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Research report

Differential display identifies neuroendocrine-specific protein-A (NSP-A) and interferon-inducible protein 10 (IP-10) as ethanol-responsive genes in the fetal rat brain

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Abstract

Fetal alcohol exposure is the most common nonhereditary cause of mental retardation in the western world. Rats prenatally treated with ethanol liquid diet exhibit extensive defects in the brain that accurately model those observed in humans. To analyze the ethanol effects on gene expression during brain development, we performed mRNA differential display and two-dimensional electrophoresis on gestational day (G) 13 and G16 brain from rats treated with ethanol liquid diet. Using mRNA differential display followed by a variety of quantitative analyses, three genes were confirmed to be ethanol-responsive. Among them was Neuroendocrine-Specific Protein-A (NSP-A), which is known to be affected by thyroid hormone in the cortex at this developmental time. However, two additional genes known to be thyroid hormone-responsive were unaffected by ethanol, indicating that interference with thyroid hormone action may not be a predominant pathway by which alcohol induces damage in the fetal brain. The observation that interferon-inducible protein-10 (IP-10) is up-regulated in ethanol-treated fetal brain may indicate the presence of a disease process recruiting CD8+ T-cells capable of interfering with myelination. The result of two-dimensional (2D) electrophoresis and Western analyses demonstrated that few changes in the abundance of individual proteins or the phosphorylation of proteins at threonine and tyrosine were induced by prenatal ethanol exposure. A critical analysis of the approaches used in the present study may be important for future studies in this field.

Theme: Cellular and molecular biology

Topic: Gene structure and function: general

Keywords: Fetal Alcohol Syndrome; Differential display; Thyroid hormone; 2D gel electrophoresis; NSP-A; Oct-1; HES-1; rpS6; Interferon-inducible protein-10 (IP-10)

1. Introduction

Fetal Alcohol Syndrome (FAS) is a cluster of physical, neurological and behavioral abnormalities in children exposed to excessive maternal alcohol consumption [1,8,13,14,26,60], and is the most common nonhereditary cause of mental retardation in the western world [60]. Experimental work indicates that specific defects in anatomy and biochemistry of the central nervous system underlie the clinical manifestations of FAS. For example, prenatal ethanol treatment in the rat affects proliferation, migration and apoptosis of cortical neurons [10,25,37], and glial proliferation and differentiation [41,63]. Because these developmental events are known to require a regulated program of gene expression, it is possible that the deleterious effects of ethanol on developing brain are mediated by, or result in, altered gene expression. Thus, identification of ethanol-responsive genes in the fetal brain may provide useful clues to understand the etiology of FAS.

The hypothesis that prenatal ethanol exposure can affect gene expression in the fetal brain is supported by a variety of focused hybridization studies, which indicate that several genes in the fetal brain are affected by maternal ethanol consumption [23,53,57,58,63]. However, there are presently no reports identifying ethanol-responsive genes

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specifically in the developing brain using broad, non-biased techniques. A non-biased approach may identify new insights into the mechanisms of ethanol exposure on brain development, and would place the present information about ethanol effects on gene expression in the fetal brain in a broader context. For example, Lee et al. [30] used mRNA differential display to identify ethanol-responsive genes in mouse fetus on gestational day 11. These authors identified three ethanol-responsive genes of an estimated 1080 genes screened. It is possible that the relative paucity of ethanol responsive genes identified by this procedure was related to the use of whole embryos to prepare the RNA and the use of an acute ethanol exposure model, which is less well characterized than a model of chronic ethanol exposure [39]. Therefore, the goal of the present study was to apply the two non-biased techniques of mRNA differential display and two-dimensional (2D) electrophoresis to identify ethanol-responsive genes at both the mRNA and protein level specifically in the fetal brain.

To screen for ethanol-responsive genes, we chose the rat model treated with an ethanol-containing liquid diet. This model is very well characterized [33,37,68] and the consequences of ethanol treatment using this model have been thoroughly studied [22,37,49,56,67,70]. Among these studies, prenatal ethanol exposure is shown to profoundly disturb the proliferation and migration of neurons in the cerebral cortex [37]. For example, the proliferation and migration of neurons were delayed by 1 day on gestational day (G) 13 and were severely decreased on G16 in the ethanol-treated cerebral cortex. Therefore, we focused our studies on these two time points to identify ethanol-responsive genes that may underlie the deleterious effects of ethanol on proliferation and migration of cortical neurons. We now report the identification of three genes, neuroendocrine specific protein-A, interferon-inducible protein-10, and ribosomal protein S6, as ethanol-responsive genes. However, overall, the effects of ethanol on gene expression in the fetal brain appeared to be quantitatively small, including effects observed on protein levels. These findings may have important implications for future identification of ethanol-responsive genes.

2. Materials and methods

2.1. Animals

All animal procedures were performed in accordance with the NIH Guidelines for animal research and were approved by the University of Massachusetts-Amherst Institutional Animal Care and Use Committee. The specific protocol for administration of ethanol was initially described extensively by Miller [34–36,38,40,41]. Two experiments were performed. In the first experiment, female Long–Evans rats ($n=18$; Charles River, Kingston, NY, USA) were maintained in the laboratory on a 12-h light, 12-h dark cycle. They were paired with a male overnight, and insemination was indicated by the presence of sperm in the vaginal smear the following morning. The day following insemination was designated as G1, and on this day, the animals were assigned to one of three treatment groups according to weight ($n=6$/group). Animals in the ethanol group were fed a liquid diet containing 2.2% (v/v) ethanol from G1 to G2, 4.5% ethanol on G3 and 6.7% ethanol from G4 to G13 (BioServ, Frenchtown, NJ, USA). Pair-fed animals were provided the amount of food consumed by their weight-paired, ethanol-fed counterpart on the same gestational day with dextrin and maltose as caloric substitutes for ethanol. To accomplish this, the pair-fed animals were mated and pair-fed the isocaloric liquid diet a day behind that of the ethanol-fed group. Fresh liquid diet was provided each day 2 h before lights off. Chow-fed animals were provided with standard laboratory rat chow and water ad libitum. Dams were sacrificed on G13.

In the second experiment, timed-pregnant Long–Evans rats (Charles River, Kingston, NY, USA) arrived in the laboratory on G2 ($n=18$), and were assigned to groups and treated as described above. To accomplish pair-feeding, again, mating was staggered for the purposes of pair-feeding. The animals were sacrificed on G16.

Dams were killed by decapitation 3 h after the onset of darkness because previous studies have determined that peak blood alcohol concentration (BAC) occurs at this time [68], and we wanted to ensure that animals achieved a BAC known to cause damage to the developing nervous system. Trunk blood was collected from dams, and BAC was determined using a diagnostics kit (Sigma, St Louis, MO, USA). The fetuses were removed from the uterus, and the cerebral cortex was hand-dissected from the brain of half the fetuses from each litter. Tissues dissected for RNA extraction were placed in a 5-ml plastic tube, snap-frozen on dry ice and stored at −80 °C; the remaining fetuses were frozen whole in pulverized dry ice and stored at −80 °C until sectioned for in situ hybridization.

2.2. RNA isolation

Total RNA was isolated by the acid–phenol extraction procedure [12], according to the manufacturer's instructions (RNAzol B, Tel-test, Friendswood, TX, USA; or Tri Reagent, Molecular Research Center, Cincinnati, OH, USA), followed by a standard phenol/chloroform extraction. The final RNA pellet was resuspended in DEPC-treated water, treated with DNAse I and repurified by phenol/chloroform extraction. The RNA was again resuspended in nuclease-free water, quantified by UV spectrophotometry, and the integrity of total RNA confirmed by gel electrophoresis. PolyA+ RNA was isolated according to the manufacture's instructions (PolyATract mRNA Isolation System III, Promega, Madison, WI, USA).

2.3. Sex determination

To preclude fetal gender as a confounding variable, we carried out mRNA differential display only on female fetuses. To identify genetic sex in G13 and G16 fetuses, we tested each fetus for the presence of the Y-specific gene, SRY, using polymerase chain reaction (PCR) on genomic DNA as follows. Five milligrams of fetal tissue was agitated in 0.5 ml lysis buffer (100 mM Tris–HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 μg/ml Proteinase K) at 55 °C for 1 h and clarified by centrifugation. The DNA was precipitated with isopropanol and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.5). PCR was performed in the presence of 1×PCR buffer, 0.8 mM dNTP, 1 μM primers (Table 1), 1.8 mM $MgCl_2$ and 0.05 U/μl AmpliTaq (Gibco–BRL, Gaithersburg, MD, USA). The thermal cycle was 91 °C for 5 min, followed by 30 cycles of 91 °C for 90 s, 60 °C for 90 s and 72 °C for 90 s, then followed by 72 °C for 10 min. The presence of the SRY PCR product, as visualized on an ethidium bromide-stained agarose gel, indicated a male. DNA from known male and female rat was run simultaneously to control for the possibility that a failed reaction would be interpreted as female DNA.

