Ethanol’s Effect on NMDAR1 Splice Variants

Differential effects of Chronic Ethanol Treatment on NMDA R1 Splice Variants in Fetal Cortical Neurons

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Summary

Functional NMDA receptors consisting of NR1 and NR2 subunits are an important site of action of ethanol. Chronic ethanol treatment increases the NR1 polypeptide levels in vivo and in vitro. Chronic ethanol treatment in vitro does not significantly alter the NR1 mRNA levels even though under similar culture conditions, ethanol (50 mM, 5 days) enhances the half-life of NR1 mRNA in fetal cortical neurons. To address this phenomenon, we determined by RT/PCR and Western blotting, whether ethanol (50 mM, 5 days) has a splice variant-specific effect on the expression of the NR1 subunit in mouse fetal cortical neurons. This report analyses for the first time the distribution of all NR1 splice variants in these neurons. Our data indicate the presence of NR1-3a,b and NR1-4a,b splice variants in cortical neurons. Chronic ethanol treatment significantly decreased the mRNA levels of exon 5 containing NR1 splice variants (NR1-3b and NR1-4b) (-E5/+E5 = 4.6 in untreated neurons, and 6.1 in ethanol treated neurons) and had no effect on the mRNA levels of NR1-3 (+E21/-E22) and NR1-4 (-E21/-E22) splice variants. At the polypeptide level, chronic ethanol treatment significantly reduced exon 5 containing splice variants (NR1-3b and NR1-4b). However, ethanol (50 mM, 5 days) induced a significant increase in polypeptide levels of NR1-4 (-E21/-E22) without any effect on NR1-3 (+E21/-E22) polypeptide levels. These results demonstrate that chronic ethanol treatment has a selective effect on the expression of NR1 splice variants both at the mRNA and polypeptide level in mouse fetal cortical neurons.
Introduction

N-methyl-D-aspartate (NMDA) receptors, the excitatory receptors in the central nervous system, are involved in a variety of physiological and pathological processes (1). Molecular cloning and functional studies reveal that NMDA receptors are heteromeric and consist of three subunits named NR1, NR2 and NR3 in the rat (2). The NR1 and NR2 subunits are named ζ and ε respectively in the mouse (3). The NR2 subunit has four members that combine with the NR1 subunit to form functional NMDA receptors with distinct pharmacological properties. Additional diversity of NMDA receptors is achieved by alternative splicing of the NR1 subunit (4). The NR1 subunit, a product of a single gene, has eight isoforms generated by alternative splicing of exons 5, 21 and 22 (5, 6). Exon 5 encodes the N1 splice cassette that lies in the extracellular amino-terminal domain of the NR1 subunit. Exons 21 and 22 encode the carboxy-terminal splice cassettes C1 and C2 respectively and are a part of the intracellular domain of the NR1 subunit. NR1 splice variants lacking exon 22 contain an additional cassette, C2’, at the carboxy terminal end. The presence or absence of the N1, C1, and C2 cassettes influence the function of the NMDA receptors (6-12).

Ethanol, one of the most abused drugs in our society, alters the expression and function of NMDA receptors in a treatment-dependent manner. Acute ethanol exposure in vivo inhibits NMDA receptor function while chronic ethanol treatment in vivo and in vitro increases the NMDA receptor number and function (13, 14). The increase in receptor number following chronic ethanol treatment of fetal cortical neurons is a result of an augmentation of NR1 and NR2B polypeptides (15) with a concomitant increase in NR2B mRNA levels (16). Although chronic ethanol treatment specifically enhances the half-life of NR1 mRNA in fetal cortical neurons (17), NR1 mRNA levels are not increased (16). The NR1 subunit has 8 splice variants. It is possible that ethanol may affect only selective NR1 splice variants resulting in no

The abbreviations used are: NMDA, N-methyl-D-aspartate receptor; NR1, NMDA R1 receptor subunit; NR2, NMDA R2 receptor subunit; 1B15, cyclophilin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPA, ribonuclease protection assay; dNTPs, deoxynucleotide triphosphates; AA, alcohol preferring; ANA, alcohol non-preferring; nNOS, neuron specific nitric oxide synthase; NO, nitric oxide.
significant overall increase in NR1 mRNA levels in fetal cortical neurons. To test this hypothesis, we examined the effect of chronic ethanol treatment on NR1 splice variants at the mRNA and polypeptide levels using our *in vitro* model system of mouse fetal cortical neurons. We performed a comprehensive analysis of all the NR1 splice variants at the mRNA and polypeptide levels in mouse fetal cortical neurons. To our knowledge, this is the first report demonstrating the simultaneous amplification of exons 21 and 22 by RT/PCR allowing the concurrent detection of NR1-1, NR1-2, NR1-3 and NR1-4 splice variants. Our results show that mouse fetal cortical neurons cultured for 5 days *in vitro* express four NR1 splice variants, NR1-3a, NR1-3b, NR1-4a, and NR1-4b. Chronic ethanol treatment decreased the expression of NR1-4b splice variant at the mRNA and polypeptide levels in fetal cortical neurons. Under similar culture conditions, ethanol (50 mM, 5 days) up regulated the polypeptide levels of NR1-4a without any effect on NR1-3 polypeptide levels. Chronic ethanol treatment thus has a differential effect on the mRNA and polypeptide levels of four NR1 splice variants expressed in fetal cortical neurons.
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Experimental Procedures

Cell culture and Ethanol Treatment

Fetal cortical neurons isolated from 14-15 days old mouse fetuses were cultured as described elsewhere (17). Time pregnant mice (strain C57 BL/6) purchased from Harlan (Indianapolis, IN) were used in accordance with institutional guidelines, and procedures were approved by the animal welfare committee.

