BRAIN RESEARCH 1458 (2012) 18–33

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BRAIN RESEARCH

Research Report

Long-term alterations to the brain transcriptome in a maternal voluntary consumption model of fetal alcohol spectrum disorders

Morgan L. Kleiber[a], Benjamin I. Laufer[a], Elise Wright[b], Eric J. Diehl[a], Shiva M. Singh[a,b,*]

[a]Molecular Genetics Unit, Department of Biology, University of Western Ontario, London, Ontario, Canada N6A 5B7
[b]Program in Neuroscience, University of Western Ontario, London, Ontario, Canada N6A 5B7

ARTICLE INFO

Article history:
Accepted 9 April 2012
Available online 17 April 2012

Keywords:
Fetal alcohol spectrum disorder
Mouse
Brain
Microarray
Gene expression
Alcohol

ABSTRACT

Many women continue to consume low to moderate quantities of alcohol during pregnancy, which can result in the variable neurobehavioural effects in the absence of physiological abnormalities that characterize fetal alcohol spectrum disorders (FASD). Previously, we reported that a mouse model for FASD based on voluntary maternal ethanol consumption throughout gestation resulted in offspring that showed mild developmental delay, anxiety-related traits, and deficits in spatial learning. Here, we extend this model by evaluating the gene expression changes that occur in the adult brain of C57BL/6J mice prenatally exposed to ethanol via maternal preference drinking. The results of two independent expression array experiments indicate that ethanol induces subtle but consistent changes to global gene expression. Gene enrichment analysis showed over-represented gene ontology classifications of cellular, embryonic, and nervous system development. Molecular network analysis supported these classifications, with significant networks related to cellular and tissue development, free radical scavenging, and small molecule metabolism. Further, a number of genes identified have previously been implicated in FASD-relevant neurobehavioural phenotypes such as cognitive function (*Ache, Bcl2, Cul4b, Dkc1, Ebp, Lcat, Nsdh1, Sstr3*), anxiety (*Bcl2*), attention deficit hyperactivity disorder (*Nsdh1*), and mood disorders (*Bcl2, Otx2, Sstr3*). The results suggest a complex residual "footprint" of neurodevelopmental ethanol exposure that may provide a new perspective for identifying mechanisms that underlie the life-long persistence of FASD-related cognitive and behavioural alterations, including potential targets for treatment.

* *Corresponding author at:* Molecular Genetics Unit, Department of Biology, University of Western Ontario, London, Ontario, Canada N6A 5B7. Fax: +1 519 661 3935.
E-mail address: ssingh@uwo.ca (S.M. Singh).

Abbreviations: FASD, fetal alcohol spectrum disorder; B6, C57BL/6J; G, gestational day; P, postnatal day; IPA, Ingenuity Pathway Analysis; GO, gene ontology

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doi:10.1016/j.brainres.2012.04.016

1. Introduction

Prenatal alcohol exposure is a well-established cause of detrimental physiological and neurological effects and remains one of the most significant causes of developmental disability in North America (Bertrand et al., 2005; Sampson et al., 1997). Yet, a substantial number of women continue to consume alcohol throughout pregnancy at low to moderate levels (Chang et al., 2011; Walker et al., 2011; Whitehead and Lipscomb, 2003). While maternal alcohol consumption in moderation may not result in the severe cognitive and craniofacial abnormalities that characterize Fetal Alcohol Syndrome (FAS), it is less clear how this drinking pattern may contribute to the more subtle and heterogeneous range of neurobehavioural effects observed in less-extreme fetal alcohol spectrum disorders (FASD).

Previously, we have described a mouse model of fetal alcohol spectrum disorders involving voluntary maternal ethanol consumption throughout gestation (Kleiber et al., 2011). Pregnant C57BL/6J (B6) mice consumed ethanol at doses representative of moderate drinking, resulting in offspring that showed a range of behavioural phenotypes relevant to FASD, including delayed neuromuscular development, anxiety, and spatial learning deficits. A similar model, including phenotypic effects, has since been repeated by Brady et al. (Brady et al., 2011). These results support that this model is relevant to human prenatal alcohol exposure and may be useful in evaluating the developmental processes involved.

Ethanol is known to disrupt neurodevelopmental gene expression (Green et al., 2007; Hard et al., 2005; Zhou et al., 2011). This is likely to lead to abnormal brain development and, subsequently, long-lasting changes to the brain's molecular architecture. Studies have explored the effect of prenatal alcohol exposure on candidate genes such as transcription factors (Aronne et al., 2008; Rifas et al., 1997), neurotransmitter-related molecules (Toso et al., 2005, 2006), and genes that control processes such as cell proliferation, survival, and maturation (Camarillo and Miranda, 2008; Fitzgerald et al., 2011; Lee et al., 2008; Miller, 2003). Many of these experiments, however, have involved high doses of alcohol at a single specific time, stressful administration procedures, and examine only the acute effects of ethanol on gene expression. Also, the relationship between gene expression changes and potential long-term effects of fetal alcohol exposure, including FASD-related phenotypes, are difficult to interpret due to variability in experimental paradigms. Examination of genome-wide changes to gene expression that are present in the brains of individuals that show phenotypic alterations may provide a better route to correlate alcohol-induced disruptions to neurodevelopmental programming with FASD behavioral outcomes.

This study seeks to extend the mouse model of FASD previously presented by our laboratory (Kleiber et al., 2011). Using two independent expression array experiments, we evaluated the genes, molecular networks, and biological functions altered in the adult brain of mice prenatally exposed to alcohol. It is hoped that these results may provide a new approach to assess the effects of ethanol on neurodevelopment by examining the long-term outcome of exposure. Finally, we suggest that ethanol causes subtle but specific changes to neurodevelopmental programming that can be associated with a number of FASD-relevant phenotypes.

2. Results

2.1. *Maternal preference drinking and offspring weight*

Ethanol consumption over gestation and parturition for dams from experiment 1 and 2 whose progeny were used for subsequent microarray analyses is shown in Fig. 1. Mean ethanol consumption of mothers from experiment 1 was 15.80 g/kg±2.89 and 15.25 g/kg±2.82 for experiment 2. Although average consumption values varied across days with a trend towards less consumption from G10 to 20 (second trimester equivalent), repeated-measures ANOVA indicated no significant difference in ethanol consumption between females in experiment 1 or 2 ($F_{1,7}=0.005$, $p=0.94$). There were no significant weight differences between ethanol and water-consuming females (data not shown). While there did appear to be a trend for ethanol-exposed litters to have less pups, the difference did not reach statistical significance (control=6.0±0.57; ethanol=4.9±0.65; $t=0.223$, $df=35$, n.s.). All pups were morphologically normal, consistent with our previous results (Kleiber et al., 2011).

2.2. *Detection and analysis of differentially expressed genes*

We examined the whole gene expression profile of adult (P70) mice that had been exposed to alcohol via maternal preference drinking in two independent experiments. The gene expression results from both experiments were characterized by subtle alterations to a number of transcripts, with few large fold-changes (Table 1). Due to the low fold-changes observed, we focused on only those genes that were identified in both independent trials and showed alterations in the same direction (upregulated or downregulated as compared to controls in both experiments). This resulted in 163 differentially expressed genes, with 73 up-regulated and 90 down-regulated transcripts (Tables 2 and 3, respectively). Fold changes ranged from 1.87 (*Vmn1r48*) to −1.80 (*Mrpl42*) in trial 1, while trial 2 showed more subtle effects with fold changes from 1.34 (*Phf11*) to −1.32 (*Zfp518a*).

To evaluate the consistency in gene expression patterns between experiments 1 and 2, a hierarchical cluster analysis was performed using Partek Genomics Suite software v.6.6 (Partek Inc., St. Louis MO, USA) using the signal ratios of the differentially expressed genes in all ten control and ethanol arrays. The heat map representing clustering by normalized probe intensity across arrays (Fig. 2) shows that the ethanol and control arrays, regardless of experiment, cluster together and show more differences between treatment groups than across within-treatment arrays. An overall pattern of differential gene expression emerges that is consistent and reproducible across the two experiments. This suggests that, although ethanol exposure by voluntary maternal consumption may be variable from litter to litter with respect to timing and dose, this model produces a subtle, reproducible, and long-lasting effect on gene expression in the adult brain.