2.4. mRNA differential display

Considering that individual fetuses, especially from different litters, may exhibit differences in gene expression unrelated to treatment, we performed mRNA differential display on RNA pools, prepared by combining equal amounts of RNA extracted from one fetus per litter within each group. Thus, a pool of RNA was created that consisted of an equal amount of RNA from six fetuses derived from the six different dams within that treatment group. The RNA pools were DNase I-treated before reverse transcription (RT) reaction, and the mRNA differential display was conducted according to the manufacturer's instructions (RNAimage, GenHunter, Nashville, TN, USA). PCR, or both RT and PCR, reactions were performed in duplicate on the same set of RNA pools extracted from G13 or G16 tissues, respectively. Because all observed gene fragments that appeared to be differentially expressed in the ethanol-treated group were extensively evaluated in follow-up experiments, we did not consider the different procedure to affect the outcome of the study. Autoradiographic bands were visually evaluated for differences in intensity between EtOH and control groups (pair-fed and chow-fed). Candidate bands were extracted from the gel, reamplified and cloned into either pBluescript KS+ II or pCRII (InVitrogen, Carlsbad, CA, USA). Sequencing was performed using ABI FS-DYE-Terminator chemistry (PE Applied Biosystems, Foster City, CA, USA).

2.5. Northern blot analysis

RNA was fractionated on a 1.2% agarose/6.5% formaldehyde gel, transferred to a nylon Zeta-Probe membrane (BioRad Laboratories, Hercules, CA, USA) and cross-linked by baking. Probes were generated with [^{32}P]dCTP using a random primer labeling kit (Boehringer-Mannheim, Indianapolis, IN, USA). Membranes were prehybridized, hybridized and washed according to the manufacturer's instructions (Zeta-Probe membrane), and exposed to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA). The resulting images were scanned into a Storm 840 Phosphorimager at 200-micron resolution and evaluated with ImageQuant (Molecular Dynamics). The ratio of the intensity of the band representing the specific mRNA to its respective internal loading control (cyclophilin) was first normalized to the median of all the

Table 1
Primers and PCR conditions

Target name	Primer sequence[a]	GenBank accession #	Length (bp) of PCR product	No. of PCR cycles
SRY[b]	5′-ATTTTTAGTGTTCAGCCCTACAGCC 5′-TAGTGTGTAGGTTGTTGTCCCATTG	X89730	459	30
CRBP-I	5′-AGTTCGAGGAGGATCTGACAGGCA 5′-GGGCCGCTCAGTGTACTTTCTTGA	M16459	207	5–23[c]
3A1	5′-TCCTCCTTGGTGTGTGCTCTCAGA 5′-GAAGTTTCAGGGGACCGCAAGTCT	BG709676	223	10–22[c]
IP-10	5′-GGTGTCTGAATCCGGAATCTGAGG 5′-AGGACTAGCCGCACACTGGGTAAA	U22520	171	15–22[c]
NSP-A	5′-ACCTAACCAGCCATCTCCTGTGGA 5′-CTTCTCGGGGATTGTCTCGTGTGT	U17604	195	23
Actin	5′-CCCTCTGAACCCTAAGGCCAACCG 5′-GTGGTGGTGAAGCTGTAGCCAC	V01217	285	–[d]

[a] For each pair, the forward primer is presented above the reverse primer.
[b] Ref. [2].
[c] The first number represents the number of cycles carried out before actin primers were added. The second number represents the number of cycles performed after actin primers were added.
[d] The number of cycles varies, as it was tailored to match the target gene.

relative intensities on the same blot. This procedure was performed to allow us to pool data from several blots. After normalizing, the data were pooled and the mean intensity ratio for each gene was analyzed using a one-way ANOVA, followed by Bonferroni's *t* as a post-hoc test.

2.6. In situ hybridization

Frozen tissues were sectioned in either sagittal or frontal plane at 12 μm in a cryostat (Reichert-Jung Frigocut 2800N, Leica, Deerfield, IL, USA). Sections were thaw-mounted onto gelatin-coated microscope slides and stored at −80 °C until hybridization. Complementary RNA probes were generated from plasmids generated by the mRNA differential display, except for IP-10, NSP-A, NSP-C, Oct-1 and HES-1 probes. IP-10 (244-925, accession no. U22520) was cloned by PCR and the authenticity was confirmed by sequencing. NSP-A (1946–2147, accession no. U17604), NSP-C (1869–1918, accession no. L49143) and Oct-1 (23–1020, accession no. U17013) constructs are described elsewhere [16]. HES-1 cDNA (12–1140, accession no. D13417) was kindly provided by Dr R. Kageyama (Kyoto University, Japan). Transcription reactions, in situ hybridization procedures and image analysis have been described previously [16,17]. The densities from the resulting films were analyzed with a one-way ANOVA among treatment groups.

2.7. Relative quantitative RT-PCR

DNase I-treated total RNA was subjected to RT using oligo $(dT)_{18}$ primer according to the manufacturer's instructions (Advantage RT-for-PCR, Clontech, Palo Alto, CA, USA). PCR was performed according to the manufacturer's instructions (Advantage 2 PCR enzyme system, Clontech, Palo Alto, CA, USA) with some modifications as follows. The PCR reaction contained 2 μl of five times diluted RT product in a 25 μl reaction volume, 1×PCR buffer, 0.2 mM each of dNTP mix, 1×Advantage 2 polymerase mix, 0.4 μM specific primers (Table 1), 0.2 μM actin primers (Table 1) and 66.7 nM α-^{33}P-dATP (3000 Ci/mmol, 10 μCi/μl, ICN, Costa Mesa, CA, USA). The common linear range of amplification cycles for each pair (actin and the specific gene) was empirically determined in a series of experiments prior to the relative quantitative PCR assay. For very low-abundant genes, PCR was conducted for several cycles with the target gene's primers before adding the actin primers, so that both amplified products would be within the similar linear range for amplification. The cycle number in the middle of the common linear range was chosen for the assay (Table 1). The resultant products were run on 6% polyacrylamide gel, exposed to a storage phosphor screen (Molecular Dynamics), and analyzed by ImageQuant software (Molecular Dynamics). The ratios of intensity of the specific gene to actin were compared between ethanol and pair-fed group using a Student's *t*-test.

2.8. Protein isolation

Proteins were isolated from frozen fetal brain by homogenizing with 250 μl osmotic lysis buffer (10 mM Tris pH 7.4, 0.3% SDS, 5 mM $MgCl_2$, 50 μg/ml RNase, 100 μg/ml DNase, 0.2 mM AEBSF (a protease inhibitor), 10 μg/ml leupeptin, 3.6 μg/ml E-64 (a protease inhibitor), 5 mM EDTA and 56 μg/ml benzamidine) per 100 mg tissue. The homogenized solutions were freeze-thawed twice, incubated on ice for 15 min, and combined with equal amounts of boiling SDS buffer (5% SDS, 10% glycerol and 60 mM Tris pH 6.8). They were then incubated in boiling water for 30 min and cooled on ice. The protein concentrations were determined by the BCA method (Pierce, Rockford, IL, USA). Protein pools were prepared by combining an equal amount of protein from each fetus per litter within one group. This extraction procedure retains the phosphorylation state of proteins.

2.9. 2D gel electrophoresis

2D gel electrophoresis was carried out according to the method of O'Farrell [45] by Kendrick Labs (Madison, WI, USA) as follows: Isoelectric focusing was carried out in glass tubes of 2.0 i.d. mm using 2.0% pH 3.5–10 ampholines (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 9600 V-h. Fifty nanograms of an IEF internal standard, tropomyosin, was added to each sample. The tube gel pH gradient was determined with a surface pH electrode. After equilibration for 10 min in buffer '0' (10% glycerol, 50 mm dithiothreitol, 2.3% SDS and 0.0625 M Tris, pH 6.8), each tube gel was sealed to the top of a stacking gel that is on top of a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 h at 12.5 mA/gel. Gels were dried between sheets of cellophane with the acid edge to the left.

2.10. Western blot

For NSP-A western, proteins were extracted simultaneously with RNA isolation using Tri Reagent (Molecular Research Center), run on SDS/8% polyacrylamide gels, and transferred to a PVDF membrane (BioRad Laboratories). After brief rinsing with 1×PBST (150 mM NaCl, 1.0 mM KH_2PO_4, 6.0 mM Na_2HPO_4 and 0.05% Tween-20), the membrane was blocked for 1 h with 5% non fat milk powder/1×PBST, and then incubated at 37 °C for 1 h with anti-NSP-A (kindly provided by Dr W.J.M. Van de Ven, Leuven, Belgium) with a dilution ratio of 1:2000. After thoroughly rinsing with 1× PBST, the membrane was incubated at 37 °C for 1 h with HRP-goat-anti-rabbit (Jackson, West Grove, PA, USA) at a dilution ratio of 1:10 000. After washing vigorously with 1×PBST, the

membrane was developed with the ECL detection kit (NEN, Boston, MA, USA) for the NSP-A signal and detected using the phosphorimager. Subsequently, the membrane was rinsed thoroughly with 1× PBST, and incubated at 37 °C for 1 h with the internal control antibody, anti-M56 (an antibody against 26S protease regulatory subunit 8, kindly provided by Lawrence M. Schwartz, Amherst, MA, USA), at a dilution ratio of 1:250. After washing with 1×PBST, the membrane was incubated with HRP-goat-anti-rabbit at a dilution ratio of 1:500, washed again, and developed with the ECL kit for M56 signal. The intensity of NSP-A, or the ratio of intensity of NSP-A to M56, was analyzed similarly to that described for the Northerns, and evaluated using a one-way ANOVA.