Isolation of Total RNA

Twenty-four hours after the last ethanol treatment, total RNA was isolated from cultured cells using Trizol (Life Technologies, Grand Island, NY). Isolated total RNA was rendered genomic DNA free by digestion with RNase-free DNase (RQ1; Promega, Madison, Wisconsin). Total RNA was purified again by organic extraction and quantified by absorbance at 260 nm. DNA-free total RNA was used for Northern blot and RT/PCR analyses and ribonuclease protection assays.

Northern Blot Analysis

Northern blot analysis was performed as described (18). Briefly, 10 µg of total RNA were electrophoresed on 1.2% formaldehyde agarose gel, transferred on to Gene Screen Plus membrane (Dupont, Boston, MA). The NR1 mRNA was detected by hybridization to a 32P-labeled mouse NR1 cDNA labeled by the random prime method using Prime-It kit (Stratagene, La Jolla, CA). The mouse NR1 cDNA was obtained from Dr. M. Mishina, Japan. Following hybridization overnight at 42°C, the membranes were washed and exposed to PhosphorImager screen. Results were analyzed on PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Ribonuclease Protection Assay

Effect of chronic ethanol treatment on the mRNA levels of β-actin, cyclophilin (IB15) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined by ribonuclease protection assay (RPA) as described previously (17). Plasmids to generate riboprobes for β-actin and GAPDH were purchased from Ambion, TX. The riboprobe for cyclophilin was the same as described elsewhere (17). Data was analyzed using the ImageQuant software.
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RT/PCR Analysis

(a) End-labeling of Forward Primers

Forward primers were end-labeled with \( \gamma^{32}\text{P}-\text{ATP} \) using T4 polynucleotide kinase as described (18). Unincorporated \( \gamma^{32}\text{P}-\text{ATP} \) was removed by filtering the reaction mix through a Sephadex G25 column. Labeled primers were further purified by the acrylamide gel (15%) purification method, suspended in water, and used within one week of \( ^{32}\text{P} \)-labeling. The specific activity of \( ^{32}\text{P} \)-labeled forward primers ranged between \( 8.6 \times 10^7 \) cpm/\( \mu \)g and \( 3.48 \times 10^8 \) cpm/\( \mu \)g.

(b) Reverse Transcription-Polymerase Chain Reaction (RT/PCR)

RT/PCR was performed using the GeneAmp RNA PCR kit from Perkin Elmer (Norwalk, CT) according to their instructions, except that AmpliTaq Gold polymerase was used. RT/PCR conditions for exon 5 and exons 21 and 22 of the NR1 subunit of the NMDA receptor were standardized using cRNA generated by \textit{in vitro} transcription of Nhe I linearized NR1-1b plasmid. The NR1-1b plasmid, obtained from Dr. M. Hollmann, Germany, was used as a positive control for PCR step of RT/PCR. One hundred nanograms (ng) of total RNA were used for RT/PCR amplification of exon 5, exons 21 and 22, and \( \beta \)-actin while 200 ng of total RNA were used for IB15 and GAPDH.

Reverse transcription for exons 21 and 22 of the NR1 subunit was performed as follows: 15 min at 25°C; 2 min at 40°C and then ramp up 2°C/min until reaching 60°C; 30 min at 60°C; 5 min at 99°C; and 5 min at 5°C. For \( \beta \)-actin, GAPDH, IB15, and exon 5 of NR1 subunit, reverse transcription was performed using the following conditions: 15 min at 25°C; 30 min at 42°C; 5 min at 99°C; and 5 min at 5°C. Total RNA (300 ng/60 \( \mu \)l for exon 5, exons 21&22 and \( \beta \)-actin, and 400 ng/40 \( \mu \)l for IB15 and GAPDH) mixed with RT master mix was reverse transcribed. The final concentration of components in 20 \( \mu \)l of RT reaction mix was 5 mM MgCl\(_2\), 1 x PCR buffer II, 1 mM each dNTPs, 20 units of RNase inhibitor, 2.5 \( \mu \)M random hexamers, and 50 units of MuLV reverse transcriptase). Following reverse transcription, RT mix was divided into 20 \( \mu \)l aliquots. Each aliquot was mixed with 80 \( \mu \)l of PCR mix (final concentration in 100 \( \mu \)l = 2 mM MgCl\(_2\), 1 x PCR buffer II, 2.5 units of AmpliTaq Gold, 125 ng each of \(^{32}\text{P}\)-end-labeled forward primer and cold reverse primer for each gene under investigation in this study). For PCR amplification of exons 21 and 22, 5% dimethyl sulfoxide was added to the PCR reaction mix. PCR was performed as follows: Step 1: 10 min at 95°C X 1 cycle, Step 2: 1 min at 95°C; 1 min at 68°C X 18 cycles
for β-actin (25 cycles for IB15; 27 cycles for exon 5 and 28 cycles for exons 21 and 22), Step 3: 7 min at 72°C X 1 cycle, and soak at 4°C. PCR amplification of GAPDH was as follows: 10 min at 95°C X 1 cycle, 30 sec at 95°C; 50 sec at 56°C; 1 min at 72°C X 25 cycles, 7 min at 72°C X 1 cycle, and soak at 4°C. RT/PCR products (5 µl/sample) were separated on 5% denaturing acrylamide gel. The gels were dried and exposed to PhosphorImager screen. Results were analyzed using the ImageQuant software.

To determine the linear range of quantitation, RT/PCR was performed using 0, 50, 100, 200, 300, 400, 500, 1,000 ng of purified total RNA from fetal cortical neurons cultured for 5 days in the absence of ethanol. The RT reaction for all genes under study was performed as above, except that each reaction was carried out in a total volume of 20 µl and PCR in a final volume of 100 µl. Results were analyzed using PhosphorImager. Following optimization of the RNA concentration, the appropriate number of PCR cycles for each gene were empirically determined to provide values that fell into both the linear range of PCR and the linear range of detection by autoradiography.