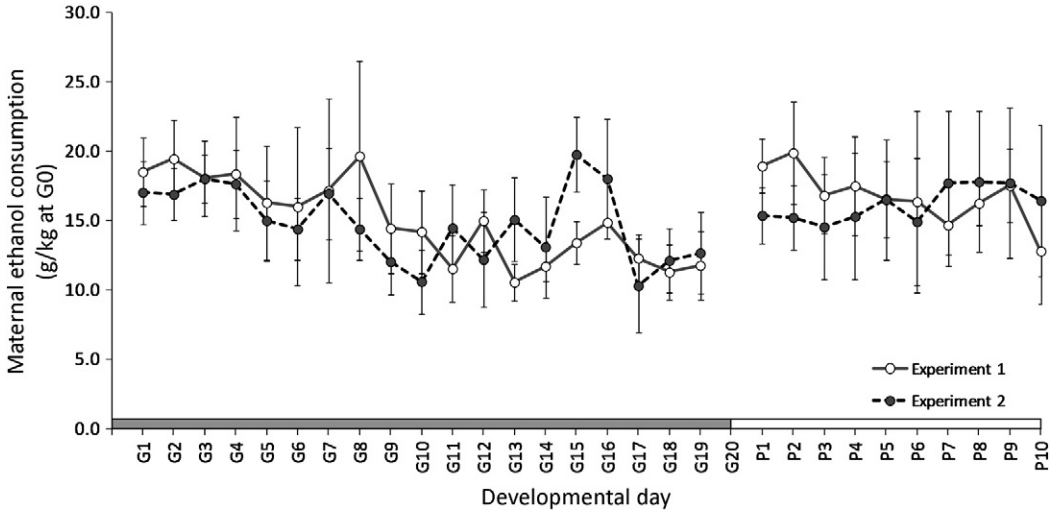

Fig. 1 – Mean daily ethanol consumption (±SEM) of pregnant mice during gestational (G) and early postpartum (P) periods for females whose offspring were used in experiment 1 (n=3) and experiment 2 (n=5).

2.3. *Gene functions and network analysis*

The overlapping genes identified from experiment one and two were used in the "core analysis" function within Ingenuity Pathway Analysis software v.9 (Ingenuity Systems, Redwood, CA, USA) to evaluate the effects of maternal ethanol consumption on gene networks, pathways, and biological processes. This analysis was used to generate a list of gene ontology processes of genes that were differentially expressed in the brains of control and neurodevelopmentally-exposed adult mice. The most significant non-redundant gene ontology terms generated are shown in Table 4. The terms suggested disruption of developmental processes including initiation of cellular differentiation, arrest in gastrulation, neurogenesis, growth of axons, and cell morphogenesis. Disturbances in genes related to molecular transport, post-transcriptional modifications (involving microRNA species), DNA replication, and vitamin and mineral metabolism were also identified. Interestingly, the largest grouping within a gene ontology category was 16 genes that were annotated to play some role in developmental disorders. We examined these genes in greater detail and found that a number of them had relevance to FASD-related endophenotypes such as cognitive function, learning, anxiety, and craniofacial and growth abnormalities (Table 5). Further, a number of these genes are already implicated in neurological features such as cognitive dysfunction, mood disorders, Alzheimer's, Parkinson's, and schizophrenia, which also possess endophenotypes that mimic some FASD-relevant abnormalities.

We further evaluated the relationship between the differentially expressed genes using a gene network analysis, which evaluates the associations between such genes as well as annotated interactions with other genes and molecules, using IPA. The top three networks identified contained 15, 14, and 13 focus molecules (differentially expressed genes). These networks also showed a great deal of overlap in terms of relevant molecules and included a number of genes with fundamental developmental and cellular processes (Fig. 3). In total, 20 of the network focus molecules were down-regulated and 22 were up-regulated. Further, examining the functions of the networks individually reinforced the gene ontology analysis, with these three networks illustrating potential disruption in gene networks important for (1) cellular development, tissue development, and embryonic development (Fig. 4A) (2) free radical scavenging, cellular growth and proliferation (Fig. 4B), (3) and lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism (Fig. 4C), respectively.

Table 1 – Number of differentially expressed transcripts identified at varying fold-change stringencies.

		Fold-change threshold		
		1.1-fold	1.2-fold	1.3-fold
$p<0.05$	Up	73	7	3
	Down	90	7	0
	Total	163	14	3
$p<0.01$	Up	21	1	1
	Down	19	2	0
	Total	40	3	1

3. Discussion

Individuals prenatally exposed to alcohol show a long-lasting phenotype of altered cognitive function that characterizes FASD (Nash et al., 2008; Riley et al., 2011; Stade et al., 2011). We have previously shown that mice exposed to alcohol throughout neurodevelopment by voluntary maternal alcohol consumption also show subtle behavioural changes relevant to FASD (Kleiber et al., 2011), supporting this as a paradigm that models the effects of moderate drinking throughout pregnancy. In this study, we show that this maternal preference drinking model of prenatal alcohol exposure leads to subtle

Table 2 – Genes showing increased transcript levels in the brain of mice exposed to ethanol during neurodevelopment by maternal continuous preference drinking.

GenBank accession no.	Gene symbol	Gene name	Chr.	Fold-change		*p*-value	
				Trial 1	Trial 2	Trial 1	Trial 2
NM_026180	Abcg8	ATP-binding cassette, sub-family G (WHITE), member 8	17: E4	1.17	1.13	0.0077	0.0023
NM_026185	Abhd15	Abhydrolase domain containing 15	11: B5	1.23	1.14	0.0122	0.0189
NM_206935	Arl9	ADP-ribosylation factor-like 9	5: C3	1.15	1.17	0.0253	0.0068
NM_024184	Asf1b	ASF1 anti-silencing function 1 homolog B (S. cerevisiae)	8: C3	1.22	1.11	0.0036	0.0096
NM_007493	Asgr2	Asialoglycoprotein receptor 2	11: B3	1.25	1.17	0.0193	0.0118
NM_001080965	Aurkc	Aurora kinase C	7: A2-A3	1.28	1.12	0.0060	0.0224
NM_009741	Bcl2	B-cell leukemia/lymphoma 2	1: E2.1	1.23	1.15	0.0067	0.0176
NM_001037939	Bglap	Bone gamma carboxyglutamate protein	3: F1	1.36	1.22	0.0215	0.0094
XR_104700	C130023O10Rik	RIKEN cDNA C130023O10 gene	2: E3	1.12	1.14	0.0402	0.0090
NM_027112	Capns2	Calpain, small subunit 2	8: C5	1.26	1.18	0.0074	0.0010
NM_009809	Casp14	Caspase 14	10: C1	1.14	1.11	0.0236	0.0081
NM_199225	Cd300c	CD300C antigen	11: E2	1.16	1.11	0.0391	0.0418
NM_027839	Ceacam20	Carcinoembryonic antigen-related cell adhesion molecule 20	7: A3	1.18	1.11	0.0057	0.0125
NM_001003951	Ces5a	Cescarboxylesterase 5A	8: C5	1.14	1.14	0.0049	0.0266
NM_009922	Cnn1	Calponin 1	9: A2-4	1.22	1.15	0.0142	0.0407
NM_001177379	Cpeb2	Cytoplasmic polyadenylation element binding protein 2	5: B	1.24	1.13	0.0167	0.0076
NM_009910	Cxcr3	Chemokine (C-X-C motif) receptor 3	X: D	1.19	1.13	0.0199	0.0252
NM_001081148	Cyp2b23	Cytochrome P450, family 2, subfamily b, polypeptide 2	7: A3	1.32	1.17	0.0083	0.0034
NM_019978	Dclk1	Doublecortin-like kinase 1	3: D	1.34	1.18	0.0066	0.0247
NM_139222	Defb15	Defensin beta 15	8: A4	1.18	1.11	0.0165	0.0005
NM_001039566	Defb30	Defensin beta 30	14: D1	1.32	1.15	0.0015	0.0244
NM_010080	Dspp	Dentin sialophosphoprotein	5: E5	1.32	1.13	0.0109	0.0010
NM_001033344	Dusp27	Dual specificity phosphatase 27 (putative)	1: H2.3	1.20	1.12	0.0305	0.0035
NM_017389	Ear14	Eosinophil-associated, ribonuclease A family, member 14	14: C1	1.13	1.11	0.0119	0.0389
NM_001033817	F730021E23Rik	RIKEN cDNA F730021E23 gene	10: A3	1.21	1.19	0.0404	0.0394
NM_029172	Fam164b	Family with sequence similarity 164, member B	10: A2	1.22	1.13	0.0099	0.0385
NM_008148	Gp5	Glycoprotein 5 (platelet)	16: B2	1.15	1.11	0.0165	0.0260
NM_001162955	Gpr15	G protein-coupled receptor 15	16: C1	1.42	1.16	0.0002	0.0100
NM_001145096	Hhla1	HERV-H LTR-associating 1	15: D1-2	1.18	1.10	0.0017	0.0159
NM_001024720	Hmcn1	Hemicentin 1	1: G1	1.14	1.13	0.0088	0.0015
NM_010469	Hoxd4	Homeobox D4	2: D	1.13	1.11	0.0485	0.0046
NM_133698	Hrnr	Hornerin	3: -	1.14	1.11	0.0226	0.0243
NM_018805	Hs3st3b1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	11: B3	1.24	1.14	0.0255	0.0104
NM_001111336	Hsd3b4	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 4	11: F2.2	1.18	1.13	0.0394	0.0215
NM_001164528.	Ildr2	Immunoglobulin-like domain containing receptor 2	1: H2.3	1.16	1.11	0.0162	0.0039
NM_001205076	Jph2	Junctophilin 2	2: H3	1.15	1.17	0.0376	0.0431
NM_008475	Krt4	Keratin 4	15: F3	1.11	1.10	0.0441	0.0003
NM_033073	Krt7	Keratin 7	15: F2	1.11	1.10	0.0282	0.0121
NM_029613	Krtap4-7	Keratin associated protein 4–7	11: D	1.35	1.10	0.0022	0.0014
NM_026253	Lrrc18	Leucine rich repeat containing 18	14: B	1.16	1.11	0.0097	0.0099
NM_144797	Metrnl	Meteorin, glial cell differentiation regulator-like	11: E2	1.10	1.10	0.0392	0.0256
NR_035481	Mir1956	MicroRNA mir-1956	-	1.16	1.11	0.0373	0.0147
NR_029721	Mir196a-1	MicroRNA 196a-1	11: -	1.35	1.15	0.0034	0.0049
NR_029722	Mir196a-2	MicroRNA 196a-2	15: -	1.72	1.16	0.0002	0.0027
NR_029591	Mir204	MicroRNA 204	19: -	1.21	1.14	0.0289	0.0149
NR_029807	Mir222	MicroRNA 222	X: -	1.14	1.11	0.0239	0.0160
NR_029961	Mir449a	MicroRNA 449a	19: -	1.37	1.24	0.0379	0.0040
NR_029818	Mir9-3	MicroRNA 9-3	7: -	1.34	1.11	0.0002	0.0352
NM_173437	Nav1	Neuron navigator 1	1: E4	1.23	1.12	0.0028	0.0047
NM_001033207	Nlrc5	NLR family, CARD domain containing 5	8: C5	1.27	1.28	0.0352	0.0135
NM_054049	Osr2	Odd-skipped related 2 (Drosophila)	15: B3.1	1.18	1.11	0.0214	0.0128
NM_172603	Phf11	PHD finger protein 11	14: C3	2.26	1.34	0.0028	0.0312
NM_008852	Pitx3	Paired-like homeodomain transcription factor 3	19: C3	1.13	1.12	0.0380	0.0239
NM_011174	Prh1	Proline rich protein HaeIII subfamily 1	6: G1	1.20	1.13	0.0093	0.0461
NM_001033343	Sec31b	Sec31 homolog B (*S. cerevisiae*)	19: C3	1.13	1.10	0.0366	0.0010
NM_147779	Sftpb	Surfactant associated protein B	6: C1	1.19	1.13	0.0056	0.0043
NM_001081370	Shank2	SH3/ankyrin domain gene 2	7: F5	1.20	1.12	0.0014	0.0068