For anti-phospho-tyrosine or -threonine blots, protein samples were electrophoresed on SDS/8% polyacrylamide gels, and transferred to a nitrocellulose membrane. The immunoblot procedures were followed according to the manufacturer's instruction for the primary antibodies (Cell Signaling, Beverly, MA, USA). Briefly, the membrane was blocked in 5% (w/v) nonfat dry milk in 1× TBST (Tris-buffered saline with 0.1% Tween-20), and then incubated with phospho-threonine (P-Thr-Polyclonal) or -tyrosine (P-Tyr-100) antibody with a dilution ratio of 1:1000 or 1:2000, respectively. After washing, the membrane was incubated with HRP-goat-anti-rabbit or HRP-goat-anti-mouse secondary antibody (Jackson) with a dilution ratio of 1:5000 or 1:2000, respectively. The membrane was developed with the ECL kit (NEN).

3. Results

3.1. Ethanol exposure

For animals sacrificed on G13, the daily ethanol consumption (DEC) increased in parallel with increased amount of ethanol in the diet during the first several gestational days (Fig. 1). Thereafter, it remained in the range of 15–17 g/kg body weight, consistent with other studies [35,37,38,40]. The maternal BAC at the time of sacrifice was 153.2±21.7 mg/dl (mean±S.E.M., $n=6$), a level of ethanol known to exert deleterious effects on brain development [54]. There was no detectable level of ethanol in the blood of pair-fed and chow-fed animals. The overall gain in maternal body weight was not different among treatment groups measured at the time of sacrifice (data not shown). These characteristics of ethanol consumption and effects on body weight were also observed for animals sacrificed on G16 (data not shown).

To obtain independent confirmation that the ethanol treatment exerted deleterious effects on the fetal brain, we carried out relative quantitative RT-PCR to test whether the abundance of Cellular Retinoic Acid Binding Protein-I (CRBP-I) mRNA was altered in G13 brain by ethanol

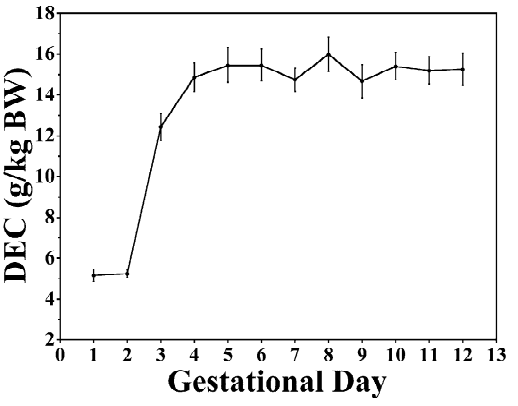

Fig. 1. Daily ethanol consumption (DEC) for ethanol-treated pregnant rats sacrificed on G13. Data represent mean±S.E.M. ($n=6$). DEC increased while the ethanol concentration increased in the liquid diet (G1–G4).

treatment as has been previously reported [23]. We found that CRBP-I mRNA was significantly higher in ethanol-exposed animals compared to pair-fed animals ($P<0.01$, Fig. 2). Thus, the experimental treatment fell within reported measures both for consumption and for effects.

3.2. Identification of putative ethanol-responsive genes by mRNA differential display

Six putative ethanol-responsive genes were identified using messenger RNA differential display on pools of total RNA prepared from G13 brain as described (Fig. 3 and Table 2). In contrast, no ethanol-responsive genes were identified from RNA prepared from G16 brain using the same 24 primer combinations. Therefore, we used 48 additional primer combinations, including 16 arbitrary forward primers (H-AP9-24) and the same three anchored primers. We identified six putative ethanol-responsive genes from G16 brain, although two gene fragments (14A and 24G) matched different regions of the same gene (Fig. 3 and Table 2). Among the putative ethanol-responsive genes from G13 and G16 brain, two (2C and 3A3) from the G13 pools appeared to be decreased by ethanol, three (9A, 13A and 14A) from the G16 pools showed obvious band-shifting in the ethanol-treated pool, and all others appeared to be increased by ethanol. Following sequence analysis, eight gene fragments exhibited matches in GenBank. They are 2C (Interferon-inducible protein; IP-10), 3A3 (phosphoribosylpyrophosphate synthetase-associated protein, PAP39), and 7C (ribosomal protein S6, rpS6) from G13 brain, and 18A1 (neuroendocrine-specific protein, NSP), 13A (16*S* rRNA), 9A (mitochondrial cytochrome *b*, Cytb), and both 14A and 24G (NADH dehydrogenase subunit 2, Nd2) from G16 brain.

Sequence analysis demonstrated that the band shifting of Cytb, 16*S* rRNA and Nd2 was due to two different mitochondrial DNA polymorphisms with an uneven distribution of these polymorphisms among the three treat-

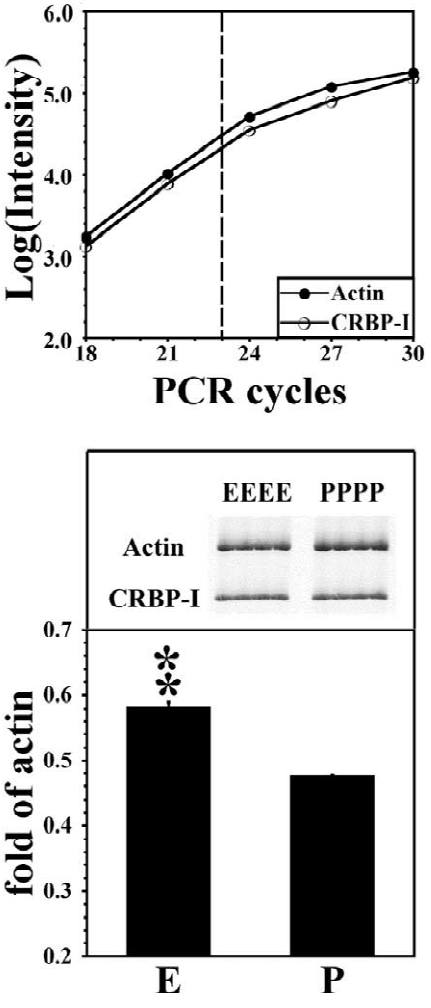

Fig. 2. Relative quantitative RT-PCR analysis for Cellular Retinoid Binding Protein-I (CRBP-I). Upper Panel. An initial experiment was performed to identify the number of cycles in the linear phase of amplification for both CRBP-I and β-actin. PCR was conducted for five cycles in the presence of only CRBP-I primers, then β-actin primers were added for 18, 21, 24, 27 and 30 cycles. Twenty-three cycles were chosen to perform the assay. Lower Panel. Effect of ethanol exposure on CRBP-I mRNA in the fetal brain. Bars represent mean band density relative to β-actin±S.E.M. **Significantly different from pair-fed group ($P<0.01$, Student's *t*-test). The inset shows the image from the scanned storage phosphor screen of the polyacrylamide gel resolving the RT-PCR products for CRBP-I and its internal control, β-actin. E, ethanol group; P, pair-fed group.

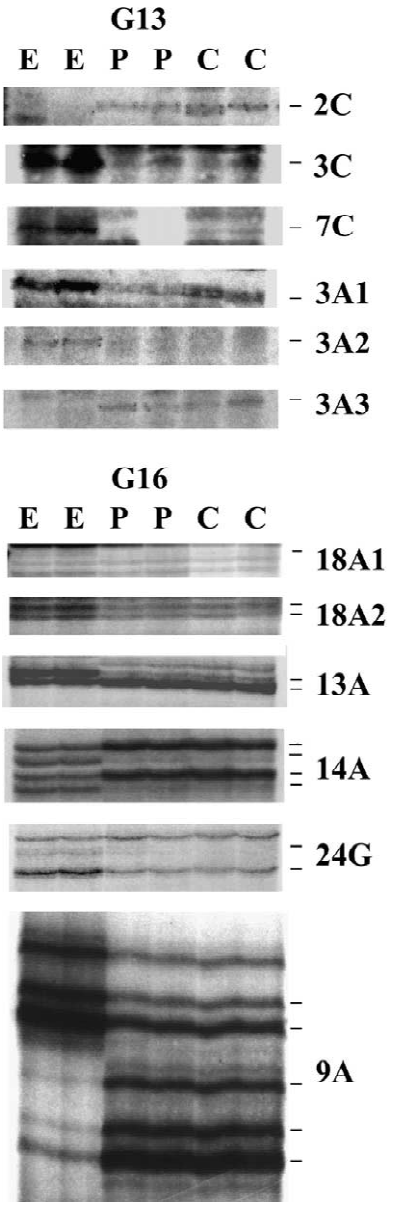

Fig. 3. Putative ethanol-responsive genes identified by mRNA differential display. The fragment identity is listed on the right. Markers on the right indicate the exact bands chosen for further analysis. Each lane corresponds to an RNA pool from five to six animals. The two lanes indicated by the same letter are duplicate PCR reactions for G13, or duplicate RT-PCR reactions for G16. The two markers for each of 18A2 and 24G indicate different parts of the same gene as revealed by sequencing. For each of 13A, 14A and 9A, the bands indicated by the markers are the same gene fragments; the difference in position is due either to conformation, or to polymorphism. E, ethanol-treated; P, pair-fed; C, chow-fed.

ment groups (Table 3). However, we considered them to be putative ethanol-responsive genes for the purpose of further analysis for the following reasons. First, other labs have found NADH dehydrogenase subunit 4 (Nd4), 12*S* rRNA and 16*S* rRNA to be ethanol-responsive genes in human alcoholic brain or rat brain by mRNA differential display [11,18]. These genes, together with Cytb and Nd2, are located on the mitochondrial genome, which exhibits a polycistronic arrangement with a single transcriptional start site. Therefore, all of these genes should, in principle, exhibit simultaneous regulation [66]. Second, two independent primer combinations identified Nd2 as a putative ethanol-responsive gene in the present study.