(c) Quantitation of RT/PCR Results

For quantitation of RT/PCR results, the signal intensity of 200 nt (-E5), 263 nt (+E5), 87 nt (-E21/-E22) and 198 nt (+E21/-E22) long DNA fragments were divided by the signal intensity of the 'ethanol-insensitive' gene (β-actin or IB15 or GAPDH) from the same RNA sample. Normalized values for -E5 (‘a’ isoforms) were divided by normalized values for +E5 (‘b’ isoforms) and expressed as ratio of –E5/+E5. Similarly, normalized values for –E21/-E22 (NR1-4) were divided by normalized values for +E21/-E22 (NR1-3) and expressed as ratio of NR1-4/NR1-3. Statistical analysis was performed using ANOVA and Fisher’s Least Significant Difference test.

(d) Restriction Analysis of RT/PCR Products

Identification of RT/PCR products was performed by restriction analysis. Unique restriction sites that lie within the amplified exons, as well as outside the amplified exons, were selected for exons 5 and 21 (Table 2). For β-actin, IB15, and GAPDH, mouse sequence obtained from GenBank was analyzed and unique restriction sites within the amplified region were selected (Table 2). RT/PCR products (10 µl) were digested with the selected restriction enzymes according to the instructions of the supplier and separated on 5% denaturing acrylamide gel. Results were analyzed using PhosphorImager.
Western Blot Analysis

Washed neurons were recovered by scraping, suspended in buffer A (50 mM Tris.HCl buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 100 µg/ml leupeptin, 100 µg/ml aprotinin, 1 mM AEBSF and 1 mM DTT) (19) and homogenized using a Dounce homogenizer with a tight-fitting pestle. Homogenates were centrifuged at 500 x g for 5 min at 4°C. Pellets containing nuclei and/or unbroken cells were discarded and supernatants (cell lysates) were recovered. Cell lysates prepared as above from cerebral cortex, hippocampus, and cerebellum of 6 months old male mice, were used as controls. The protein concentration in the cell lysates was determined by the Bradford method (20). Cell lysates were mixed with detergents (Triton X100 to 1%, Sodium deoxycholate to 1%, NP40 to 1% and sodium dodecyl sulfate (SDS) to 2%), and boiled for 2 min.

Western blot analysis was performed as described elsewhere (15). Proteins (10 µg protein for cultured cells and 5 µg protein for samples from adult brain) were separated on 8.5% SDS-PAGE and transferred electrophoretically onto Hybond ECL-pure nitrocellulose membrane (Amersham, Inc., Arlington Heights, IL). Membranes were incubated overnight at 4 °C with one of the following primary antibodies: mouse anti-NMDAR1 monoclonal antibody (NR1pan) (Chemicon, Temecula, CA); rabbit anti-NMDAR1 N1 splice variant polyclonal antibody (Chemicon); rabbit anti-NMDAR1 C1 splice variant antibody (Zymed, San Francisco, CA); rabbit anti-rat NR1 alternative CT (C2’) polyclonal antibody (Upstate Biotechnology, Lake Placid, NY). Following incubation with appropriate horseradish peroxidase-conjugated secondary antibody, immunoreactive bands were visualized on X-ray film using the ECL detection system (Amersham).

To determine the linear range of detection of immunoreactive bands, Western blot analysis was performed as above using 2.5, 5, 7.5, 10, 15, 20, 25, and 30 µg of cell lysate from fetal cortical neurons grown in the absence of ethanol for 5 days. The concentrations of antibodies as well as the exposure time to X-ray film were optimized to provide density values that fell into linear range of detection. Similar analysis was also performed for cell lysates prepared from cerebral cortex (CC), hippocampus (H) and cerebellum (CB) of adult mouse.
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Quantitation of Western Blot Results

The relative changes in the NR1 splice variants were measured by quantifying the intensity of immunoreactive bands and Coomassie blue-stained protein bands using the NIH Image software (version 1.61, NIH, Bethesda, MD). Following transfer of proteins to nitrocellulose membrane, gels were stained with Coomassie B. blue R250. A consistent protein band present in all the lanes was used to control for equal loading. To normalize the Western blot results, the density values of the immunoreactive bands were divided by the density values of Coomassie blue-stained protein band from the corresponding gel lanes. Results are expressed as percent of control. Statistical analysis was performed using ANOVA and Fisher’s Least Significant Difference Test.
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Results

Effect of Ethanol on NR1 mRNA Size

Effect of chronic ethanol treatment on the size of the NR1 mRNA in cultured fetal cortical neurons was determined by Northern blot analysis. Analysis of results indicated no apparent change in the size of the NR1 mRNA in fetal cortical neurons grown in the presence of ethanol as compared to the untreated controls (data not shown).

Effect of Ethanol on the mRNA Levels of β-Actin, Cyclophilin and GAPDH

Three genes, β-actin, IB15, and GAPDH, were selected as internal controls for quantitative RT/PCR analysis because their expression is not altered by chronic ethanol treatment both in vivo and in vitro (16, 21-25). In this study, these genes are referred to as 'ethanol-insensitive' genes. To confirm that chronic ethanol treatment has no effect on the mRNA levels of these three genes under our cell culture conditions, we performed RPAs using appropriate antisense riboprobes. Results shown in Table 3 indicated that chronic ethanol treatment had no significant effect on the mRNA levels of β-actin, IB15, and GAPDH in fetal cortical neurons as compared to untreated controls.

Detection of NR1 Splice Variants in Cultured Fetal Cortical Neurons

Expression of NR1 splice variants at the mRNA level was examined by RT/PCR in fetal cortical neurons cultured for 5 days in the absence of ethanol. Amplification of exons 21 (E21) and 22 (E22) using primers encompassing these two exons (Table 1) was performed to determine the presence or absence of NR1-1, NR1-2, NR1-3 and NR1-4 while amplification of exon 5 (E5) allowed the detection of ‘a’ (-E5) and ‘b’ (+E5) isoforms of the NR1 subunit. The classification of NR1 splice variants used here is adapted from Hollmann and colleagues (6).