(continued on next page)

Table 2 (continued)

GenBank accession no.	Gene symbol	Gene name	Chr.	Fold-change		p-value	
				Trial 1	Trial 2	Trial 1	Trial 2
NM_001042760	Slc22a18	Solute carrier family 22 (organic cation transporter), member 18	7: F5	1.20	1.13	0.0340	0.0351
NM_009218	Sstr3	Somatostatin receptor 3	15: E1	1.33	1.15	0.0118	0.0108
NM_198170	Szt2	Seizure threshold 2	4: D2.1	1.15	1.14	0.0371	0.0066
NM_207028	Tas2r126	Taste receptor, type 2, member 126	6: B2.1	1.24	1.24	0.0203	0.0358
NM_009325	Tbxa2r	Thromboxane A2 receptor	10: C1	1.29	1.13	0.0065	0.0163
NM_009347	Tecta	Tectorin alpha	9: A5.1	1.17	1.12	0.0481	0.0338
NM_153109	Tgif2lx1	TGFB-induced factor homeobox 2-like, X-linked 1	X: E1	1.11	1.12	0.0490	0.0025
NM_009405	Tnni2	Troponin I, skeletal, fast 2	7: F5	1.20	1.17	0.0069	0.0342
NM_029726	Trdn	Triadin	10: A4	1.53	1.31	0.0408	0.0445
NM_053218	Vmn1r48	Vomeronasal 1 receptor 48	6: D1	1.87	1.14	0.0001	0.0090
NM_001081449	Vmn2r54	Vomeronasal 2, receptor 54	7: A1	1.47	1.33	0.0042	0.0014
BC050813	Vwa3b	von Willebrand factor A domain containing 3B	1: B	1.17	1.14	0.0022	0.0080
NM_009522	Wnt3a	Wingless-related MMTV integration site 3A	11: B1.3	1.22	1.10	0.0001	0.0313
NM_144783	Wt1	Wilms tumor 1 homolog	2: E	1.32	1.10	0.0016	0.0037
NM_001080943	Zdhhc22	Zinc finger, DHHC-type containing 22	12: D2	1.13	1.10	0.0194	0.0049
NM_001167936	Zyg11a	zyg-11 homolog A (C. elegans)	4: C7	1.19	1.12	0.0302	0.0190

Fold-change represents ethanol-exposed transcript levels to water-only controls; Chr. = chromosomal location.

yet long-term alterations to brain gene expression that can be associated with FASD-related phenotypes.

The ethanol consumption of the dams used in this study was consistent with values for C57BL/6J (B6) mice and previous findings involving maternal consumption models (Allan et al., 2003; Boehm et al., 2008; Brady et al., 2011; Kleiber et al., 2011; Rodgers and McClearn, 1962; Yoneyama et al., 2008). The dosage of 10% ethanol consumed daily was also consistent with volumes consumed by non-pregnant B6 mice (Kleiber et al., 2011), which has been established to represent alcohol preference values (% ethanol as a proportion of total liquid consumed) of 75–80% (Yoneyama et al., 2008). Although precise blood alcohol concentrations (BAC) were not determined in this study due to variations in consumption patterns over the 24 hour period and to avoid additional stress to pregnant dams, the volume of alcohol consumed by the females was comparable to other consumption models that produce peak BACs of approximately 80–120 mg/dl per day (Allan et al., 2003; Boehm et al., 2008; Brady et al., 2011). As B6 mice are a high-preferring inbred strain, they will typically consume enough ethanol to produce significant neurocellular damage to a developing fetus and be relevant to children who are "high-risk" for FASD (Coles et al., 1985; Young and Olney, 2006). Further, it is likely that the BACs reached by the female mice are pharmologically relevant given the behavioural alterations observed in the offspring of these mice (Kleiber et al., 2011).

Since the phenotypic effects on the offspring obtained using these models are variable and modest, we expected that ethanol's effect on the transcript levels within the brain of the adult offspring would be as well. We acknowledge that any analysis of brain gene expression following experimental manipulation during neurodevelopment may be influenced by changes in maternal behaviour, which has been shown to be able to cause long-term changes in behaviourally-relevant genes (Champagne et al., 2003; Weaver et al., 2006). We chose to continue ethanol availability until P10 to reduce the potential effect of ethanol withdrawal on maternal behaviours, given that this is after the peak of synapotgenesis (Dobbing and Sands, 1979; Ikonomidou et al., 2000) and the time when maternal care has shown to be the most predictable and critical (Champagne et al., 2003, 2007). While we and others have not found differences in maternal behaviour between ethanol-consuming and control mice using voluntary consumption models (Allan et al., 2003; Boehm et al., 2008; Kleiber et al., 2011), this factor remains as a potential source of some of the observed changes in gene expression in the adult offspring of ethanol-consuming females. Also, despite studies that suggest that mice given a choice between 10% ethanol and water do not differ from control (water-only) in their caloric intake (Allan et al., 2003; Fernandez et al., 1983; Wiebold and Becker, 1987), it is also possible that nutritional differences may have affected neurodevelopment and, subsequently, adult brain gene expression.

To facilitate the identification of altered transcripts, we adopted a strategy of pooling RNA samples to reduce litter and individual-specific noise, replicating our experiment using two populations of ethanol-exposed and control offspring generated by different researchers at different times, and focusing our attention on those genes that were identified by both independent experiments. Our results suggest that the maternal voluntary preference drinking paradigm is responsible for distinct but subtle changes in global gene expression in the young adult (P70) brain of ethanol-exposed offspring (Table 1, Fig. 2). There were also a number of differences in the genes identified in each independent experiment, possibly due a number of variables such as variability in ethanol dosage and timing of exposure, the timing of our analysis (during adulthood rather than immediate), and the use of whole brain tissue, which can make the detection of region-specific or low-expression transcripts more difficult. While these factors introduced variability between the two experiments, we were able to identify a number of genes that were

Table 3 – Genes showing decreased transcript levels in the brain of mice exposed to ethanol during neurodevelopment by maternal continuous preference drinking.

