptor (NMDAR) in the hippocampus of control mice but not in Fyn-deficient mice. An acute tolerance to ethanol inhibition of NMDAR-mediated excitatory postsynaptic potentials in hippocampal slices developed in control mice but not in Fyn-deficient mice. These results indicate that Fyn affects behavioral, biochemical, and physiological responses to ethanol.

Ethanol (EtOH) is among the most widely abused drugs in the world, yet the neural mechanisms responsible for EtOH intoxication and dependence are largely unknown. Genetic factors affect the determination of the behavioral responses to EtOH in rodents and humans (*1*), but few specific genes that increase or decrease the drug actions have been reported (*2*, *3*).

Tyrosine kinases phosphorylate *N*-methyl-D-aspartate (NMDA) and γ-aminobutyric acid A ($GABA_A$) receptors and modulate the electrophysiological function of these receptors (*4*–*6*). The function of these receptors is also modulated by EtOH, and they are hypothesized to be targets

through which EtOH exerts its behavioral effects (*7*). To investigate the possible involvement of Fyn tyrosine kinase in the behavioral sensitivity to EtOH, we assessed the hypnotic effect of EtOH on Fyn-deficient mice (*8*). At all doses tested, the duration of the loss of righting reflex (LORR) after EtOH administration was significantly longer for Fyn-deficient (homozygous Fyn-deficient, fyn^z/fyn^z) mice than for control (heterozygous Fyn-deficient, $+/fyn^z$) mice (Fig. 1A). It is unlikely that the difference between the two groups was due to a difference in the general excitability of the central nervous system or in the general ability of a mouse to right itself, as no significant differences were found in the duration of the LORR induced by flurazepam (a benzodiazepine derivative) at any dose (Fig. 1B). Analysis of the blood EtOH concentration curve (*8*, 9) revealed no significant differences between the two groups (Fig. 1C). The enhanced sensitivity to EtOH is, therefore, likely due to changes in the sensitivity of the central nervous system rather than to changes in pharmacokinetic or metabolic factors. Thus, lack of Fyn tyrosine kinase exacerbates the hypnotic effects of EtOH.

Because EtOH enhances tyrosine phosphorylation in A431 cells (a human epidermal carcinoma) (*10*) and in neural cell line PC12 cells (*11*), we examined whether such modulation of tyrosine phosphorylation by EtOH also occurred in the brains of $+/fyn^z$ and fyn^z/fyn^z mice (*12*). The level of tyrosine phosphorylation after saline treatment was not significantly different between the two groups. A significant enhancement in tyrosine phosphorylation of a 180-kD protein (p180) 5 min after EtOH administration was observed in the hippocampus of $+/fyn^z$ mice but not in the hippocampus of fyn^z/fyn^z mice (Fig. 2, A and B). The enhancement was also observed in C57BL/6, a standard inbred mouse strain. The lack of this up-regulation in fyn^z/fyn^z mice indicates that it is mainly mediated by Fyn tyrosine kinase.

In the central nervous system of the rats, a tyrosine-phosphorylated protein band of molecular mass 180 kD in the postsynaptic