3.3. Confirmation of ethanol regulation

To confirm the effects of ethanol on the expression of the genes identified by mRNA differential display, each gene was subjected first to Northern analysis. We used total

Table 2
Candidate ethanol-responsive genes identified by mRNA differential display

CDNA fragment	Match in GenBank	GenBank accession #	% DNA identity	Length (bp)
G13				
2C	Interferon inducible protein 10 (IP-10)	U22520	99	226
3A3	Phosphoribosylpyrophosphate synthetase-associated protein (PAP39)	D26073	100	147
7C	Ribosomal protein S6 (rpS6)	M29358	100	175
3A1	None	BG709676[a]	N/A	297
3C	None	BG709678[a]	N/A	107
3A2	None	BG709677[a]	N/A	194
G16				
18A1	Neuroendocrine-specific protein (NSP)	U17604	98	256
18A2	None	BG709679[a]	N/A	163
9A	Mitochondria cytochrome *b* (Cytb)	X14848	99	213
13A	16*S* ribosomal RNA (16*S* rRNA)	X14848	91	264
14A	NADH dehydrogenase subunit 2 (Nd2)	X14848	94	250
24G	NADH dehydrogenase subunit 2 (Nd2)	X14848	99	285

cDNA fragments were named according to the RNAimage primers by which they were generated. Number before letter indicates H-AP arbitrary primer; Letter indicates H-T_{11}M primer; Number after letter indicates different fragments generated by the same pair of primers. N/A, not applicable.
[a] Submitted to GenBank at the time of manuscript submission.

RNA prepared from littermates of the fetuses from which the original pools were prepared for mRNA differential display. Only rpS6 and PAP39 from G13 brain were detectable using 20 μg total RNA, while all the putative ethanol-responsive genes from G16 brain were detectable using 5–30 μg total RNA (Fig. 4 and Table 4). All probes hybridized to the size-class of RNA predicted by their sequence identities, except for 18A2, which had no sequence identity available (Table 4) [3,17,28,44]. Because 14A and 24G hybridized to the same size class of RNA on successive blots, only 14A was used for further Northern analysis of Nd2. Quantitative analysis of these Northern blots revealed that rpS6 mRNA exhibited a significant increase in the ethanol-treated group compared to the pair-fed group (Table 4). Therefore, we considered rpS6 as an ethanol-responsive gene. The four genes that remained undetectable using 20 μg total RNA were hybridized to 3 μg polyA+ RNA. 3A1 hybridized weakly to a band at 7.8 kb (Fig. 4), but others exhibited no specific hybridization signals. To produce a probe with higher specific activity for IP-10, a larger fragment (244–925, accession no. U22520) was cloned and was found to hybridize weakly to 3 μg polyA+ RNA at about 1.5 kb (Fig. 4 and Table 4). However, quantitative Northern analysis was not performed for 3A1 or IP-10, because sufficient material was not available to extract 3 μg polyA+ RNA from each treated group.

A potential weakness of Northern analysis carried out on genes expressed in the brain is that it may not detect differences in abundance when these differences are limited to specific neuroanatomical regions. Therefore, we performed quantitative in situ hybridization for eight putative ethanol-responsive genes. We did not perform this analysis on the putative mitochondrial genes because they were too abundant and ubiquitously expressed to be accurately analyzed using quantitative in situ hybridization. The cDNA fragments cloned from mRNA differential display were used as probes, with the exception that specific probes were used for NSP-A and NSP-C as previously described [16]. Seven of the probes exhibited specific hybridization signals in the tissues from the same gestation day from which they were originally identified (Fig. 5). However, IP-10 and 3A1 were not detectable. Quantitative analysis of the resulting films was performed over the primordial areas of cortex, cerebellum, medulla and pons for tissues at G13, and over the primordial areas

Table 3
Structural characteristics of the two mitochondrial polymorphisms identified by ddPCR and their distribution among the three treatment groups

Gene product	Position	Polymorphism A	Polymorphism B
16*S* rRNA	1659–1660	AC	Deletion
	1714	T	C
	1825	G	A
Nd2	4802	C/threonine	T/isoleucine
	4844	A/–	ACCA/histidine
Cytb	15 176	T	C
	15 197	T	C

Group name	Polymorphism A	Polymorphism B
Ethanol	4[a]	2
Pair-fed	2	4
Chow-fed	1	5

Only the amino acids that are different between the two polymorphisms are listed behind their nucleotide sequence. The numbering is according to the sequence of rat mitochondrial genome (GenBank accession no. X14848). –, no nucleotide or amino acid at that position.
[a] Numbers represent the number of dams exhibiting a specific polymorphism.

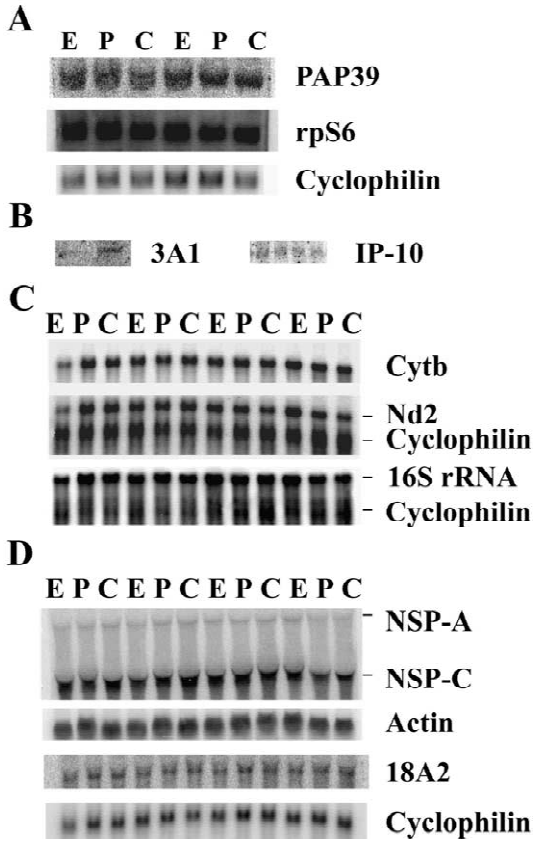

Fig. 4. Images of Northern blots hybridized to probes obtained from mRNA differential display (except IP-10, see Table 4). (A) An example of Northern blots for PAP39 and rpS6. 20 μg total RNA per lane from G13 brains of different treatment groups were electrophoresed as described; the two lanes indicated by the same treatment group are duplicate RNA pools. The same experiment was run at least three times. rpS6 was shown to be increased by ethanol treatment compared to pair-fed group (see Table 4). (B) Northern blots for 3A1 and IP-10. Three micrograms of polyA+ RNA per lane from G13 brains were electrophoresed as described, but the hybridization signal was very low. (C) Northern blots for Cytb, Nd2 and 16*S* rRNA. Five micrograms per lane of total RNA from G16 brains of different treatment groups were electrophoresed and hybridized as described in the text. The lanes indicated as the same treatment group were independent RNA pools from the group. (D) Northern blots for NSP and 18A2. Thirty or 15 μg per lane of total RNA from G16 brains of different treatment groups were hybridized with NSP probe or 18A2, respectively. The lanes indicated as the same treatment group were independent RNA pools from the group. Each lane (in A–D) corresponds to RNA pool from four to six animals. Cyclophilin and actin served loading controls. Because 30 μg per lane of total RNA was out of the linear range of the cyclophilin probe on Northern blot, actin was used as loading control instead. E, ethanol-treated; P, pair-fed; C, chow-fed.

of cortex and midbrain for tissues at G16. NSP-A mRNA was found to be significantly elevated in the cortex of fetuses derived from ethanol-treated dams compared to fetuses derived from pair-fed dams. The others were not found to be differentially expressed among treatment groups using in situ hybridization (Table 5).

Because the expression of IP-10 and 3A1 was too low to be analyzed by either Northern analysis or in situ hybridization, we performed relative quantitative RT-PCR with total RNA from G13 brain using beta-actin as an internal control. Both 3A1 and IP-10 were shown to be significantly increased by ethanol ($P<0.05$, Fig. 6), but IP-10 was in the opposite direction from that shown by the mRNA differential display. To further confirm the effect of ethanol on NSP-A expression, relative quantitative RT-PCR was also conducted with total RNA from G16 brain. However, we observed no differences of NSP-A expression between ethanol and pair-fed groups (Fig. 6).