Genomic DNA-free total RNA (500 ng) isolated from fetal cortical neurons was reverse transcribed and amplified by PCR using primers spanning exon 5 of the NR1 subunit (Table 1). Two DNA fragments of 200 (-E5) and 263 (+E5) nucleotides (nt) in length were amplified using total RNA from cortical neurons (Fig. 1A: Lane F). Using NR1-1b cRNA, a single DNA band of 263 nt (+E5) was amplified (Fig. 1A: Lane D). The NR1-1b cRNA contains exons 1-22 including exon 5 (6). These results suggested the presence of both ‘a’ and ‘b’ isoforms of the NR1 subunit in fetal cortical neurons. We observed that the intensity of 263 nt long DNA fragment was less than the 200 nt long DNA fragment. To
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ensure that this difference in intensities of DNA fragments was not due to differences in amplification efficiency, DNA-free total RNA from the cerebellum of adult mouse was used as a control for RT/PCR. RT/PCR results in Figure 1A (Lane CB) showed the amplification of 200 and 263 nt long DNA fragments in equal proportion as has been reported previously (26, 27). These results confirmed the differential expression of NR1 ‘a’ and NR1 ‘b’ isoforms in cultured fetal cortical neurons.

Expression of NR1-1, NR1-2, NR1-3, and NR1-4 in fetal cortical neurons was detected by RT/PCR using primers spanning exons 21 and 22. RT/PCR products (10 µl) separated on 2% agarose gels (3:1 agarose, high resolution blend) were visualized by ethidium bromide staining (Fig. 1B). Two DNA fragments of 198 (+E21/-E22) and 87 (-E21/-E22) base pairs (bp) were amplified using total RNA isolated from fetal cortical neurons cultured in the absence or presence of ethanol (Fig. 1B: Lanes C, E). Using NR1-1b cRNA and cDNA, a single band of 552 bp (+E21/+E22 = NR1-1) was amplified (Fig. 1B: Lanes C1 and C2). This data suggested that cultured fetal cortical neurons express NR1-3 (198 bp) and NR1-4 (87 bp) splice variants only. Similar results were obtained using total RNA isolated from cerebral cortex, hippocampus, and cerebellum of adult mouse (Fig. 1B: Lanes CC, H, CB). Detection of both ‘a’ and ‘b’ isoforms of the NR1 subunit as well as NR1-3 and NR1-4 indicated the presence of NR1-3a, NR1-3b, NR1-4a and NR1-4b splice variants in cultured fetal cortical neurons, regardless of ethanol treatment.

Restriction analyses of DNA fragments amplified by RT/PCR were performed to verify the amplified DNA sequences. Unique restriction sites that lie within the amplified exons as well as outside the amplified exons were selected for exons 5 and 21 (Table 2). The size of DNA fragments obtained following restriction digestion with appropriate restriction enzymes (Figs. 2A, 2B) were identical to the predicted size (Table 2) demonstrating that appropriate exons of the NR1 subunit were amplified using the primer pairs shown in Table 1. Restriction analysis was also performed for β-actin, IB15, and GAPDH (internal controls) using one restriction site in the amplified DNA sequence (Table 2). Analysis of results showed that the appropriate gene products were amplified for each of the internal controls (Table 2; Fig. 2C).

Effect of Ethanol on the mRNA Levels of NR1 Splice Variants in Fetal Cortical Neurons

Quantitative RT/PCR was performed to determine the effect of chronic ethanol treatment on the expression of NR1-3a,b and NR1-4a,b splice variants in fetal cortical neurons. Total RNA isolated from
cortical neurons grown in the presence of ethanol (50 mM, 5 days) was amplified using primers spanning exon 5, and exons 21 and 22 (Table 1). Total RNA isolated from cortical neurons grown in the absence of ethanol served as untreated controls. Three 'ethanol-insensitive' genes, β-actin, IB15, and GAPDH, were employed as internal controls because chronic ethanol treatment did not significantly alter their mRNA levels in fetal cortical neurons (Table 3).

The autoradiogram in Figure 3 shows the RT/PCR results for internal controls while Figures 4 and 5 show the RT/PCR results for exons 21 and 22, and exon 5 of the NR1 subunit respectively. The RT/PCR results were quantitated as described in experimental procedures. Because similar results were obtained with all the internal controls, only results normalized with β-actin were plotted (Fig. 6). Fetal cortical neurons express NR1-3 (+E21/-E22) and NR1-4 (-E21/-E22) splice variants regardless of ethanol treatment (Fig. 4). Quantitative analysis of results indicated that the expression of NR1-4 splice variant was greater than the NR1-3 splice variant in fetal cortical neurons grown in the absence of ethanol as the ratio of NR1-4/NR1-3 was 2.66±0.14 (Figs. 4 and 6). Chronic ethanol treatment had no significant effect on the expression of NR1-3 and NR1-4 splice variants (NR1-4/NR1-3 = 2.27±0.09) in fetal cortical neurons (Figs. 4 and 6). Fetal cortical neurons cultured in the absence or presence of ethanol express both 'a' isoforms (-E5) and 'b' isoforms (+E5) of the NR1 subunit (Fig. 5). Quantitative analysis of results indicated that the expression of 'a' isoform was greater than the expression of 'b' isoform in fetal cortical neurons grown in the absence of ethanol as the ratio of -E5/+E5 was 4.63±0.53 (Figs. 5 and 6). A significant decrease in the expression of 'b' isoform of the NR1 subunit (+E5) was observed following chronic ethanol treatment of fetal cortical neurons. Specifically, the ratio of -E5/+E5 increased from 4.63±0.53 in the control to 6.14±0.17 in ethanol treated cortical neurons (Fig. 6).