GenBank accession no.	Gene symbol	Gene name	Chr.	Fold-change		*p*-value	
				Trial 1	Trial 2	Trial 1	Trial 2
NM_025862	Acad8	Acyl-Coenzyme A dehydrogenase family, member 8	9: A4	−1.17	−1.18	0.0142	0.0058
NM_009599	Ache	Acetylcholinesterase	5: G2	−1.31	−1.15	0.0164	0.0464
NM_026792	Agpat5	1-Acylglycerol-3-phosphate O-acyltransferase 5 (lysophosphatidic acid acyltransferase, epsilon)	8: A3	−1.18	−1.11	0.0070	0.0274
NM_133981	Alg9	Asparagine-linked glycosylation 9 homolog (yeast, alpha 1,2 mannosyltransferase)	9: A5.3	−1.22	−1.16	0.0019	0.0153
NM_023210	Anp32e	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	3: F2.1	−1.21	−1.13	0.0194	0.0263
NM_026512	Bphl	Biphenyl hydrolase-like (serine hydrolase, breast epithelial mucin-associated antigen)	13: A4	−1.22	−1.12	0.0135	0.0098
NM_025686	Brf2	BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like	8: A2	−1.16	−1.21	0.0200	0.0237
NM_029951	C330007P06Rik	RIKEN cDNA C330007P06 gene	X: A3.3	−1.13	−1.14	0.0314	0.0020
NM_145595	Cbr4	Carbonyl reductase 4	8: B3.1	−1.18	−1.16	0.0272	0.0191
NM_001025576	Ccdc141	Coiled-coil domain containing 141	2: C3	−1.20	−1.13	0.0071	0.0314
NM_023731	Ccdc86	Coiled-coil domain containing 86	19: B	−1.39	−1.14	0.0022	0.0064
NM_028771	Ccdc97	Coiled-coil domain containing 97	7: A3	−1.16	−1.12	0.0326	0.0073
NM_139291	Cdc26	Cell division cycle 26	4: B3	−1.18	−1.12	0.0088	0.0077
NM_145825	Cetn4	Centrin 4	3: B	−1.25	−1.17	0.0368	0.0040
NM_181588	Cmbl	Carboxymethylenebutenolidase-like (Pseudomonas)	15: B2	−1.42	−1.19	0.0031	0.0195
NM_016856	Cpsf2	Cleavage and polyadenylation specific factor 2	12: F1	−1.15	−1.14	0.0239	0.0014
NM_212450	Ctdspl2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase like 2	2: E5	−1.39	−1.11	0.0000	0.0480
NM_001110142	Cul4b	Cullin 4B	X: A2	−1.21	−1.13	0.0004	0.0077
NM_023118	Dab2	Disabled homolog 2 (Drosophila)	15: A	−1.18	−1.21	0.0461	0.0011
NM_007833	Dcn	Decorin	10: C3	−1.12	−1.18	0.0308	0.0050
NM_001166597	Dgkz	Diacylglycerol kinase zeta	2: E1	−1.35	−1.11	0.0040	0.0132
NM_177564	Dhrs11	Dehydrogenase/reductase (SDR family) member 11	11: C	−1.12	−1.11	0.0209	0.0006
NM_001030307	Dkc1	Dyskeratosis congenita 1, dyskerin homolog (human)	X: A7.3	−1.15	−1.20	0.0209	0.0048
NM_026332	Dnajc19	DnaJ (Hsp40) homolog, subfamily C, member 19	3: F1	−1.15	−1.15	0.0407	0.0014
NM_172508	Dse	Dermatan sulfate epimerase	10: B1	−1.16	−1.10	0.0245	0.0144
NM_026981	Dtwd1	DTW domain containing 1	2: F2	−1.18	−1.12	0.0377	0.0203
NM_007897	Ebf1	Early B-cell factor 1	11: B1.1	−1.16	−1.14	0.0366	0.0108
NM_007898	Ebp	Phenylalkylamine Ca2+ antagonist (emopamil) binding protein	X: A1.1	−1.23	−1.13	0.0013	0.0072
NM_001146350	Eng	Endoglin	2: B	−1.21	−1.18	0.0399	0.0034
NM_015744	Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	15: D2	−1.11	−1.13	0.0395	0.0471
NM_010222	Fkbp7	FK506 binding protein 7	2: C3	−1.18	−1.15	0.0037	0.0066
NM_021355	Fmod	Fibromodulin	1: E4	−1.17	−1.17	0.0123	0.0109
NM_008043	Frat1	Frequently rearranged in advanced T-cell lymphomas	19: C3	−1.22	−1.16	0.0333	0.0283
NM_028058	Fundc1	FUN14 domain containing 1	X: A2	−1.30	−1.11	0.0064	0.0162
NM_008095	Gbas	Glioblastoma amplified sequence	5: G1.3	−1.13	−1.11	0.0467	0.0034
NM_198169	Gmeb2	Glucocorticoid modulatory element binding protein 2	2: H4	−1.19	−1.13	0.0305	0.0012
NM_001038015	Gnpda2	Glucosamine-6-phosphate deaminase 2	5: D	−1.10	−1.11	0.0248	0.0004
NM_010271	Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	15: F1-3	−1.12	−1.12	0.0096	0.0013
NM_144788	Hectd1	HECT domain containing 1	12: C1	−1.21	−1.13	0.0139	0.0426
NM_172535	Iqub	IQ motif and ubiquitin domain containing	6: A3.1	−1.13	−1.20	0.0121	0.0124
NM_011317	Khdrbs1	KH domain containing, RNA binding, signal transduction associated 1	4: D2.2	−1.10	−1.11	0.0081	0.0073
NM_172781	Klhl4	Kelch-like 4 (Drosophila)	X: E1	−1.42	−1.19	0.0006	0.0347
NM_010655	Kpna2	Karyopherin (importin) alpha 2	11: E1	−1.20	−1.13	0.0341	0.0059
NM_015771	Lats2	Large tumor suppressor 2	14: C3	−1.21	−1.15	0.0272	0.0245
NM_008490	Lcat	Lecithin cholesterol acyltransferase	8: D3	−1.11	−1.11	0.0380	0.0197
NM_025681	Lix1	Limb expression 1 homolog (chicken)	17: A3.1	−1.19	−1.11	0.0333	0.0481
NM_001007573	Maneal	Mannosidase, endo-alpha-like	4: D2.2	−1.13	−1.10	0.0423	0.0430
NM_026310	Mrpl18	Mitochondrial ribosomal protein L18	17: A2	−1.27	−1.13	0.0195	0.0023
NM_026065	Mrpl42	Mitochondrial ribosomal protein L42	10: C2	−1.80	−1.24	0.0024	0.0176
NM_025450	Mrps17	Mitochondrial ribosomal protein S17	5: F	−1.31	−1.21	0.0198	0.0188
NM_010270	Mrps33	Mitochondrial ribosomal protein S33	6: B4	−1.14	−1.14	0.0439	0.0125
NM_028024	Nkiras2	NFKB inhibitor interacting Ras-like protein 2	11: D	−1.16	−1.13	0.0291	0.0053
NM_010941	Nsdhl	NAD(P) dependent steroid dehydrogenase-like	X: A7.3	−1.16	−1.13	0.0250	0.0469

(continued on next page)

Table 3 (continued)