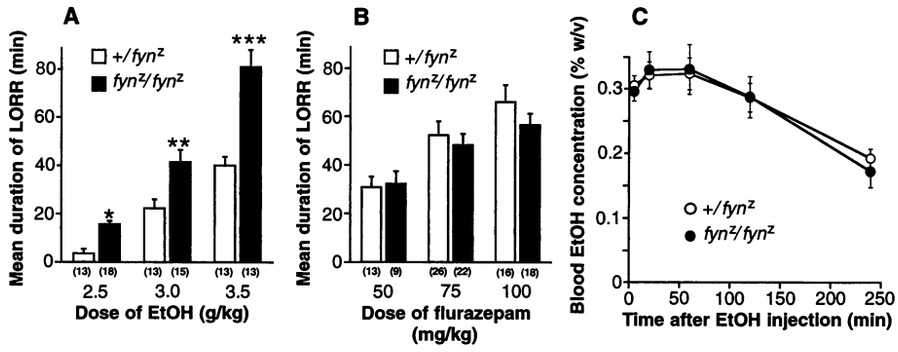

Fig. 1. Enhanced sensitivity to the hypnotic effect of EtOH in Fyn-deficient mice. Sensitivity was evaluated by measuring the duration of LORR after administration of (**A**) EtOH or (**B**) flurazepam (doses measured per kilogram of mouse body weight). Two-way analysis of variance (ANOVA) showed a significant difference [$F(1,81) = 5.78$, $P < 0.02$] in the sensitivity to EtOH. There was no significant effect in the sensitivity to flurazepam [$F(1,100) = 0.39$, $P > 0.50$]. The number of animals tested is shown in parentheses below each column. Asterisks indicate a significant difference between $+/fyn^z$ and fyn^z/fyn^z mice at each dose (simple main effect; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$). (**C**) Blood EtOH concentration curve in $+/fyn^z$ and fyn^z/fyn^z mice (seven mice of each genotype). There were no statistically significant differences between the two genotypes (two-way repeated-measure ANOVA).