Western blot analysis was conducted to determine whether NSP-A protein was affected by ethanol treatment. NSP-A antibody revealed a single protein band at about 145 kDa, consistent with the known size of NSP-A in rat [64]. We used an antiserum against M56 (26S protease regulatory subunit 8) as an internal loading control. Pilot experiments demonstrated that the linear range of Western blot conditions for both NSP-A and M56 was up to 20 μg protein per lane (data not shown). Quantitative Western analysis for 15 μg protein per lane did not reveal differences in the amount of NSP-A protein in G16 brain among treatment groups whether we normalized the intensity of NSP-A using M56 as a loading control or not (Fig. 7).

3.4. Does fetal alcohol exposure affect the expression of additional thyroid hormone-responsive genes in the G16 brain?

Considering that NSP-A is a thyroid hormone-responsive gene in the G16 fetal cortex [16,17], and that it was identified in the present screen for ethanol-responsive genes, we tested whether additional known thyroid hormone-responsive genes are affected by ethanol in the G16 cortex. These genes included Oct-1 [17] and HES-1 [24].

The in situ hybridization for Oct-1 mRNA exhibited widespread expression in the brain and somatic tissues, while HES-1 mRNA exhibited intense labeling over the ventricular zone of the telencephalon and less intense labeling over the ventricular zone of midbrain (Fig. 8). These observations are similar to that previously reported [5,17,27]. The specificity of the signals for both genes was confirmed in that the sense probe exhibited no signal (data not shown). Quantitative analysis for Oct-1 over the entire region of the midbrain, anterior and posterior telencephalon and for HES-1 over the ventricular zone of the same three brain regions revealed no significant differences among the three treatment groups (Fig. 8).

3.5. Ethanol effects on protein abundance and phosphorylation in fetal rat brain

2D gels of total protein isolated from fetal brains were silver-stained to compare the abundance of proteins between ethanol and pair-fed groups (Fig. 9). One band at about 16 kDa exhibited a larger range of isoelectric point (p*I*) in the proteins extracted from G13 ethanol-treated brain, indicating that this protein has more heterogeneity of

Table 4
Analysis of ethanol effects using Northern analysis

Target mRNA	Total RNA (μg)	3 μg polyA+ RNA	mRNA size (kb)	Ethanol[a]	Pair-fed	Chow-fed
G13						
PAP39	20	NP	2.0	105.8±10.5(3)	100.9±8.5(3)	100.0±9.6(3)
rpS6	20	NP	0.9	109.5±0.9(4)[b]	98.7±2.0(4)	100.0±9.6(4)
3A1	–	+	7.8	N/A	N/A	N/A
IP-10 (2C)	–	–	N/A	N/A	N/A	N/A
IP-10	–	+	1.5	N/A	N/A	N/A
3C	–	–	N/A	N/A	N/A	N/A
3A2	–	–	N/A	N/A	N/A	N/A
G16						
Cytb	5	NP	1.1	103.6±28.7(4)	106.5±2.6(4)	100.0±8.9(4)
Nd2	5	NP	1.0	114.0±28.4(4)	118.7±5.0(4)	100.0±13.5(4)
16*S* rRNA	5	NP	1.5	113.4±34.3(4)	102.3±3.9(4)	100.0±16.7(4)
NSP-A	30	NP	3.5	91.3±3.0(4)	86.8±2.4(4)	100.0±5.3(4)
NSP-C	30	NP	1.5	91.7±5.6(4)	85.0±1.6(4)	100.0±5.2(4)
18A2	15	NP	1.7	93.5±17.2(4)	81.0±2.3(4)	100.0±12.9(4)

[a] Values shown represent mean±S.E.M. of the ratio of target band density to loading control (converted to % chow-fed). The numbers in parentheses are the numbers of pools used in the comparison. Each pool is from four to six animals. +, detectable; –, undetectable; np, not performed; N/A, not applicable.
[b] Ethanol group was significantly different from pair-fed group ($P<0.01$).

charge in the ethanol group. This may be due to different protein glycosylation. All other proteins from G13 or G16 samples exhibited similar patterns between the two groups, suggesting that no other large differences in protein abundance were detectable between the ethanol and pair-fed groups.

To test whether ethanol treatment alters the phosphorylation status of total protein in fetal brain, Western analysis was carried out using anti-phospho-threonine and anti-phospho-tyrosine antibodies (Fig. 10). The same protein pools used in 2D electrophoresis were run on 8% SDS–PAGE. Each antibody detected eight bands. However, we observed no differences in the densities of each band between the ethanol and pair-fed groups using either anti-phospho-threonine or -tyrosine (data not shown).

4. Discussion

The goal of the present study was to identify ethanol-responsive genes in the fetal brain. We employed a well characterized chronic ethanol treatment paradigm and we confirmed that the amount of ethanol consumed fell within the range reported to produce neural damage (see Introduction). Moreover, we confirmed that this treatment paradigm altered the expression of CRBP-I as reported by others [7]. Thus, we confirmed that ethanol affected the developing brain in this experiment. To identify novel ethanol-responsive genes, we used the relatively non-biased methods of mRNA differential display and 2D gel electrophoresis, and focused on the cerebral cortex at G13 and G16 because these times coincide with known periods of deleterious effects of ethanol on the cerebral cortex. Although we identified a number of putative ethanol-responsive genes in the fetal cortex, those identified on G13 were not identified also on G16. Among the ethanol-responsive genes identified was NSP-A, which was elevated in the cortex of ethanol-exposed fetuses on G16. Considering that NSP-A expression is suppressed by thyroid hormone in the G16 fetus [17], we tested whether ethanol exposure affects the expression of other thyroid hormone-responsive genes in the G16 fetal cortex. However, ethanol exposure did not affect the expression of either Oct-1 or HES-1, two known thyroid hormone-responsive genes in the G16 cortex, indicating that interference with thyroid hormone action in the G16 brain is not likely to be a major contributing factor in the etiology of FAS. Although several ethanol-responsive genes were identified and confirmed in the present experiments, prenatal ethanol exposure produced very limited effects on gene expression as visualized by mRNA differential display. A consideration of the factors contributing to the limited number of genes identified in the present studies should help guide future experiments with similar goals.

It is surprising that genes identified on G13 as ethanol-responsive were not also found on G16. In particular, in the case of rpS6, one would predict that its functional role would likely be the same at both G13 and G16 and would likely be similarly regulated. There are two potential explanations for this observation. First, it is well known that the specific neurotoxic effects of ethanol depend on the developmental events occurring at the time of exposure [4]. Therefore, it is possible that the developmental events occurring in the cortex on G13 are more vulnerable to the deleterious ethanol exposure than those occurring on G16. However, this interpretation does not seem particularly compelling because the developmental events occurring on G13 are not entirely different from those occurring on

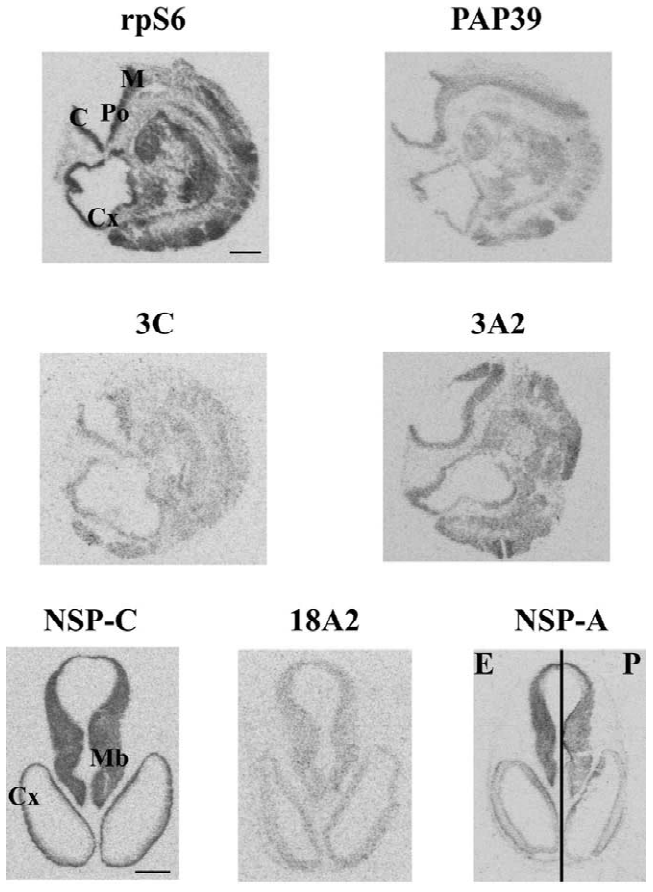

Fig. 5. Quantitative in situ hybridization to confirm the effect of prenatal ethanol on the expression of genes identified by mRNA differential display. Each panel except NSP-A represents the image on film following in situ hybridization on the section from the chow-fed group using the antisense probe targeting the mRNA labeled above the panel. The panel for NSP-A is a composite showing the ethanol-induced increase in its expression (see Table 5). Sense probes were applied to adjacent sections and produced negligible hybridization signal (data not shown). The regions indicated by letters were where the densities were measured and analyzed. Cx, cerebral cortex; C, cerebellum; Po, pons; M, medulla; Mb, midbrain; E, ethanol-treated; P, pair-fed. Scale bar=1 mm.