GenBank accession no.	Gene symbol	Gene name	Chr.	Fold-change		p-value	
				Trial 1	Trial 2	Trial 1	Trial 2
NM_028091	Osgepl1	O-sialoglycoprotein endopeptidase-like 1	1: C1.1	−1.27	−1.12	0.0055	0.0144
NM_144841	Otx2	Orthodenticle homolog 2 (Drosophila)	14: C1	−1.37	−1.26	0.0052	0.0158
NM_025571	Pam16	Presequence translocase-asssociated motor 16 homolog (S. cerevisiae)	16: A1	−1.34	−1.13	0.0070	0.0024
NM_011864	Papss2	3′-phosphoadenosine 5′-phosphosulfate synthase 2	19: C1	−1.32	−1.15	0.0078	0.0451
NM_028376	Pfn4	Profilin family, member 4	12: A1.1	−1.13	−1.15	0.0498	0.0355
NM_001081274	Pgd	Phosphogluconate dehydrogenase	4: E2	−1.17	−1.18	0.0271	0.0057
NM_023625	Plbd2	Phospholipase B domain containing 2	5: F	−1.31	−1.12	0.0000	0.0214
NM_027002	Polr2d	Polymerase (RNA) II (DNA directed) polypeptide D	18: B2	−1.31	−1.25	0.0203	0.0002
NM_146141	Ppa2	Pyrophosphatase (inorganic) 2	3: G3	−1.23	−1.14	0.0135	0.0059
NM_011224	Pygm	Muscle glycogen phosphorylase	19: A	−1.21	−1.14	0.0493	0.0085
NM_009708	Rnd2	Rho family GTPase 2	11: C-D	−1.22	−1.12	0.0230	0.0212
NM_011277	Rnf2	Ring finger protein 2	1: G2	−1.13	−1.11	0.0287	0.0237
NM_026991	Sat2	Spermidine/spermine N1-acetyl transferase 2	11: B4	−1.18	−1.16	0.0418	0.0007
NM_153053	Sf3b4	Splicing factor 3b, subunit 4	3: F2.1	−1.20	−1.11	0.0225	0.0006
NM_011774	Slc30a4	Solute carrier family 30 (zinc transporter), member 4	2: E5	−1.12	−1.12	0.0064	0.0186
NM_175090	Slc31a1	Solute carrier family 31, member 1	4: C1-2	−1.19	−1.11	0.0122	0.0167
NM_175283	Srd5a1	Steroid 5 alpha-reductase 1	13: C1	−1.15	−1.19	0.0190	0.0306
NM_019635	Stk3	Serine/threonine kinase 3 (Ste20, yeast homolog)	15: B3.3	−1.27	−1.13	0.0011	0.0013
NM_008188	Thumpd3	THUMP domain containing 3	6: E3	−1.36	−1.14	0.0018	0.0227
NM_026433	Tmem100	Transmembrane protein 100	11: C	−1.14	−1.12	0.0108	0.0144
NM_199199	Tmem199	Transmembrane protein 199	11: B5	−1.29	−1.11	0.0061	0.0221
NM_001101483	Tmem22	Transmembrane protein 22	9: E3.3-4	−1.26	−1.17	0.0010	0.0456
NM_178745	Tmem229b	Transmembrane protein 229B	12: C3	−1.25	−1.27	0.0314	0.0005
NM_177601	Tmem60	Transmembrane protein 60	5: A3	−1.20	−1.20	0.0244	0.0095
NM_015767	Ttpa	Tocopherol (alpha) transfer protein	4: A3	−1.31	−1.17	0.0026	0.0371
NM_025773	Ube2w	Ubiquitin-conjugating enzyme E2W (putative)	1: A3	−1.20	−1.11	0.0006	0.0192
NM_011671	Ucp2	Uncoupling protein 2 (mitochondrial, proton carrier)	7: E3	−1.26	−1.17	0.0009	0.0056
NM_001033202	Usp30	Ubiquitin specific peptidase 30	5: F	−1.15	−1.12	0.0205	0.0123
NM_011515	Vamp7	Vesicle-associated membrane protein 7	X: -	−1.19	−1.13	0.0355	0.0484
NM_026664	Vps53	Vacuolar protein sorting 53 (yeast)	11: B4	−1.10	−1.12	0.0278	0.0146
NM_134139	Wdr74	WD repeat domain 74	17: A	−1.13	−1.10	0.0092	0.0024
NM_028118	Wdsub1	WD repeat, SAM and U-box domain containing 1	2: C3	−1.22	−1.11	0.0160	0.0268
NM_009533	Xrcc5	X-ray repair complementing defective repair in Chinese hamster cells 5	1: E	−1.27	−1.11	0.0027	0.0307
NM_009544	Zfp105	Zinc finger protein 105	9: F4	−1.29	−1.20	0.0133	0.0227
NM_028319	Zfp518a	Zinc finger protein 518A	19: D1	−1.27	−1.32	0.0159	0.0019
NM_001001187	Zfp738	Zinc finger protein 738	13: B3	−1.34	−1.12	0.0056	0.0054
NM_207541	Zfp81	Zinc finger protein 81	17: B1	−1.18	−1.18	0.0144	0.0212

Fold-change represents ethanol-exposed transcript levels to water-only controls; Chr. = chromosomal location.

differentially expressed in both trials one and two. These genes may point to more broad categories of processes that are both directly and indirectly disrupted by prenatal ethanol exposure. We evaluated these genes and their biological functions using gene ontology (GO), gene network, and pathway analysis.

Results indicating long-term and genome-wide changes to specific genes upon fetal alcohol exposure are not well-documented, but it appears that the genes identified by both trials do not represent a random distribution of biological functions. There is an over-representation of genes that function in neurodevelopmental processes (Table 4), which supports that they may reflect pathways that are disrupted by prenatal ethanol exposure, even at low doses. Specifically, the identified GO categories involve central nervous system development, as well as RNA and DNA regulation and small molecule metabolism. The functional annotations of the genes are also pertinent to physiological abnormalities observed in the brain of individuals with FASD, such as disruptions of neurogenesis, axonal development, and cell differentiation (Bartlett et al., 1994; Guerri, 1998; Klintsova et al., 2007; Lindsley et al., 2003; Rice and Barone, 2000). Interestingly, several of these genes are known to contribute to neural system development and maintenance, with the disruption of their expression or function associated with cognitive phenotypes in mice and humans (Table 5). Disruptions in a number of these genes are implicated in neurodevelopmental disorders, leading to some endophenotypes that are relevant to FASD. For example, alterations in *Ache, Bcl2, Cul4b, Dkc1, Nsdhl,* and *Sstr3* have been implicated in learning, memory, and general cognitive dysfunction or delay (du Souich et al., 2009; Einstein et al., 2010; Knight et al., 1999; Niu et al., 2007; Tarpey et al., 2009; Xiao et al., 2011). Also, many genes have been associated with human psychopathologies such as anxiety and bi-polar disorders (*Bcl2*), attention deficit hyperactivity disorder (*Nsdh1*), Alzheimer's disease (*Ache*, *Lcat*, *Sstr3*, *Wt1*) and schizophrenia (*Pitx3*) (Bergman et al., 2010; Demeester et al., 2000; du Souich et al., 2009; Einat et

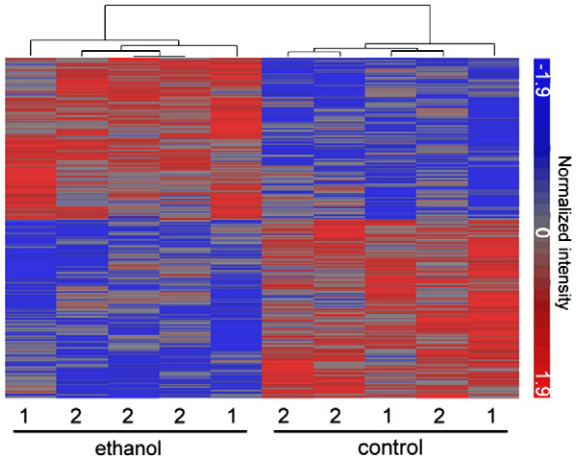

Fig. 2 – Hierarchical clustering of differentially expressed genes based on normalized intensity of the probe sets. Arrays generated from control and ethanol-exposed offspring are indicated, as is the experiment (1 or 2).

al., 2005; Grouselle et al., 1998; Lovell et al., 2003; Machado-Vieira et al., 2011; Schliebs and Arendt, 2010). While each of these genes show correlations to FASD-relevant phenotypes in both mice and humans, their specific roles in neurodevelopment, including how they may be disrupted by alcohol and the specific consequences of this, remain to be evaluated.

The results from the Ingenuity® Pathway network analysis support the GO classifications. The 'cellular, tissue, and embryonic development' gene network (Fig. 3A) is centralized around the gene *wingless-related MMTV integration site 3A* (*Wnt3a*), which acts in the canonical Wnt/β-catenin pathway to regulate neural development (David et al., 2010; Munji et al., 2011) including axon guidance and hippocampal formation (Lee et al., 2000; Purro et al., 2008). Also interesting are *dyskeratosis congenita 1, dyskerin homolog (human)* (*Dkc1*) (Fig. 3B), known for its involvement in a number of disease phenotypes (see Table 5), and *ectonucleotide pyrophosphatase/phosphodiesterase 2* (*Enpp2*), essential for cranial neural tube closure and defining the midbrain-hindbrain boundary (Koike et al., 2011). Fig. 3C includes a number of developmentally important molecules that were also identified within the GO 'developmental disorder' cluster, such as *Bcl2*, *Ebp*, and *Wt1*. Indeed, a number of genes within this group have well-established roles neuronal differentiation such as *homeobox D4* (*Hoxd4*) (Kobrossy et al., 2006), *ring finger protein 2* (*Rnf2*) (Roman-Trufero et al., 2009), and *Rho family GTPase 2* (*Rnd2*), a protein also essential for cortical neuron migration (Heng et al., 2008). The complementary function of many of these genes within the three gene networks support that these networks have collaborative and even overlapping functions and can be merged together to provide a more comprehensive picture of the interactions between a number of ethanol-affected genes and the molecules that act with them (Fig. 2).