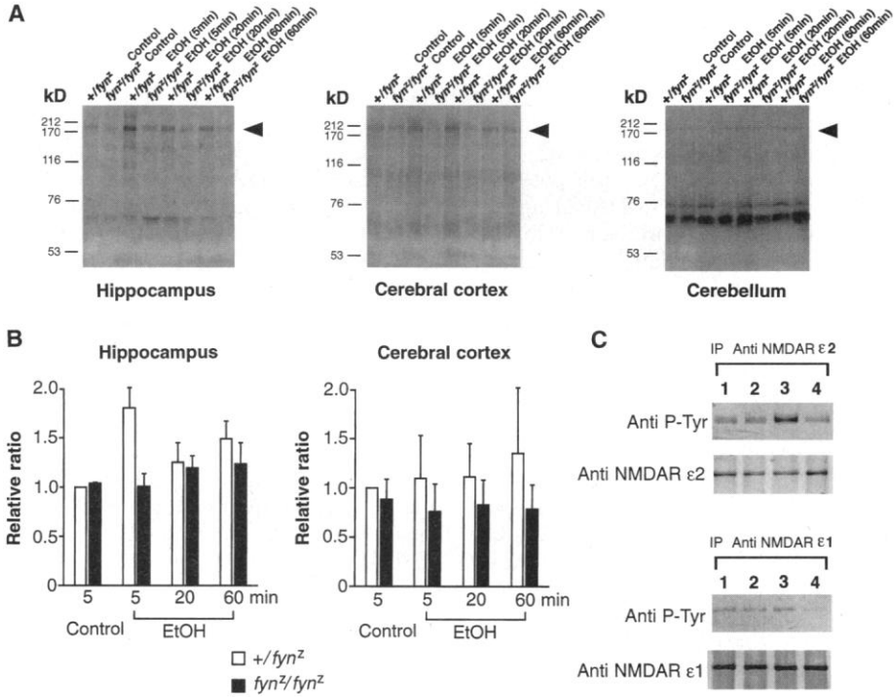

Fig. 2. Up-regulation of protein tyrosine phosphorylation after EtOH administration. (**A**) Representative immunoblots of extracts prepared after the administration of saline or EtOH with a phosphotyrosine-specific antibody. Arrowheads indicate p180. (**B**) Quantification of the level of phosphotyrosine at 180 kD. Two-way ANOVA showed that the effect of gene and treatment×gene interaction were significant [$F(1,32) = 5.88$, $P < 0.05$; $F(3,32) = 2.96$, $P < 0.05$]. A significant enhancement in tyrosine phosphorylation was observed 5 min after EtOH administration in $+/fyn^z$ mice [Tukey's honestly significant difference (HSD) test, $\alpha = 0.05$] but not in fyn^z/fyn^z mice. (**C**) Tyrosine phosphorylation of NMDARε1 and ε2 and the amount of NMDARε1 and ε2 protein in the hippocampus 5 min after the administration of saline (lane 1, $+/fyn^z$; lane 2, fyn^z/fyn^z) or EtOH (lane 3, $+/fyn^z$; lane 4, fyn^z/fyn^z). Each sample with the same number was obtained from the same animal. Group effect was significant in tyrosine phosphorylation of NMDARε2 [$F(3,20) = 4.18$, $P < 0.02$]. It was significantly greater 5 min after the administration of EtOH than it was 5 min after saline administration [control, 1.00 ± 0.00; EtOH, 2.89 ± 0.58; Tukey's HSD test, $\alpha = 0.05$] in $+/fyn^z$ mice but not in fyn^z/fyn^z mice [control, 0.84 ± 0.35; EtOH, 1.09 ± 0.66]. There were no significant group effects in the tyrosine phosphorylation of NMDARε1 [1.00 ± 0.00, 0.61 ± 0.12, 1.33 ± 0.25, and 0.67 ± 0.33, in the same order as that of the representative blots] nor in the amounts of the NMDARε1 [1.09 ± 0.11, 1.12 ± 0.25, 1.25 ± 0.21, and 1.15 ± 0.12] and NMDARε2 [1.06 ± 0.05, 1.00 ± 0.07, 0.97 ± 0.06, and 1.12 ± 0.11] proteins ($P > 0.05$).

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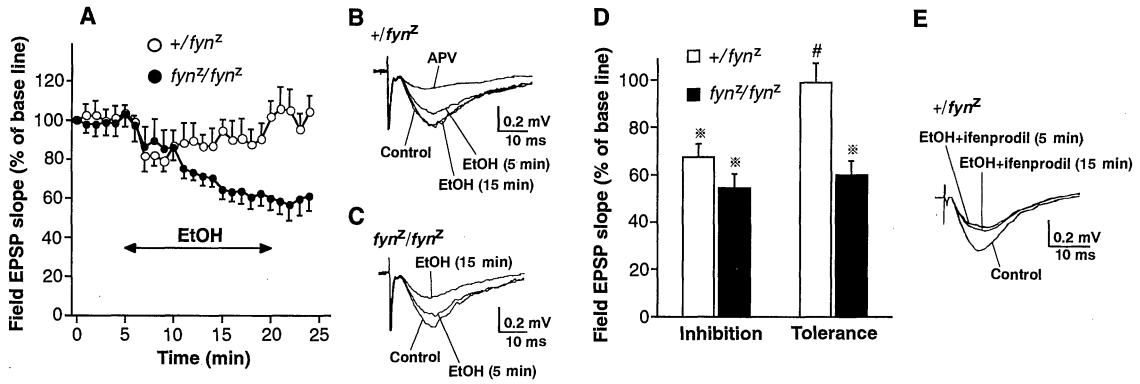

Fig. 3. Acute tolerance to EtOH inhibition of NMDAR-mediated EPSPs. (**A**) Time course of maximal slope for EPSPs in the hippocampus of +/*fyn*^{z} and *fyn*^{z}/*fyn*^{z} mice. The EtOH was bath-applied during the time indicated by the arrow. Five slices from four +/*fyn*^{z} mice and seven slices from seven *fyn*^{z}/*fyn*^{z} mice were used. Representative traces of EPSPs in +/*fyn*^{z} mice (**B**) and *fyn*^{z}/*fyn*^{z} mice (**C**) during the control period and 5 and 15 min after the start of EtOH application. These EPSPs were almost completely abolished by APV (10 μM). (**D**) Comparison of peak inhibition of EPSPs during the 15 min of EtOH application (Inhibition) and maximum reduction of inhibition after the peak inhibition of each slice (Tolerance) in +/*fyn*^{z} and *fyn*^{z}/*fyn*^{z} mice. Two-way repeated-measure ANOVA showed a significant gene effect [$F(1, 10) = 9.78$, $P < 0.02$], tolerance effect [$F(1,10) = 18.33$, $P < 0.005$], and tolerance×gene interaction [$F(1,10) = 9.07$, $P < 0.02$]. Means with * are significantly different from the mean with # (Tukey's HSD test, $\alpha = 0.05$). (**E**) Elimination of acute tolerance to EtOH inhibition by ifenprodil in +/*fyn*^{z} mice. NMDAR-mediated EPSPs were inhibited by application of EtOH and ifenprodil (10 μM) in +/*fyn*^{z} mice [$n = 6$, repeated-measure ANOVA, $F(2, 15) = 13.39$, $P < 0.0001$], but no significant difference was noted between the EPSP amplitudes at 5 min and those at 15 min after the start of the application (Tukey's HSD test, $\alpha = 0.05$).