G16. At G13, the rat cerebral cortex is made up largely of the ventricular zone where cells are proliferating to expand the population of stem cells [9,61,62]. On G16, the ventricular zone is larger as a result of cell proliferation, and a larger proportion of cells are beginning to leave the cell cycle and migrate radially [31,62], but it is part of the same process that was initiated earlier.

In contrast, there may be two practical explanations for the differences we observed in the genes identified on G13 and G16. First, it is possible that the model of chronic ethanol exposure we employed produces a degree of tolerance that reduced the number of genes we identified during a single developmental window. For example, acute ethanol administration can alter the expression of proopiomelanocortin in the adult rat brain, but this effect disappears with chronic exposure [69]. Likewise, acute ethanol administration alters the expression of the gene encoding thyrotropin-releasing hormone in the hypothalamus, but this effect diminishes with chronic exposure [72,73]. Thus, it is possible that during the early stages of cortical development that occurs from about G13 to G16, the effect of chronic ethanol exposure on gene expression may diminish, though the ability of gene expression to respond to signaling events may be compromised. If this concept is correct, it would suggest that, in the presence of chronic alcohol exposure, specific developmental events may undergo a process of tolerance independent of tolerance that had been established in other brain regions or for other developmental processes. Second, it is possible that the increased cellular heterogeneity of the G16 cortex, compared to the G13 cortex, limits the ability of mRNA differential display to identify ethanol-responsive genes. These are not mutually exclusive possibilities, and they serve to illustrate that identifying ethanol-responsive genes in a developing tissue presents a number of theoretical and practical challenges.

It is potentially important that NSP-A expression is elevated in fetal brains exposed to alcohol for two reasons. First, this gene has been identified as an ethanol-responsive gene in adult brain by a number of groups working with rodents and humans. Schafer et al. found, using mRNA differential display followed by Northern analysis, that NSP-A is elevated by 26% in whole mouse brain exposed to ethanol vapors for 72 h [51]. Moreover, this effect was observed in a strain of mice genetically selected to be highly vulnerable to handling-induced seizures during ethanol withdrawal and was not observed in a resistant strain. Therefore, it is possible that the up-regulation of this gene by ethanol is related to the acquisition of physical dependence. In a follow-up study, this same group found that NSP-A (3.0 kb), and the splice variant NSP-C (1.4 kb), exhibit different responses to ethanol exposure in hippocampus, cerebellum and cortex [50]. These studies indicate that the specific effect of ethanol exposure on NSP expression is dependent upon genotype, brain region, and splice variant. Finally, Lewohl et al. [32] found, using a cDNA microarray, that NSP-A (reticulon) expression is about 40% higher in the postmortem brains of alcoholics compared to matched controls. Because several groups have identified NSP as an ethanol-responsive gene in the brain, it is possible that NSP will provide important insight into the deleterious effects of ethanol on brain function. Moreover, because these effects have been observed in both experimental animals and humans, it is possible that this gene will provide information that will have both experimental and clinical significance.

The function of NSP in the developing or adult brain is poorly understood; however, several observations about its expression and regulation may provide some insight. For example, NSP mRNA is localized to the axonal pole of neuronal cell bodies in the cerebral cortex [6,44]. This localization may serve to distribute the protein within large polarized cells, a phenomenon that is important for ner-

Table 5
Ethanol effects on gene expression using in situ hybridization

Probe	Region	Ethanol	Pair-fed	Chow-fed
G13				
rpS6	Cortex	102.3±6.7(5)	107.5±6.3(4)	100.0±2.0(5)
	Cerebellum	92.6±8.0(5)	94.4±6.8(4)	100.0±2.6(5)
	Pons	101.4±8.5(5)	101.4±8.1(4)	100.0±3.0(5)
	Medulla	96.5±9.2(5)	95.0±6.8(4)	100.0±3.4(5)
PAP39	Cortex	100.8±3.0(5)	95.7±5.7(5)	100.0±2.3(5)
	Cerebellum	97.0±9.9(4)	91.4±2.9(5)	100.0±2.1(4)
	Pons	104.3±3.8(5)	87.2±4.9(5)	100.0±6.8(5)
	Medulla	108.9±7.7(5)	105.0±4.7(5)	100.0±6.3(4)
3C	Cortex	92.8±8.2(5)	80.4±6.4(5)	100.0±6.9(5)
	Cerebellum	92.5±11.7(5)	85.1±7.2(5)	100.0±5.9(5)
	Pons	98.5±15.5(5)	82.0±9.8(5)	100.0±6.0(5)
	Medulla	103.8±13.6(5)	77.5±8.8(4)	100.0±4.3(5)
3A2	Cortex	109.3±3.7(5)	107.9±5.1(5)	100.0±4.7(5)
	Cerebellum	100.1±6.8(4)	111.5±7.6(5)	100.0±6.1(5)
	Pons	98.4±5.9(5)	99.1±5.6(5)	100.0±7.6(5)
	Medulla	95.5±4.8(5)	96.3±2.8(5)	100.0±6.9(5)
G16				
NSP-A	Cortex	108.6±5.1(6)[a]	91.6±4.2(7)	100.0±11.2(7)
	Midbrain	103.3±3.3(7)	89.0±5.4(7)	100.0±4.6(7)
NSP-C	Cortex	94.0±4.8(6)	91.7±6.5(6)	100.0±4.4(6)
	Midbrain	95.1±2.2(6)	97.5±2.2(6)	100.0±2.3(6)
18A2	Cortex	111.4±4.4(6)	91.3±5.6(6)	100.0±4.2(6)
	Midbrain	107.4±4.2(6)	94.4±4.5(6)	100.0±8.3(6)

Values shown represent mean±S.E.M. of film density (converted to % chow-fed). The numbers in parentheses are the numbers of fetuses in each group used in the comparison.
[a] Ethanol group was significantly different from pair-fed group ($P<0.05$).

vous system developmental and neuronal plasticity [59]. In addition, the carboxy-terminus of the NSP protein is integrated into the neuronal endoplasmic reticulum [55,64,65], indicating that NSP may be important for the formation of synaptic vesicles and the packaging or trafficking of secretory products [55]. Finally, NSP-A immunostaining has been found to be robust in growth cones, indicating that it may be involved in the rapid anterograde transport of membrane proteins [55]. Taken together, these observations suggest that NSP plays an important role in elements of neuronal development and function that may be deleterious if impacted by ethanol exposure.

In the present study, ethanol-induced changes in the abundance of NSP-A mRNA were revealed by in situ hybridization, but not by Northern analysis or by relative quantitative RT-PCR. We speculate that this apparent lack of consistency is due to the restricted spatial pattern of NSP-A expression in the early cortex. Specifically, NSP-A is selectively expressed in the ventricular zone of the fetal cortex [16]. However, the tissue collected for Northern analysis and for relative quantitative RT-PCR contained both ventricular zone and intermediate zone. This may have confounded our measurements because we used loading controls (actin or cyclophilin) that are expressed in both ventricular and intermediate zones. Therefore, the denominator we used reflects total tissue rather than ventricular zone; thus, different ratios of ventricular zone to total tissue in each of the dissected brains may have increased the variance and obscured effects of ethanol on NSP-A. This may also have obscured effects of ethanol on NSP-A using RT-PCR and Western analysis.

Considering that NSP-A expression in the G16 cortex is elevated in rat fetuses derived from dams made hypothyroid [16,17], it is possible that ethanol blocked thyroid hormone action, thus increasing NSP-A expression. Several independent lines of evidence support this possibility. Ethanol administration causes a decline in circulating levels of maternal thyroid hormone [15,43,46], thus, depriving the fetus of the sole source of thyroid hormone until the fetal thyroid begins to function on G17 [20]. Additionally, chronic ethanol exposure reduces the expression of thyroid hormone receptor (TRα1) mRNA in the fetal brain [52]. Finally, exogenous thyroxine can ameliorate the effects of chronic ethanol on the organization of cerebellar Purkinje cells [42]. Considering these observations, we independently tested whether ethanol could affect the expression of other, known, thyroid hormone-responsive genes in the G16 cortex in a manner consistent with the hypothesis that ethanol blocks thyroid hormone action globally. We focused on the genes encoding Oct-1 [17] and HES-1 [24]. However, because neither of these genes was affected by ethanol exposure, we conclude that there is no formal evidence that the deleterious effects of ethanol on