Due to the subtlety of the observed gene expression changes (less than 1.3-fold), we opted to use a fold-change stringency that, we acknowledge, is unconventionally low. However, the replication of the experiment in two populations of mice and the results of the gene ontology and pathway analyses suggest that the genes identified may represent some of the developmental processes that can be disrupted by moderate maternal alcohol consumption. Also, while this model seeks to replicate chronic but moderate maternal alcohol consumption throughout all three trimesters, the third human trimester equivalent occurs postnatally in mice. This likely means fetuses were subjected to higher blood alcohol concentrations during human equivalent trimesters one and two than three, given that ethanol transfer through milk feeding is inefficient (Swiatek et al., 1986). These results, then, may reflect more disruption of earlier neurodevelopmental processes (neurulation, migration, differentiation) than later processes (synaptogenesis) (Rice and Barone, 2000).

It should be noted that the observed expression changes are the sum of primary and secondary effects of ethanol exposure, and there are several potential mechanisms that may facilitate altered gene expression in response to maternal

Table 4 – Significant gene ontology (GO) categories and molecular function annotations for genes differentially expressed in the brains of mice exposed to ethanol by maternal continuous preference drinking versus controls at postnatal day 70.

GO Category	Functions annotation	*p*-value	Genes
Cellular development	Initiation of differentiation of cell lines	0.00017	*Ebf1, Wt1*
Embyonic development	Arrest in gastrulation of mice	0.00034	*Dab2, Rnf2*
Molecular transport	Transport of drug	0.00085	*Ebp, Slc22a18*
RNA Post-transcriptional modification	Binding of RNA	0.00158	*Khdrbs1, Wt1*
Nervous system development and function	Neurogenesis	0.00200	*Blc2, Dclk1 Otx2, Vamp7, Wnt3a, Wt1, Xrcc5*
Nervous system development and function	Growth of axons	0.00224	*Bcl2, Vamp7, Wnt3a, Wt1*
Organ development	Morphogenesis of organ	0.00407	*Bcl2, Dcn, Eng, Otx2, Pitx3, Sftpb, Wnt3a, Wt1*
DNA replication, recombination, and repair	Double-stranded DNA break repair of tumor cell lines	0.00571	*Kpna2, Xrcc5*
Developmental disorder	Developmental disorder	0.00584	*Ache, Bcl2, Cnn1, Cul4b, Dkc1, Ebp, Lcat, Nsdhl, Osr2, Otx2, Papss2, Pitx3, Slc31a1, Sstr3, Stk3, Wt1*
Visual system development and function	Differentiation of retinal cells	0.00732	*Ebf1, Otx2*
Vitamin and mineral metabolism	Synthesis of sterol	0.00734	*Abcg8, Ebp, Lcat*
Vitamin and mineral metabolism	Metabolic process of terpenoid	0.00754	*Abcg8, Ebp, Lcat, Nsdhl, Srd5a1, Ttpa*

Table 5 – FASD-relevant phenotypes associated with alterations in genes categorized with a developmental disorder involvement.

Symbol	Gene name	Direction of change	Associated phenotype	References
Ache	Acetylcholinesterase	↓	Learning, spatial memory, neuronal degeneration, Alzheimer's disease	(Schliebs and Arendt, 2010; Xiao et al., 2011)
Bcl2	B-cell leukemia/lymphoma 2	↑	Learning, bi-polar disorder, anxiety	(Einat et al., 2005; Machado-Vieira et al., 2011; Niu et al., 2007)
Cnn1	Calponin 1	↑	Locomotor activity, neuron density	Bannai et al. (2003)
Cul4b	Cullin 4B	↓	X-linked intellectual disability	Tarpey et al. (2009)
Dkc1	Dyskeratosis congenital 1, dyskerin homolog (human)	↓	Learning, growth retardation, ataxia, mental retardation, developmental delay, microcephaly, cerebellar hypoplasia, telomere dysfunction (Hoyeraal–Hreidarsson syndrome, X-linked dyskeratosis congenital)	(Aalfs et al., 1995; Hreidarsson et al., 1988; Knight et al., 1999)
Ebp	Phenylalkylamine Ca2+ antagonist (emopamil) binding protein	↓	Growth abnormalities, craniofacial defects decreased cognitive function (X-linked chondrodysplasia punctata)	(Derry et al., 1999; Sutphen et al., 1995)
Lcat	Lecithin cholesterol acyltransferase	↓	Lipid metabolism, proteinuria (Norum disease), cognitive function, dementia, Alzheimer's disease, multiple sclerosis	(Albers et al., 1992; Demeester et al., 2000; Holleboom et al., 2011)
Nsdh1	NAD(P) dependent steroid dehydrogenase-like	↓	X-linked intellectual disability, aggression, attention deficit hyperactivity disorder, irritability, cerebral cortical malformations, microcephaly, craniofacial malformations, seizures (CK syndrome), CHILD syndrome	(du Souich et al., 2009; Konig et al., 2000)
Osr2	Odd-skipped related 2 (Drosophila)	↑	Facial clefts, palate morphogenesis	Lan et al. (2004)
Otx2	Orthodenticle homolog 2 (Drosophila)	↓	Brain malformations, craniofacial malformations, microphthalmia, pituitary hypoplasia, bipolar disorder	(Acampora et al., 1995; Nolen et al., 2006; Sabunciyan et al., 2007; Simeone et al., 1993)
Papss2	3′-phosphoadenosine 5′-phosphosulfate synthase 2	↓	Skeletal development (spondyloepimetaphyseal dysplasia), adrenocortical dysfunction	(Faiyaz ul Haque et al., 1998; Noordam et al., 2009)
Pitx3	Paired-like homeodomain transcription factor 3	↑	Dopamine neuron development, Parkinson's disease, schizophrenia	(Ardayfio et al., 2008; Bergman et al., 2010; Fuchs et al., 2009; Wallen and Perlmann, 2003)
Slc31a1	Solute carrier family 31, member 1	↓	Neural tissue cell fate determination	Haremaki et al. (2007)
Sstr3	Somatostatin receptor 3	↑	Cognitive function, memory impairment, depression, Alzheimer's disease	(Agren and Lundqvist, 1984; Einstein et al., 2010; Grouselle et al., 1998)
Stk3	Serine/threonine kinase 3 (Ste20, yeast homolog)	↓	Regulation of apoptosis, cell prolifieration, mitotic chromosome alignment	(Chiba et al., 2009; O'Neill et al., 2004)
Wt1	Wilms tumor 1 homolog	↑	Neuronal degeneration, Alzheimer's disease	Lovell et al. (2003)

drinking. The first is that ethanol induces a change in the cellular composition of the brain, resulting from ethanol-induced apoptotic neurodegeneration of susceptible cell types (Ikonomidou et al., 2000; Olney et al., 2002). Given that our results represent whole brain changes, this cannot be ruled out and would represent an indirect effect resulting from an altered balance of brain cell types. However, due to the low ethanol dosages achieved via voluntary maternal consumption, as well as evidence that individuals both with and without neurophysiological abnormalities can be equally cognitively impaired (Mattson et al., 1998; Spadoni et al., 2007), it is likely that this is not the only explanation for ethanol's long-term effects on brain gene expression. Alternatively, ethanol may act to alter processes such as proliferation, migration, differentiation, and cell-cell signaling. The results from the gene network analysis support this, and there is substantial evidence in the literature that ethanol is able to disrupt these processes in developing brain tissue without inducing cell death (Camarillo and Miranda, 2008; Guerri, 1998; Rice and Barone, 2000). If this is the case, then the behavioural and gene expression changes observed are indirect: a residual "footprint" of a neurodevelopmental disturbance. This is not a new idea and similar hypotheses have been suggested for a number of other neurodevelopmental disorders such as schizophrenia and autism (Demjaha et al., 2011; Dufour-Rainfray et al., 2011). This idea is appealing in the case of "spectrum" disorders, since it is reasonable that variations in the timing and extent of neurodevelopmental disruption could lead to a range of phenotypes of varying severity. Finally, it is possible that ethanol affects neurodevelopmental epigenetic patterning, resulting in long-term regulatory changes that may persist over the lifetime of an individual. This would also suggest changes in methylation status, chromatin configuration, or microRNA (miRNA)