density fraction contains NMDAR2B (corresponding to NMDARε2 in mice) (*13*) and NMDAR2A (NMDARε1) (*5*). The tyrosine phosphorylation of NMDAR2B is up-regulated in response to lesions with 6-OH-dopamine (*14*), taste learning (*15*), and the induction of long-term potentiation (*16*). In addition, NMDARε1 and ε2 are colocalized with Fyn in the postsynaptic density and phosphorylated by Fyn (*5*). Consequently, we investigated the level of tyrosine phosphorylation of NMDARε1 and ε2 after saline and EtOH treatment in the hippocampus, where the up-regulation occurred (*17*). Enhanced tyrosine phosphorylation of NMDARε2 5 min after EtOH treatment was observed in +/*fyn*^{z} mice but not in *fyn*^{z}/*fyn*^{z} mice (Fig. 2C, first row). On the other hand, +/*fyn*^{z} mice and *fyn*^{z}/*fyn*^{z} mice had similar amounts of NMDARε2 after saline and EtOH administration (Fig. 2C, second row). Concerning NMDARε1, neither tyrosine phosphorylation nor the amount of receptor protein was up-regulated (Fig. 2C, third and fourth rows). It is therefore likely that the enhanced tyrosine phosphorylation of NMDARε2 after EtOH treatment accounts for the enhancement of tyrosine phosphorylation of p180.

The inhibition of NMDAR-mediated excitatory postsynaptic potentials (EPSPs) by EtOH is gradually reduced during the period of EtOH exposure in hippocampal slices (acute tolerance) (*18*). This acute tolerance might be caused by the up-regulation of tyrosine phosphorylation of NMDAR subunits, because NMDAR currents are potentiated by tyrosine kinases in hippocampal neurons (*4*). To test this possibility, we compared the effects of EtOH on NMDA-mediated EPSPs in the CA1 hippocampal neurons of +/*fyn*^{z} and *fyn*^{z}/*fyn*^{z} mice (*19*). Bath application of EtOH initially suppressed NMDA-mediated EPSPs, but the amplitude of the EPSPs gradually recovered in +/*fyn*^{z} mice during the application of EtOH, showing the development of acute tolerance (Fig. 3, A, B, and D). By contrast, in *fyn*^{z}/*fyn*^{z} mice, EtOH suppressed NMDA-mediated EPSPs with little sign of development of tolerance (Fig. 3, A, C, and D), and EPSPs recovered their original amplitude after EtOH was removed. Thus, modulation of NMDAR function by Fyn seems to be involved in the development of acute tolerance to EtOH. Furthermore, the acute tolerance was eliminated when EtOH was applied together with ifenprodil, an agent considered to be a selective antagonist of NMDAR containing NMDARε2 (*20*) (Fig. 3E). These findings are consistent with the notion that enhancement of tyrosine phosphorylation of NMDARε2 is a basis of the acute tolerance (*21*).

Data have been accumulated to support the hypothesis that the inhibition of NMDA-mediated currents underlies the behavioral effects of EtOH (*22*). Although it is not certain whether the hypnotic effects of EtOH are directly mediated by the hippocampus (from which some of the data, including ours, were derived), the hypothesis is further supported by our results: Fyn-deficient mice showed abnormalities in their behavioral sensitivity to EtOH together with abnormal responses of NMDAR to EtOH.

Mice lacking the γ isoform of protein kinase C (PKCγ) are more resistant to the behavioral effects of EtOH, and these effects could be mediated by modulation of $GABA_A$ receptor function (*3*). These findings and our present results indicate that kinases may regulate behavioral EtOH sensitivity by modulating the function of receptors that are targets of EtOH, such as NMDA and $GABA_A$ receptors.