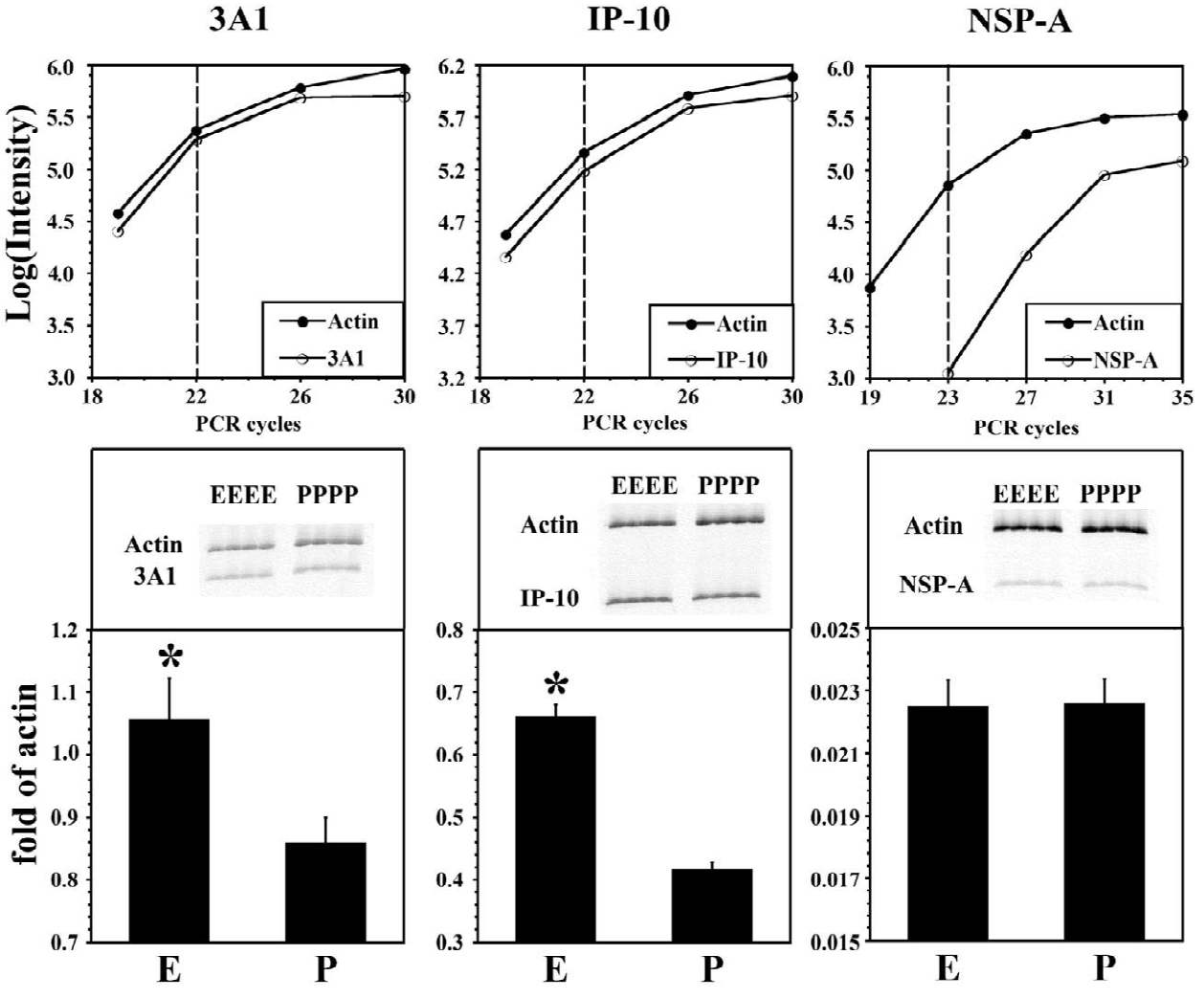

Fig. 6. Relative quantitative RT-PCR analysis for 3A1, IP-10 and NSP-A. Upper panel. An initial experiment was carried out to identify the number of cycles in the linear quantitative phase to amplify both target and actin. The numbers of cycles indicated by the dashed vertical lines were chosen for analysis (see Table 1). Middle panel. Polyacrylamide gels of the relative quantitative RT-PCR products. Each lane corresponds to a RNA pool from three animals. The lanes indicated as the same treatment group were duplicate RT-PCR reactions. Each experiment was run at least three times. Lower panel. Bar charts for the relative quantitative RT-PCR analysis. Bars represent mean band density relative to β-actin±S.E.M. *Significantly different from pair-fed group ($P<0.05$). E, ethanol-treated; P, pair-fed.

brain development are mediated broadly by interference with thyroid hormone action.

The observation that the mRNA encoding rpS6 is elevated by ethanol exposure in the fetal brain is potentially important considering the report of Lang et al. [29] who showed that acute ethanol exposure reduced the abundance of rpS6 protein in rat myocardium. Thus, the increase in rpS6 mRNA induced by chronic ethanol in the present study suggests that rpS6 may be engaged in neuroadaptation to ethanol. However, the effect of ethanol on rpS6 expression is also quite modest, exhibiting an 11% increase revealed by Northern analysis. Our failure to confirm the effect of ethanol on rpS6 expression by in situ hybridization suggests the possibility that the ethanol effect is region-specific within the developing brain and the mid-sagittal sections employed in the present study did not intersect with the regions affected. The ribosomal S6 protein is a phosphoprotein important in regulating translation efficiency [19]. Our observation that rpS6 mRNA is elevated in fetal brain following chronic ethanol exposure may indicate that protein translation is enhanced. However, this protein is activated by a kinase (eIF4F), the activity of which is inhibited by ethanol [29]. These observations lead us to suggest the possibility that the increased expression of rpS6 may be related to a compensatory mechanism following chronic ethanol exposure.

Two reports demonstrate that three mitochondrial genes, Nd4, 12*S* rRNA and 16*S* rRNA, are ethanol-responsive in human or rat brain [11,18]. However, the present study indicated that Cytb, 16*S* rRNA and ND2 may not be ethanol-responsive in the entire fetal brain, because we had considerable difficulty in confirming ethanol regulation

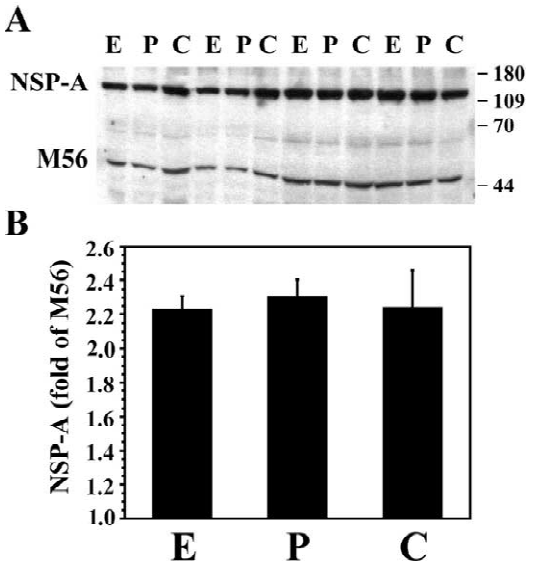

Fig. 7. NSP-A Western analysis. (A) Film autoradiogram of NSP-A Western blot. The bands for NSP-A and M56 are indicated on the left. Each lane corresponds to 15 μg of protein pooled from four to six animals. The lanes indicated as the same treatment group are independent protein pools from the group. Molecular weight standards are indicated on the right. The light bands between NSP-A and M56 bands may be caused by non-specific binding of NSP-A antibody or the secondary antibody. (B) Quantification of band density shown in (A) using NIH image 1.62. Bars represent mean band density relative to M56±S.E.M. No significant effect of treatment was observed. E, ethanol-treated; P, pair-fed; C, chow-fed.

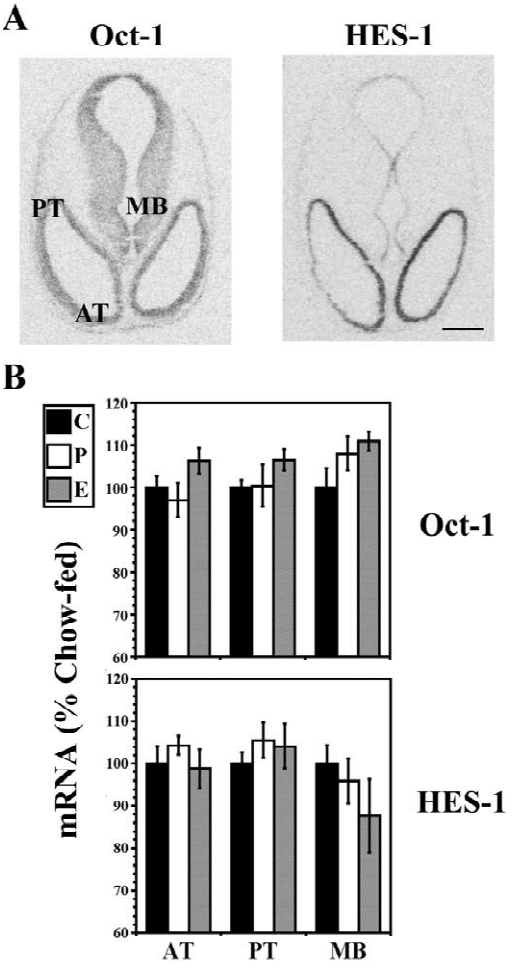

Fig. 8. In situ hybridization analysis of Oct-1 and HES-1 expression. (A) Film autoradiograms following in situ hybridization on G16 brain sections from chow-fed group using the cRNA probes for Oct-1 and HES-1. Letters highlight the regions in which density was measured and analyzed. (B) Quantitative analysis of the in situ hybridization of Oct-1 and HES-1. Bars represent mean film density±S.E.M. (converted to % chow-fed group for comparisons). The animal number was six for each group. Data were analyzed using a one-way analysis of variance for each brain area. AT, anterior telencephalon; PT, posterior telencephalon; MB, midbrain; E, ethanol group; P, pair-fed group; C, chow-fed group. Scale bar=1 mm.

using focused hybridization techniques. It is also important to recognize our finding that these genes exhibit specific polymorphisms in DNA sequence, that they were distributed non-randomly in G16 animals, and that these genetic polymorphisms were identified by the mRNA differential display. Thus, it is reasonable to be cautious in interpreting the effects of fetal alcohol exposure on mitochondrial gene expression. Fortunately, the two mitochondrial polymorphisms found in the present study were not large and did not likely produce physiological differences among animals in the different groups.