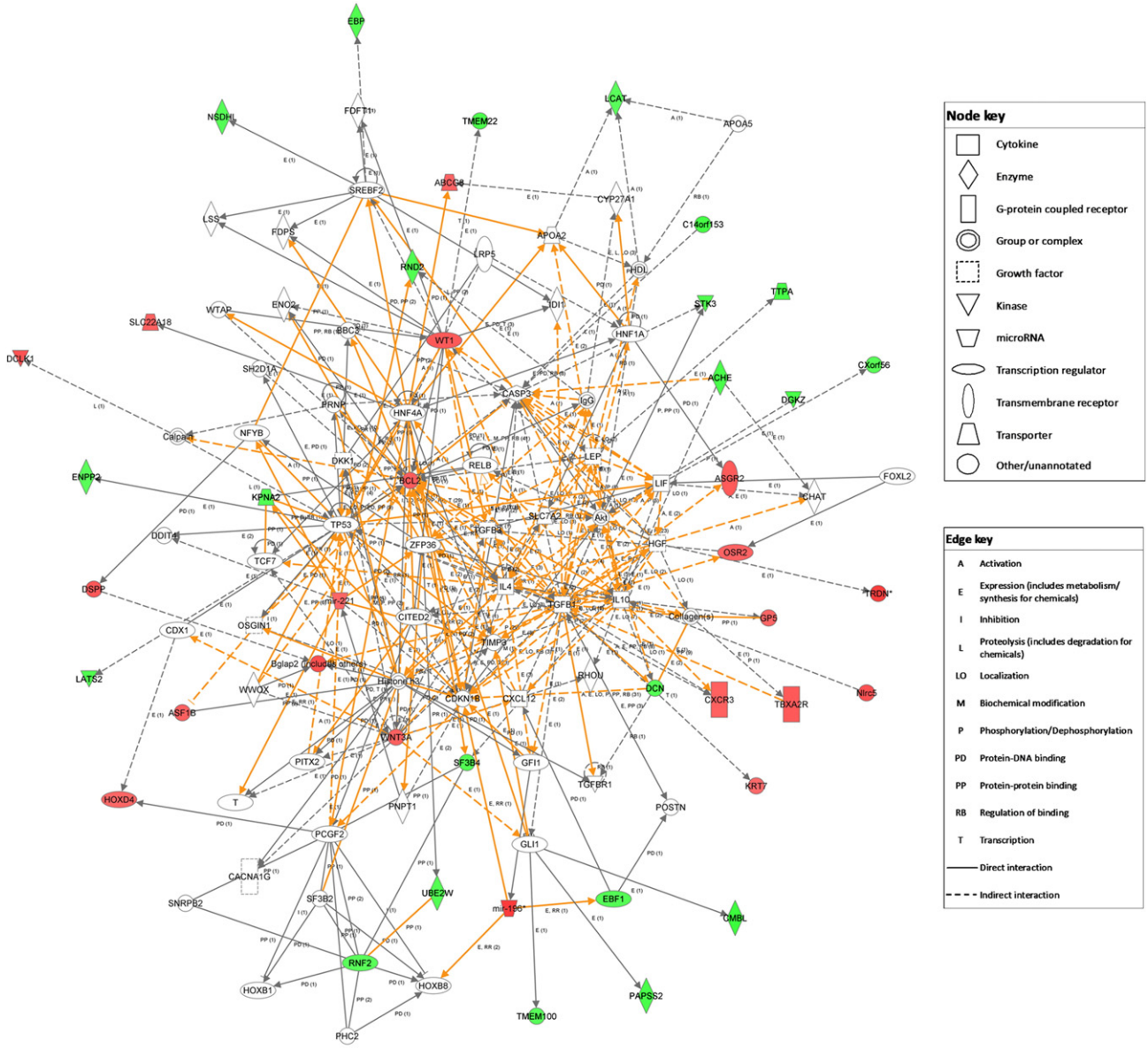

Fig. 3 – Ingenuity Pathway Analysis (IPA) network diagram illustrating annotated interactions between genes affected by prenatal alcohol exposure. Network represents the merged view of the three significant subnetworks categorized by IPA function: "Cellular Development, Tissue Development, Embryonic Development," "Free Radical Scavenging, Cellular Growth and Proliferation," and "Lipid metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism." Molecules identified as upregulated (red) or downregulated (green) in ethanol-exposed mice as compared to controls are indicated. Full gene names for gene symbols are as listed in Tables 2 and 3. Number of reports supporting interactions indicated on edges are in parentheses following the interaction abbreviation.

expression that correlate with the gene expression changes in the adult brain. Our results include a number of miRNA transcripts, which play critical roles in neurodevelopment (Gao, 2010; Krichevsky et al., 2003; Saba and Schratt, 2010). Further, it has been shown that prenatal ethanol exposure can alter miRNA expression in the developing brain (Sathyan et al., 2007). Ethanol can also alter genomic methylation profiles (Liu et al., 2009). This effect is persistent, with changes to methylation profiles of specific genes able to last into adulthood and exert phenotypic effects (Kaminen-Ahola et al., 2010). How these changes occur and are maintained is unknown, but epigenetic alterations are an interesting avenue for further research. Not only would this have implications for the persistence of certain phenotypes across the life of an individual prenatally exposed to alcohol, but it introduces the possibility of transgenerational effects.

We have demonstrated that voluntary, continuous preference drinking by pregnant C57BL/6J mice has a subtle yet long-term effect on the neurotranscriptome of alcohol-exposed offspring. We have identified a set of genes and pathways that

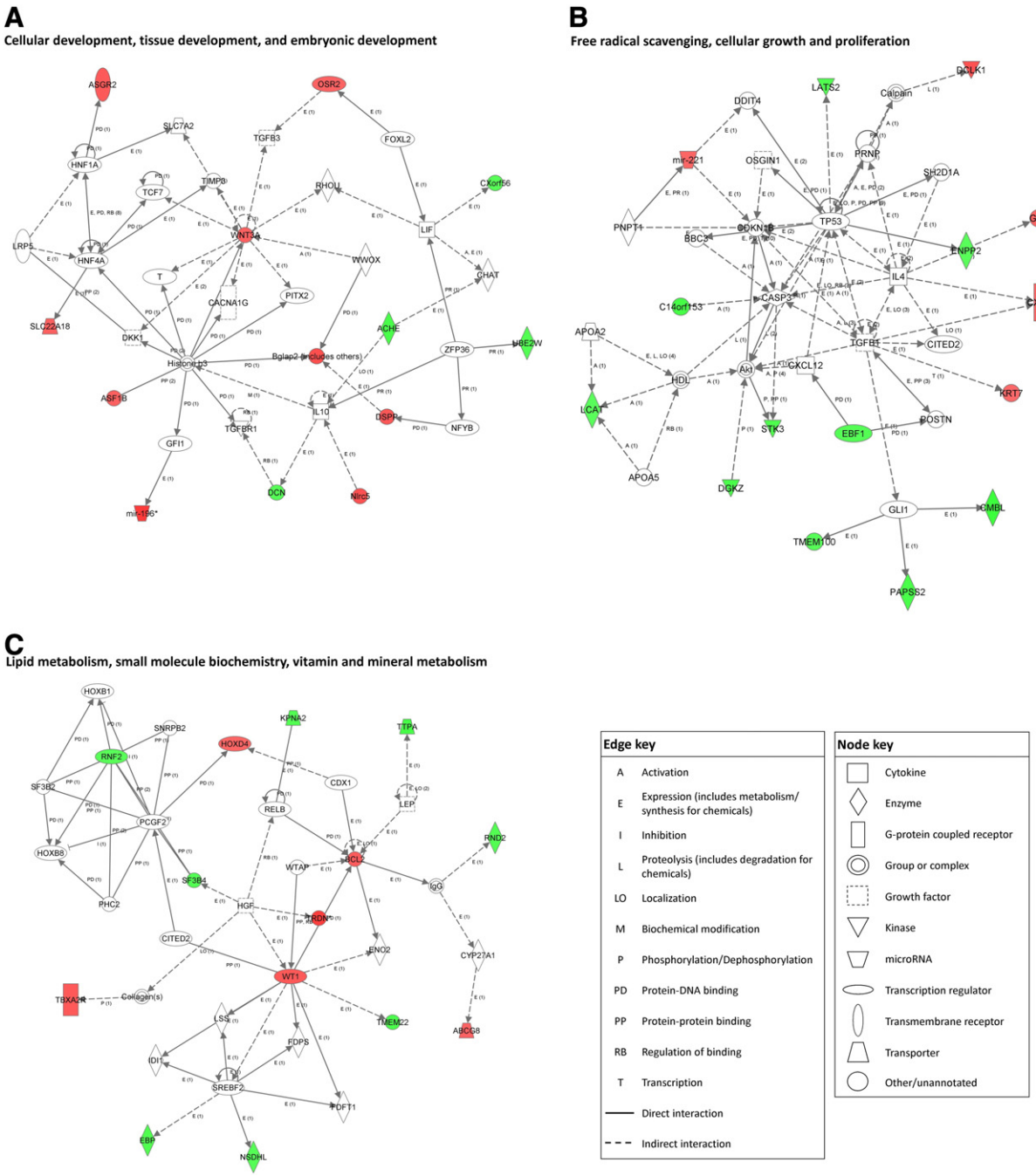

Fig. 4 – Abridged Ingenuity Pathway Analysis (IPA) networks showing interactions between molecules within specific IPA functions: (A) Cellular Development, Tissue Development, Embryonic Development, (B) Free Radical Scavenging, Cellular Growth and Proliferation, (C) Lipid metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism. Molecules identified as upregulated (red) or downregulated (green) in ethanol-exposed mice as compared to controls are indicated. Full gene names for gene symbols are as listed in Tables 2 and 3. Number of reports supporting interactions indicated on edges are in parentheses following the interaction abbreviation.