**Effect of Ethanol on the Polypeptide Levels of NR1 Splice Variants in Fetal Cortical Neurons**

Western blot analysis using cell lysates was performed to determine the distribution of NR1 splice variants at the polypeptide level as well as to detect ethanol-mediated alterations in their expression in fetal cortical neurons. The NR1 protein was detected using NR1pan, a polyclonal antibody raised against 1-564 amino acids of the NR1 subunit. NR1-3b/NR1-4b protein was identified using the N1 antibody raised against 21 amino acids encoded by exon 5. Expression of NR1-3 and NR1-3/NR1-4 polypeptides were determined by C1 and C2’ antibodies respectively.
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Immunoblot studies showed the presence of NR1 splice variants containing the N1, C1, and C2’ cassettes in fetal cortical neurons cultured in the absence of ethanol (Fig. 7B-D: Lanes C). Using the NR1pan antibody, a significant increase in the polypeptide levels of the NR1 subunit (58.75% above control levels) was detected in fetal cortical neurons following chronic ethanol treatment (Fig. 7A: Lanes E; and Fig. 8). A similar increase (53.66% above control levels) was also observed for NR1 splice variants containing C2’ cassette (NR1-3 and NR1-4) in cortical neurons following exposure to ethanol (50 mM, 5 days) (Fig. 7D: Lanes E; and Fig. 8). Using an antibody specific for the N1 cassette, a significant reduction (28% below control levels) of NR1 splice variants containing the N1 cassette (NR1-3b and NR1-4b) was seen in fetal cortical neurons following chronic ethanol treatment (50 mM, 5 days) (Fig. 7B: Lanes E; and Fig. 8). No significant change in the expression of NR1 splice variants containing C1 cassette (NR1-3) (3.67% above control values) was observed (Fig. 7C: Lanes E; and Fig. 8).

In adult mouse, the expression of NR1 polypeptide was found to vary in a region specific manner in the brain. The maximum levels of NR1 protein were observed in the hippocampus and the least levels were observed in the cerebellum (hippocampus>cerebral cortex>cerebellum) (Fig. 7A: Lanes CC, H, and CB). A similar pattern of expression was seen when C2’ antibody was used (Fig. 7D: Lanes CC, H, and CB). Expression of N1 containing splice variants was identical in all brain regions examined (Fig. 7B: Lanes CC, H, and CB). Surprisingly, the C1 cassette containing NR1 splice variant was absent in cerebellum (Fig. 7C: Lane CB) even though NR1 mRNAs containing exon 21 were detected in adult cerebellum by RT/PCR (data not shown).
Discussion

In the present study, we have investigated by RT/PCR and Western blotting, the effect of chronic ethanol treatment on the expression of NR1 splice variants in cultured mouse fetal cortical neurons, our in vitro model system. Our data showed that fetal cortical neurons cultured for five days in the absence of ethanol expressed four (NR1-3a, NR1-3b, NR1-4a, NR1-4b) out of eight splice variants of the NR1 subunit of the NMDA receptor. Chronic ethanol treatment (50 mM, 5 days) significantly down-regulated the mRNA and polypeptide levels of NR1-3b and NR1-4b (splice variants containing exon 5) in cultured fetal cortical neurons. Under identical culture conditions, ethanol had no effect on the mRNA levels of NR1-3 (+E21/-E22) and NR1-4 (–E21/-E22) splice variants. At the protein level, however, a significant increase in NR1 splice variants containing the C2’ cassette (NR1-3 and NR1-4) was observed without any effect on the NR1 splice variant containing the C1 cassette (NR1-3). These results suggest that chronic ethanol treatment (50 mM, 5 days) had a differential effect on the expression of NR1 splice variants both at the mRNA and polypeptide level in cultured fetal cortical neurons.

The NR1 subunit is an essential subunit of the NMDA receptor (14, 28, 29). It has eight splice variants that result from alternative splicing of exons 5, 21 and 22 (3, 5, 6, 30). During postnatal development, the NR1 subunit is widely expressed in mouse brain (3). However, a closer examination using a diverse array of techniques reveals a spatiotemporal pattern of expression of NR1 splice variants in the central nervous system of rat (26, 27, 31, 32). Since the expression of NR1 splice variants in fetal mouse brain has not been examined, we first determined by RT/PCR and Western blotting, the distribution of various NR1 splice variants in cultured mouse fetal cortical neurons. RT/PCR of total RNA from cultured fetal cortical neurons using primers spanning exon 5 amplified two DNA bands of 200 (-E5) and 263 (+E5) bp in length indicating the presence of both exon 5 lacking (‘a’ isoforms) and exon 5 containing (‘b’ isoforms) NR1 splice variants respectively. In a similar manner, analysis of DNA bands obtained by RT/PCR of total RNA isolated from cultured neurons using primers encompassing exons 21 and 22 revealed the presence of NR1-3 (+E21/-E22 = 198 bp) and NR1-4 (-E21/-E22 = 87 bp) splice variants. The absence of DNA fragments of 552 or 442 bp in length suggested the absence of NR1 splice variants
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containing exon 22 [NR1-1 (+E21/+E22) and NR1-2 (-E21/+E22)] in cultured fetal cortical neurons. Western blot analysis using commercially available NR1 splice variant-specific antibodies confirmed the presence of NR1 splice variants containing N1 (+E5), C1 (+E21) and C2’ (+E21/-E22 and/or –E21/-E22) amino acid cassettes. Thus RT/PCR and Western blot analysis results showed for the first time that fetal cortical neurons express NR1-3a, NR1-3b, NR1-4a, and NR1-4b splice variants. These results also demonstrated the absence of exon 22 containing NR1 splice variants in cultured fetal cortical neurons. In this regard, it is interesting to note that the human cerebral cortex also expresses only hNR1-3a, hNR1-3b, hNR1-4a, and hNR1-4b splice variants (33). More recently, a complete absence of exon 22 is reported in the central nervous system of *Apteronotus leptorhynchus* (34).