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8. Fyn-deficient mice were produced as described [T. Yagi *et al.*, *Nature* **366**, 742 (1993)]. Experimentally naïve male mice were tested at 70 to 80 days of age. Heterozygotes were used as control subjects, as they are a theoretically more appropriate control than wild-type animals (+/+) in the strain used [T. Miyakawa, T. Yagi, K. Tateishi, H. Niki, *Neuroreport* **7,** 2723 (1996)], and no significant difference between +/+ and +/*fyn*^{z} mice was found in the duration of LORR induced by EtOH in our pilot study [dose, 3.5 g of EtOH per kilogram of mouse body weight (3.5 g/kg EtOH); duration for +/+ mice, 31.8 ± 5.6 min; for +/*fyn*^{z} mice, 34.8 ± 3.2 min; $P > 0.05$]. Testing was double-blind [T. Miyakawa, T. Yagi, S. Watanabe, H. Niki, *Mol. Brain Res.* **27,** 179 (1994)]. Averaged values in the text and figures are mean ± SEM, unless otherwise noted. The EtOH (Wako Pure Chemicals), Osaka, Japan was dissolved in physiological saline [20% (v/v)] and administered intraperitoneally in all behavioral and biochemical experiments. Flurazepam was dissolved in 0.2 N HCl, diluted with saline, and administered intraperitoneally. The duration of LORR was measured as described (*3*).
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17. Cell lysates from hippocampus obtained 5 min after saline or EtOH (3.5 g/kg) administration were homogenized in lysis buffer [10 mM tris-HCl (pH 7.4), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitors as listed in (*12*)], sonicated, centrifuged to remove insoluble material, and precleared with protein G–Sepharose. Samples were incubated for 1 hour at 4°C with antibody to NMDARε1 or antibody to NMDARε2 and then incubated for 1 hour at 4°C with added protein G–Sepharose. After five washes, pellets were boiled for 5 min in 2× SDS sample buffer, subjected to SDS-PAGE, immunoblotted, and quantified as described above with antibody to NMDARε1, to NMDARε2, or to P-Tyr. Six animals per condition were used.
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19. Slice preparations of the hippocampus were made in a manner similar to that described [H. Tsubokawa *et al.*, *Neuroscience* **59**, 291 (1994)]. The bathing solution had the same composition as that described except the concentration of $MgSO_4$ (0.1 mM). Schaffer collateral/commissural afferents were stimulated with 0.1-Hz pulses. The extracellular field EPSPs were recorded in the stratum radiatum of the CA1 subfield, with glass micropipettes filled with Ringer solution. The maximal rate of change in field EPSP within a time window selected around the rising phase was calculated. To obtain NMDA-mediated EPSPs, we perfused slices with 10 μM 6-cyano-7-nitroquinoxaline-2.3-dione (CNQX) (Tocris, Bristol, UK). The EtOH (60 to 70 mM), DL-2-aminophosphonovalerate (APV) (Sigma, 10 μM), and ifenprodil (Wako, 10 μM) were applied in the perfusing medium. Little or no inhibitory effect of EtOH on EPSPs mediated by non-NMDA receptors could be noted in the presence of APV, as reported (*7*).
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21. The tolerance to EtOH inhibition seemed longer lasting in physiological data (Fig. 3) than the up-regulation of tyrosine phosphorylation (Fig. 2). This discrepancy may be due to the difference in the preparation used (whole tissues versus slices) or in EtOH administration procedures. The other possibility is that the up-regulation of tyrosine phosphorylation is a trigger for acute tolerance to EtOH. Concerning tyrosine phosphorylation of the NMDARζ1 subunit, 115-kd bands immunoprecipitated with antibody to NMDARζ1 (Santa Cruz Biotechnology, Santa Cruz, CA) were scarcely stained by anti P-Tyr in our supplementary experiments, as others reported [L.-F. Lau and R. L. Huganir, *J. Biol. Chem.* **270**, 20036 (1995)]. No changes in this hardly detectable band nor in the amount of NMDARζ1 protein were found by EtOH administration.
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