are altered in the brains of prenatally exposed adults, indicating that even mild prenatal alcohol exposure may result in long-term changes to neural function. Some of the genes identified have been implicated in endophenotypes similar to those present in FASD, including anxiety, mood disorders, and cognitive deficits. The sum and interaction of these gene expression changes, although individually slight, have the potential to explain at least some of these phenotypic features. Ultimately, these results demonstrate that the transcriptomic footprint of prenatal alcohol exposure is complex and likely explained by ethanol's ability to pleiotropically disrupt a number of neurodevelopmental mechanisms,

including apoptosis, cellular differentiation and maturation, and, intriguingly, the epigenetic programming that may be established as the brain develops.

4. Experimental procedures

4.1. *Animals and ethanol treatment model*

All protocols complied with ethical standards established by the Canadian Council on Animal Care and were approved by Animal Use Subcommittee at the University of Western Ontario. Male and female C57BL/6J (B6) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and bred and maintained in the Health Sciences Animal Care Facility at the University of Western Ontario. Mice were housed in same-sex colonies with *ad libitum* access to water and food. Environmental factors (such as cage type and size, colony size, bedding, nestlets, and environmental enrichment) were standardized between cages. Colony rooms were maintained in a controlled environment on a 14/10-hour light/dark cycle with 40% to 60% humidity and a temperature range of 21 °C to 24 °C.

The maternal voluntary drinking model has been described previously (Kleiber et al., 2011). Briefly, female B6 mice of approximately eight weeks of age were individually housed and given free access to both 10% ethanol in water and water, or water alone (control) in modified serological pipettes fitted with 2.5-inch stainless steel sipper tubes. Females were introduced to ethanol in a step-wise fashion with increasing concentrations of 2%, 5%, and 10% ethanol in water, each introduced after a 48 h acclimation to the previous concentration. Consumption of 10% ethanol was evaluated using a modified two-bottle choice protocol previously described by our laboratory (Loney et al., 2006; Weng et al., 2009). Mice were exposed to the 10% ethanol and water sipping tubes for a 14-day period to establish stable drinking patterns. Following this acclimation period, females were mated overnight with only water available, and ethanol was re-introduced following the removal of the male from the cage. Pregnant experimental mice (total n=8) were given free-access to ethanol throughout gestation day 0 (G0) and postpartum until postnatal day 10 (P10). Control dams (total n=8) had access to water only. The volume of ethanol and water was recorded daily from G0 to P10 and the tubes were rotated every second day to balance position effects. Females drinking less than 2 ml per day were excluded from the study. Resulting pups were weaned at P21 and housed in same-sex colony cages of two to four mice. This procedure was used to generate two independent populations of mice at different time points.

4.2. *Microarray hybridization and analysis*

At P70, male offspring from ethanol-consuming or control dams were sacrificed by carbon dioxide asphyxiation and whole brain tissue was isolated, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation. Total RNA was isolated from whole brain using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) according to the directions of the manufacturer and cleaned using RNeasy Mini kit (QIAGEN, Valencia, CA, USA). The quality of the RNA was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) for 28S and 18S quantitation and a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) to ensure all samples showed an OD_{260}/OD_{280} nm absorption ratio within the range of 1.9–2.1. Due to the subtlety of the model with respect to behavioral differences (Kleiber et al., 2011), and given that we chose to assess long-term alterations in gene expression, two independent microarray experiments were performed using the offspring from different ethanol-consuming and control dams. In experiment 1, RNA samples from three non-littermate male mice were pooled per array to reduce litter effects. Two biological replicate arrays were performed per treatment group (total n=12 mice and 4 arrays). For experiment 2, RNA from two non-littermate male mice were pooled per array again to reduce litter effects, and three biological replicates were performed (total n=12 mice and 6 arrays). Average RNA integrity numbers (RIN) for unpooled RNA samples used were 9.0±0.1 (n=12) for experiment 1 and 8.4±0.08 (n=12) for experiment 2. All pooled RNA samples were processed at the London Regional Genomics Centre (Robarts Research Institute, London, ON, CA). Single-stranded complementary DNA (sscDNA) was synthesized using 200 ng of total RNA using the Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript WT Expression Arrays (Applied Biosystems, Calsbad, CA, USA) and the Affymetrix GeneChip WT Terminal Labeling kit and hybridization user manual (Affymetrix, Santa Clara, CA, USA). First-cycle cDNA was transcribed *in vitro* to cRNA, and used to synthesize 5.5 μg of sscDNA that was subsequently end-labeled and hybridized for 16 hours at 45 °C to Affymetrix Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA). Liquid-handling steps were performed by a GeneChip Fluidics Station 450 and arrays were scanned using the GeneChip Scanner 3000 using Command Console v1.1 (Affymetrix, Santa Clara, CA, USA).

4.3. *Microarray data analysis*

Probe level (.CEL file) data from experiments 1 and 2 were generated using Affymetrix Command Console v1.1. Probes were summarized to gene-level data using Partek Genomics Suite software v.6.6 (Partek Inc., St. Louis MO, USA) where they were quantile-normalized using the GeneChip-Robust Multi-array Averaging (GC-RMA) algorithm (Irizarry et al., 2003) and $\log_2$-transformed. The Partek Genomics Suite (Partek Inc.) was also used to batch-correct the data from experiment 1 and 2 for different scan dates and determine gene-level ANOVA *p*-values and fold changes. Given that this model of ethanol exposure is moderate and that we wanted to assess gene expression in ethanol-exposed animals during early adulthood, we accepted a fold-change of 1.1 and a p-value<0.05 in our initial analysis to maximize the number of genes identified. These significant genes were subjected to a hierarchical clustering analysis using Euclidean distance and average linkage to assess consistency in ethanol-response between experiment 1 and 2. To then increase the stringency of the analysis, we then intersected the gene lists from experiment 1 and 2 and used only those genes that were consistently altered in both trials, including directionality

of fold-change, for further study. Unannotated genes and standards were edited from gene lists used for clustering and pathway analyses.

4.4. *Gene pathway and biological function analysis*

Ingenuity Pathway Analysis v.9 (IPA) (Ingenuity Systems, Redwood, CA, USA, www.ingenuity.com) was used to identify the primary biological functions and associated gene networks altered by neurodevelopmental ethanol exposure. Average fold-change and p-value of experiments 1 and 2 were used with the gene identifiers. Up and down-regulated genes were included together in the analysis and mapped to functional networks in the IPA Knowledge Base. Only those biological functions annotations that contained two or more molecules with a $p<0.05$ were included. Gene networks were created from genes that have annotated functional relationships with other genes, proteins, and molecules using a cut-off significance score of 3 ($p<0.001$).

4.5. *Statistical analysis*

All data are reported as mean±standard error of the mean. Data were analyzed with SPSS v.16 (SPSS Inc, Chicago, IL, USA) using analysis of variance (ANOVA) methods depending on relevant independent variables, or as indicated in the text using relevant array-based or pathway-based software.

Author's Note

Array data has been submitted to Gene Expression Omnibus (GEO) at the National Center for Biotechnical Information (http://www.ncbi.nlm.nih.gov/geo/), GEO ID: GSE34305.

Acknowledgments

This work was funded by a grant to SMS and a Postgraduate Scholarship to EJD from the Natural Sciences and Engineering Research Council, and an Ontario Graduate Scholarship in Science and Technology to MLK. The authors thank David Carter and the London Regional Genomics Centre for the array hybridization and technical assistance.

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