The NMDA receptor system is an important site for the action of ethanol. At the receptor subunit level, chronic ethanol treatment (50 mM, 5 days) enhances the half-life of the NR1 mRNA in fetal cortical neurons (17) without altering the NR1 mRNA levels (16) and the size of NR1 mRNA (present study). In this study, we observed by quantitative RT/PCR analyses that chronic ethanol treatment specifically down-regulated the mRNA levels of exon 5 containing NR1 splice variants (NR1-3b and NR1-4b). The ratio of -Exon 5/+Exon 5 changed from 4.6 in the control to 6.1 in ethanol treated (50 mM, 5 days) fetal cortical neurons. A similar decrease in NR1 splice variants containing exon 5 is seen in the cortex of adult male Wistar rats following 16 days of exposure to ethanol (35). Further, our quantitative RT/PCR results showed no significant change in the mRNA levels of NR1-3 (+E21/-E22) and NR1-4 (−E21/-E22) splice variants in fetal cortical neurons cultured in the presence of 50 mM ethanol for 5 days. In an independent study using alcohol preferring (AA) and alcohol non-preferring (ANA) rats, Winkler et al. (36) also found no change, by RT/PCR, in the NR1-4 mRNA levels in hippocampus of AA rats as compared to ANA rats following 30 days of exposure to ethanol. In contrast to the quantitative RT/PCR studies, *in situ* hybridization results show down-regulation of NR1-2 and NR1-4 mRNAs (NR1 splice variants lacking exon 22) following 8 days of ethanol treatment of adult male Wistar rats (37). Our quantitative RT/PCR analyses provide evidence for a selective effect of chronic ethanol treatment on the
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mRNA levels of specific NR1 splice variants because this treatment does not change the NR1 mRNA levels on the whole (16), but specifically decreased the mRNA levels of exon 5 containing NR1 splice variants (present study). Thus, these results may explain why no significant increase in NR1 mRNA levels is detected following chronic ethanol treatment (16) even though similar ethanol treatment increases the half-life of NR1 mRNA in fetal cortical neurons (17).

Chronic ethanol treatment in vivo up regulates NR1 immunoreactivity by 65% in the rat hippocampus (38). A similar up regulation of NR1 polypeptide levels occurs in cultured fetal cortical neurons following 5 days of ethanol treatment (50 mM) and NR1 polypeptide levels return to control levels following 48 hours of ethanol withdrawal (15). In the present study, we observed ~58% increase in NR1 subunit polypeptide levels in ethanol treated fetal cortical neurons using the NR1pan antibody that detects all the NR1 splice variants. Using an antibody specific for the C2′ cassette, we found ~53% increase in the polypeptide levels of NR1 splice variants lacking exon 22 (NR1-3 and NR1-4) in fetal cortical neurons following chronic ethanol treatment. However, no change in the expression of C1 (+E21) containing splice variants (NR1-3) was observed suggesting that the increase in polypeptide levels of exon 22-lacking NR1 splice variants (NR1-4 and NR1-3) was mainly contributed by up regulation of NR1-4 and not by the NR1-3 splice variant. Interestingly, a significant increase in the levels of NR1-3/NR1-4 protein has been reported in alcohol preferring (AA) adult rats following 30 days of exposure to ethanol (31). Using the N1 cassette - specific antibody, we detected ~28% decrease in the expression of N1 (+E5) containing NR1 splice variants (NR1-3b and NR1-4b). This decrease in N1 (+E5) containing NR1 polypeptide levels may be due to a selective reduction in the mRNA levels of exon 5 containing NR1 splice variants. Antibodies specific for N1-lacking (-E5) NR1 splice variants are currently not available. Therefore, we were unable to determine ethanol-mediated effects on the polypeptide levels of NR1-3a/NR1-4a splice variants (-E5). Despite a reduction in polypeptide levels of exon 5 containing NR1 splice variants, a significant increase (58%) in NR1 polypeptide levels was detected using NR1pan antibody. A similar increase (53%) was also observed using an antibody specific for C2′ cassette. These
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observations imply that the overall increase in NR1 polypeptide levels detected by NR1pan was due to an increase in polypeptide levels of C2’ cassette - containing NR1 splice variant (NR1-4a). Because no increase in mRNA levels of NR1-4 was observed, it is likely that the increase in NR1 polypeptide levels seen in fetal cortical neurons following chronic ethanol treatment may be a result of mechanisms(s) operating at the post-transcriptional level.

NMDA receptors containing NR1 splice variants lacking exon 5 (N1 cassette) exhibit higher affinity for NMDA receptor agonists, display relatively small currents and show marked potentiation by spermine and micromolar concentrations of Zn$^{2+}$ (4, 6, 7, 11). Our data suggest that chronic ethanol treatment augmented the expression of NR1 splice variants lacking exon 5 in fetal cortical neurons. It is possible that following chronic ethanol treatment, the majority of NMDA receptors in fetal cortical neurons contain NR1 subunits which lack the N1 cassette (exon 5). It is thus reasonable to speculate that in fetal cortical neurons, NMDA receptors will have a different physiological and pharmacological profile following chronic ethanol treatment. Confirmation of this notion, however, will have to await further experimentation. Interestingly, Okabe and colleagues recently demonstrated that NR1-4 splice variants show the highest cell surface expression both in cultured hippocampal neurons and transfected fibroblasts (34). It is also pertinent to note that nNOS – positive neurons are enriched with the NR1-4 splice variants, and lack NR1-1 and NR1-3 splice variants (35). This corollary observation suggests that NO-mediated signaling system may be exceptionally important in ethanol treated cortical neurons.