Our finding that chronic ethanol exposure increased the expression of IP-10 in the fetal rat brain on G16 may be quite significant. Specifically, IP-10 is expressed selectively in CD8+ T-cells that infiltrate the central nervous system during periods of a variety of pathological situations. For example, IP-10 is elevated in scrapie-infected brain tissue [48]. In addition, IP-10 is elevated in the serum and CSF of patients with active multiple sclerosis [21]. IP-10 may be a chemoattractant for myelin protein-specific CD4+ T-cells. Thus, the observation that IP-10 is elevated in fetal brain following chronic exposure to ethanol indicates that a pathological processes is occurring and this could, in itself, indicate a mechanism by which alcohol exposure interferes with myelination during development [71].

Considering the profound and stereotypic effects of fetal ethanol exposure on brain development in the rodent model, we were surprised to find such limited effects of fetal ethanol exposure on gene expression using mRNA differential display. Therefore, we considered the possibility that ethanol exposure has more robust effects on expression of protein, using 2D gel electrophoresis. Again, we found virtually no evidence that chronic ethanol exposure altered protein levels in the fetal cortex. Thus, considering the well-known effects of ethanol on cell signaling such as cAMP [47], we evaluated the effects of ethanol on the abundance of phosphorylated proteins. Similarly, we found no evidence that chronic ethanol exposure affected protein phosphorylation.

Several aspects of the present results suggest that the

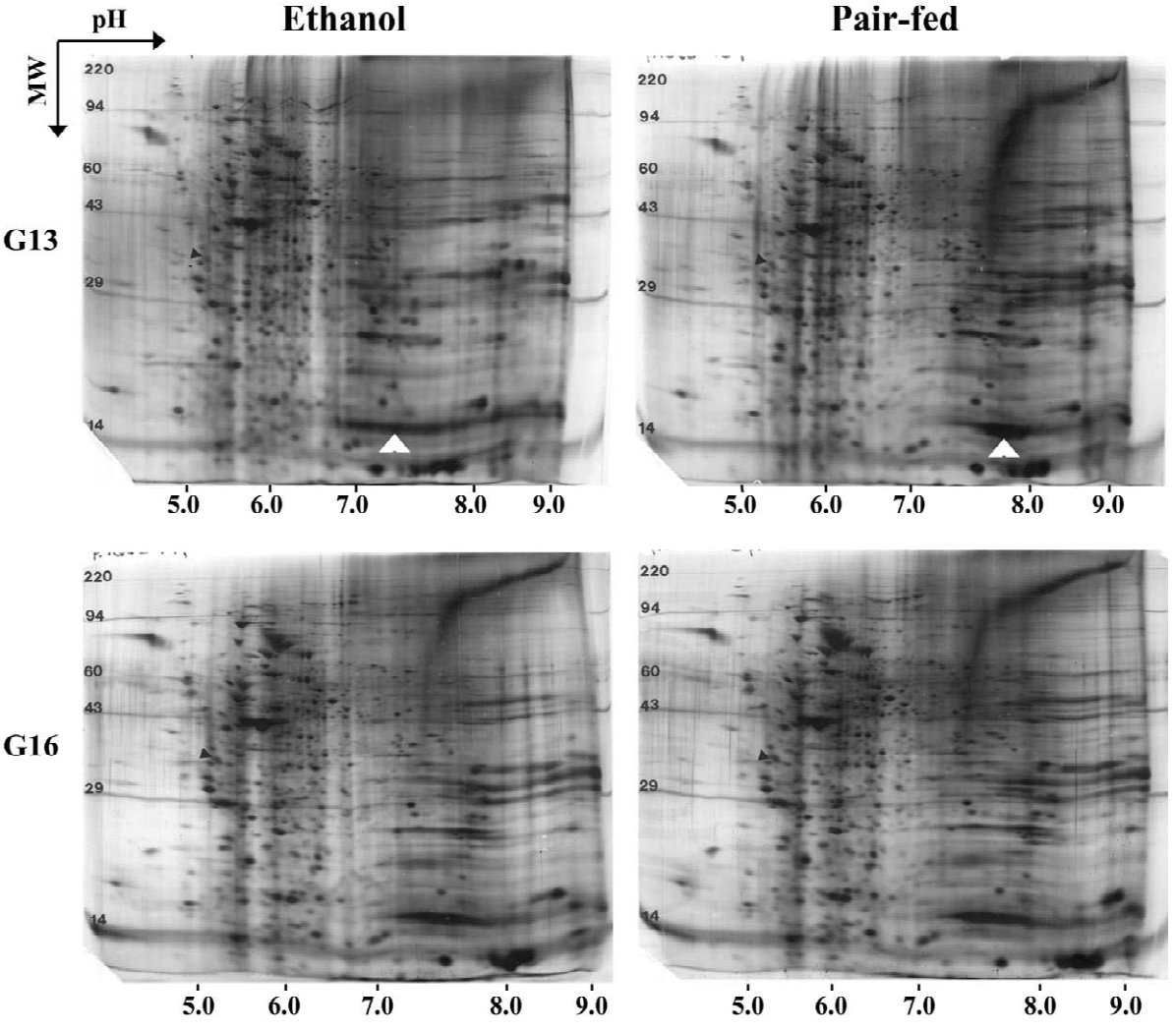

Fig. 9. Silver-stained 2D gels of proteins isolated from G13 or G16 brains; 50 μg of protein pooled from three animals was loaded to each gel. The white arrowheads highlight bands of different p*I* ranges between ethanol and the pair-fed group. In each gel, the black arrowhead illustrates tropomyosin (MW 33 000, p*I* 5.2) used as an internal control. Molecular weight standards and pH gradient are indicated on the left and bottom, respectively.

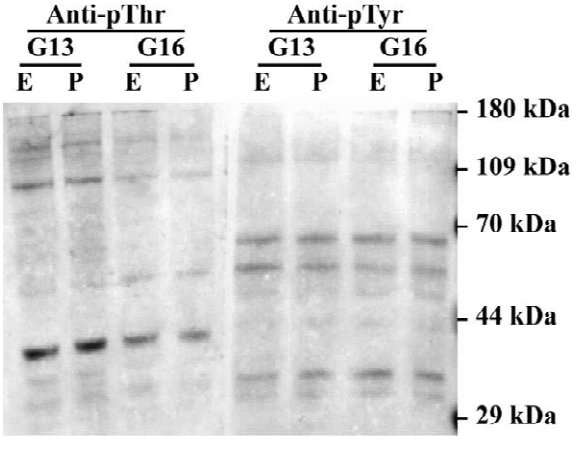

Fig. 10. Film autoradiogram of Western blots with either anti-phosphor-threonine (Anti-pThr) or -tyrosine (Anti-pTyr). Molecular weight standards are indicated on the right. Each lane represents 15 μg of protein pooled from three animals. E, ethanol-treated; P, pair-fed.

limited effects of ethanol exposure are a feature of ethanol exposure itself and not the mRNA differential display or protein analyses per se. Specifically, in the present study, although we sampled only about 10% (G13) and 30% (G16) of the rat genome, based on the number of primers used, the rate at which gene fragments appeared to be differentially represented among treatment groups on the initial gel was approximately 0.01–0.03%. This estimate is based on the observation that our acrylamide gel resolved about 75 gene fragments for each primer pair. This rate is over 10-fold lower than our previous study in which we used mRNA differential display to identify thyroid hormone responsive genes in the G16 cortex using the same sets of primers [17]. Moreover, other labs appear to report a similarly low percentage of putative ethanol-responsive genes. For example, Lee et al. [30] reported about a 0.09% rate of appearance of ethanol-responsive genes in G11 mouse embryo using an acute ethanol treatment, and Fan et

al. [18] reported a rate of about 0.03% in human alcoholics.

Taken together, these data suggest that mRNA differential display identifies a low number of differentially expressed genes following ethanol exposure. We predict that a significant cause is that chronic ethanol exposure may produce a limited quantitative effect on gene expression, which may be obscured by the 40 cycles of amplification during the mRNA differential display procedure. Ethanol appears to exert effects that are less than twofold [23,53,57,58,63] and they are brain region-specific [50,53]. Therefore, the challenge for future studies will be to perform non-biased genomic methods on selected brain areas without using amplification. However, it is important to recognize that these studies were performed selectively on female fetuses and therefore, we have not precluded that these effects are sex-specific.

Acknowledgements

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