To summarize, this is the first report examining the presence of all NR1 splice variants in mouse fetal cortical neurons. We also examined the effect of chronic ethanol treatment on NR1 splice variants at the mRNA and polypeptide level. Our results suggest that chronic ethanol treatment of mouse fetal cortical neurons selectively decreased the expression of the NR1-4b splice variant both at the mRNA and polypeptide level. At the same time, chronic ethanol treatment up regulated the polypeptide levels of NR1-4a without any effect on the mRNA level.
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Legends to Figures

Figure 1: Distribution of NR1 splice variants in cultured fetal cortical neurons. A: A representative gel autoradiogram showing RT/PCR amplification of exon 5-containing/lacking NR1 splice variants using DNA-free total RNA isolated from fetal cortical neurons (F), adult cerebellum (CB), and a positive control - cRNA of NheI linearized NR1-1b plasmid (D). RT/PCR products, separated on denaturing acrylamide gels, were detected using PhosphorImager. DNA bands of 263 and 200 nucleotides in length (arrows on the left) show the presence (+E5/’b’ isoform) and absence (-E5/’a’ isoform) of exon 5 respectively (see Table 1). Lanes M: end labeled markers, φX174/Hinf I digested (numbers on the right indicate nucleotides). B: A representative gel autoradiogram showing RT/PCR amplification of exons 21 and 22 using total RNA isolated from fetal cortical neurons grown in the absence (C) or presence (E) of ethanol, adult cerebral cortex (CC), adult hippocampus (H), adult cerebellum (CB), and a positive control - cRNA of NheI linearized NR1-1b (C2). Lane C1: a positive control for PCR step of RT/PCR, DNA fragment amplified by PCR using NR1-1b cDNA as template. Amplification products separated on 2% agarose gel were visualized by ethidium bromide staining. The 198 bp and 87 bp long DNA bands (open arrows) indicate the presence of NR1-3 (+E21/-E22) and NR1-4 (-E21/-E22) splice variants. The 552 bp DNA band in lanes C1 and C2 (open arrow) indicate the amplification of cDNA and cRNA sequences spanning exons 21 and 22 (NR1-1 splice variant) respectively. Lanes M: 100 bp DNA ladder (numbers on the left indicate nucleotides); Lane λ: DNA markers, Lambda DNA / Hind III digested, 564 bp band is marked on the right.

Figure 2: Restriction analysis of RT/PCR products. RT/PCR products were digested with appropriate restriction enzymes, separated on denaturing gels and analyzed using PhosphorImager. Numbers on the right indicate the size of the undigested (open arrows) and restricted DNA fragments (filled arrows). The size of restricted DNA bands for all the genes amplified in this study was the same as predicted (Table 2). A: A gel autoradiogram showing results for exon 5 of the NR1 subunit. Lane 1: end-labeled marker, φX174/Hinf I digested (numbers on the left indicate nucleotides); Lane 2: undigested RT/PCR products (open arrows); Lanes 3 and 4: RT/PCR products digested with Taq I and Pst I respectively (filled arrows). B: A gel autoradiogram showing results for exons 21 and 22 of the NR1 subunit. Lane 1: end-labeled marker, φX174/Hinf I digested (numbers on the left indicate nucleotides); Lanes 2 and 3: RT/PCR
products digested with \textit{Pst I} and \textit{EcoO109 I} respectively (filled arrows); Lane 4: undigested RT/PCR products (open arrows). C: A gel autoradiogram showing results of restriction analysis of RT/PCR products amplified using primers spanning IB15, β-actin, and GAPDH. Lanes 1 and 8: end-labeled marker, φX174/Hinf I digested (numbers on the left indicate nucleotides); Lanes 2, 4 and 6: the undigested RT/PCR products for IB15, β-actin and GAPDH respectively; Lane 3: IB15 RT/PCR product restricted with \textit{EcoO109 I}; Lane 5: β-actin RT/PCR product restricted with \textit{Msp I}; Lane 7: GAPDH RT/PCR product restricted with \textit{EcoO109 I}.

\textbf{Figure 3: Amplification of β-actin, GAPDH, and IB15 by RT/PCR.} Internal controls were amplified by RT/PCR using total RNA isolated from fetal cortical neurons grown in the absence (C) and presence (E) of ethanol and primer pairs shown in Table 1. RT/PCR products separated on denaturing acrylamide gel were analyzed using PhosphorImager. Lanes M: end-labeled marker, φX174/Hinf I digested (numbers on the right indicate nucleotides).

\textbf{Figure 4: Effect of chronic ethanol treatment on the mRNA levels of NR1-3 and NR1-4 splice variants in fetal cortical neurons:} Total RNA isolated from fetal cortical neurons grown in the absence (C) and presence (E) of ethanol was analyzed by quantitative RT/PCR. The primer pair shown in Table 1 were used to simultaneously amplify exons 21 and 22 of the NR1 subunit mRNA. RT/PCR products separated on denaturing acrylamide gels were analyzed using PhosphorImager. The 198 nt (NR1-3 = +E21/-E22) and 87 nt (NR1-4 = -E21/-E22) long DNA fragments are indicated by arrowheads on the left. Lanes M: end-labeled marker, φX174/Hinf I digested (numbers on the right indicate nucleotides).

\textbf{Figure 5: Effect of chronic ethanol treatment on the mRNA levels of splice variants containing (+E5) or lacking exon 5 (-E5) in fetal cortical neurons:} Total RNA isolated from fetal cortical neurons grown in the absence (C) and presence (E) of ethanol (50 mM, 5 days) was analyzed by quantitative RT/PCR using primers shown in Table 1. RT/PCR products separated on denaturing acrylamide gel were analyzed using PhosphorImager. Arrows on the left point to 200 nt (-E5 = 'a' isoform) and 263 nt (+E5 = 'b' isoform) long DNA fragments showing amplification of exon 5 lacking and exon 5 containing NR1 splice variants respectively. Lanes M: end-labeled marker, φX174/Hinf I digested (numbers on the right indicate nucleotides).
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Figure 6: **Quantitative analysis of effect of chronic ethanol treatment on the mRNA levels of NR1 splice variants expressed in fetal cortical neurons:** RT/PCR results were quantified using the ImageQuant software (see experimental procedures). Following normalization with β-actin, results were expressed as ratios of –E5/+E5 and NR1-4/NR1-3. The data are expressed as percent of control (mean ± SE of four separate experiments). Statistical analysis was performed using ANOVA and Fisher’s Least Significant Difference Test, *p < 0.02.

Figure 7: **Effect of chronic ethanol treatment on the polypeptide levels of NR1 splice variants.** Western blot analysis was performed to detect the distribution of NR1 splice variants as well as to determine the effect of chronic ethanol treatment on these splice variants in fetal cortical neurons. Cell lysates, Kaleidoscope pre-stained protein markers, and broad range biotinylated SDS-PAGE markers were separated on SDS-PAGE, blotted onto nitrocellulose and probed with appropriate primary antibody. NR1pan antibody raised against 1-564 amino acids was used to detect all splice variants of the NR1 subunit. A-D: Representative immunoblots showing results obtained with antibodies specific for NR1pan, and N1, C1 and C2′ amino acid cassettes. A 116 kd protein band indicated by an arrowhead was detected with the antibodies employed in this study. E: A representative coomassie blue-stained gel; arrowhead points to the band whose intensity was used to normalize Western blot results (see experimental procedures). Lanes C: cortical neurons grown in the absence of ethanol, Lanes E: cortical neurons grown in the presence of ethanol (50 mM, 5 days), Lane CC: cerebral cortex, Lane H: hippocampus and Lane CB: cerebellum of adult mouse; Lane M: broad range biotinylated SDS-PAGE markers. Numbers on the left indicate molecular weight of biotinylated SDS-PAGE markers in kilodaltons.

Figure 8: **Quantitative analysis of effect of chronic ethanol treatment on the polypeptide levels of NR1 splice variants.** The relative changes in the NR1 splice variants were quantified using NIH Image software version 1.61 (see experimental procedures). Results were normalized and the data are expressed as percent of control (mean ± SE of three separate experiments). Statistical analysis was performed using ANOVA and Fisher’s Least Significant Difference Test, *p < 0.02.
Table 1: Primers used for RT/PCR.

| Gene    | Primers (5’ to 3’). Upstream/downstream | Bases Spanned (bp) | Product Size | Reference        |
|---------|-----------------------------------------|-------------------|--------------|------------------|
| NR1     | GTACCACCTACTCCACCAGTCC                  | 854-1117          | 263 (+E5)    | Vezzani et al. (1995) |
|         | GGAACCAAGAATGTGACGGCTCTG                |                   | 200 (-E5)    |                  |
|         | CAGATGCAGCTGGCTTTTGCAG                  | 3035-3587         | 552 (+E21/+E22) | Hollman et al. (1993) |
|         | TGATATCACGGGCCCGCTCAA                    |                   | 442 (-E21/+E22) |                  |
|         |                                         |                   | 198 (+E21/-E22) |                  |
|         |                                         |                   | 87 (-E21/-E22)  |                  |
| Cyclophilin | GTCTGCTTCGAGCTGTTGACGAGGCAGCAC        | 98-601            | 503          | Vezzani et al. (1995) |
| GAPDH   | ACCACAGTCCATGCCATCAC                    | 566-1017          | 451          | Clontech         |
| β-Actin | TACATGAAGTGTGACGTTGACATCCGT             | 925-1209          | 285          | Promega          |
|         | CATCGTGACCGCAAATGTTCTAGG                |                   |              |                  |
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Table 2: Restriction analysis of RT/PCR products. Table shows the predicted size of DNA fragments following restriction digestion of RT/PCR products with the respective restriction enzymes. * indicates radiolabeled RT/PCR products and DNA fragments obtained following restriction digestion.

| Gene/Exon | RT/PCR Product Size | Restriction Enzyme | Predicted Size of DNA Bands following Restriction Digestion | Restriction Enzyme | Predicted Size of DNA Bands following Restriction Digestion |
|-----------|---------------------|--------------------|-------------------------------------------------------------|--------------------|-------------------------------------------------------------|
| NR1       |                     |                    |                                                             |                    |                                                             |
| +E5 ('b' isoform) | *263              | Taq I  | *176 87                                      | Pst I  | *230 33                                   |
| -E5 ('a' isoform)  | *200              | Taq I  | *200                                        | Pst I  | *166 34                                   |
| NR1       |                     |                    |                                                             |                    |                                                             |
| +E21/-E22 (NR1-3) | *198              | EcoO109 I   | *146 52                                       | Pst I  | *50 148                                   |
| -E21/-E22 (NR1-4)  | *87               | EcoO109 I | *87                                          | Pst I  | *50 37                                    |
| IB15      |                     |                    |                                                             |                    |                                                             |
| β-Actin   | *285               | Msp I   | *250                                        |                    |                                                             |
| GAPDH     | *451               | EcoO109 I | *275 176                                    |                    |                                                             |
Table 3: Effect of chronic ethanol treatment on the mRNA levels of three housekeeping genes in fetal cortical neurons

| Gene    | mRNA levels (% of control) |
|---------|-----------------------------|
| β-Actin | 101.04 ± 4.8                |
| IB15    | 105.36 ± 3.6                |
| GAPDH   | 99.26 ± 3.8                 |

Results are expressed as percent of control (mean ± SEM; n = 10).
Figure 2
Figure 3
NR1-3 (+E21/-E22)

NR1-4 (-E21/-E22)
Figure 6
Figure 8

Effect of Ethanol on Polypeptide Levels of NR1 Splice Variants (% of Control)
Differential effects of chronic ethanol treatment on NMDA R1 splice variant in fetal cortical neurons
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