Published in final edited form as:

Brain Res. 2009 February 3; 1252: 105–116. doi:10.1016/j.brainres.2008.11.023.

Gestational ethanol exposure alters the behavioral response to ethanol odor and the expression of neurotransmission genes in the olfactory bulb of adolescent rats

Frank A. Middleton^{a,b}, Kellyn Carrierfenster^{a,b}, Sandra M. Mooney^{a,b}, and Steven L. Youngentob^{a,b,*}

^aDepartment of Neuroscience and Physiology, SUNY Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210, USA

^bThe SUNY Developmental Exposure Alcohol Research Center, SUNY Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210, USA

Abstract

Fetal exposure to ethanol is highly predictive of the propensity to ingest ethanol during adolescence and *in utero* chemosensory plasticity has been implicated as a contributing factor in this process. Recent rodent studies have shown that fetal ethanol exposure results in a tuned unconditioned sniffing and neurophysiological olfactory response to ethanol odor in infant animals. Importantly, a significant proportion of increased ethanol avidity at this age can be attributed to the tuned behavioral response to ethanol odor. These effects are absent in adults. Using behavioral methods and comprehensive gene expression profiling to screen for robust transcriptional differences induced in the olfactory bulb, we examined whether ethanol exposure via maternal diet results in an altered responsiveness to ethanol odor that persists into late adolescence and, if so, the molecular mechanisms that may be associated with such effects. Compared to controls, fetal exposure altered: the adolescent sniffing response to ethanol odor consistent with the previously observed changes in infant animals; and the expression of genes involved in synaptic transmission and plasticity as well as neuronal development (both cell fate and axon/neurite outgrowth). These data provide evidence for a persistence of olfactory-mediated responsiveness to ethanol into the period of adolescence. Further, they provide insight into an important relationship between fetal exposure to ethanol, adolescent odor responsiveness to the drug and potential underlying molecular mechanisms for the odor-guided behavioral response.

Keywords

Gene expression; Fetal ethanol exposure; Olfactory bulb; Odor preference

1. Introduction

There is an extensive animal and human literature that addresses the role that fetal and postnatal learning about ethanol's chemosensory attributes plays in later perception, preference and intake of ethanol (Abate et al., 2000, 2001; Arias and Chotro, 2005; Chotro and Arias, 2003; Faas et al., 2000; Mennella, 1999, 2001; Mennella and Beauchamp, 1998; Molina et al., 1995, 1999; Spear and Molina, 2001, 2005). With specific respect to *in utero*

exposure, when ethanol is present as a stimulus in the amniotic fluid of the developing fetus its presence can later lead to a memory of ethanol's sensory-related cues as well as an association between both its sensory attributes and its unconditioned properties (Abate et al., 2000, 2001). Young postnatal rodents (between postnatal day [P] 8–15) elicit an orienting response to (Chotro and Molina, 1992; Chotro et al., 1996) and enhanced preference for ethanol odor (Chotro and Molina, 1990; Chotro et al., 1996), as well as enhanced ethanol consumption (Chotro and Arias, 2003; Chotro and Molina, 1990; Dominguez et al., 1993; Molina et al., 1995; Youngentob et al., 2007b). Many of these effects, and in particular enhanced ethanol intake, have been uniformly shown to persist through weaning and into adolescence (e.g., Chotro et al., 2007; Molina et al., 2007; Spear and Molina, 2005). In contrast, intake acceptance studies in adult rodents have been quite varied, ranging from enhanced acceptance (e.g., Bond and Di Giusto, 1976; Phillips and Stainbrook, 1976) to no observed changes as a consequence of fetal exposure (Abel and York 1979; McGivern et al., 1984). Consistent with these latter negative results, Youngentob et al. (2007b) also found an absence of enhanced intake acceptance in adult animals as a consequence of exposure to ethanol throughout gestation.

It has been suggested that *in utero* experience-induced olfactory plasticity in response to ethanol alters the preference response to ethanol odor and the perception of ethanol's flavor (the integration of olfaction, taste and somatosensation) important to acceptance patterns of the drug (Youngentob et al., 2007b). Consistent with this proposition, prenatal ethanol exposure results in a "tuned" olfactory-mediated response to ethanol odor that is manifested both behaviorally and neurophysiologically in the early postnatal animal (Youngentob et al., 2007a). Specifically, compared to control animals (both iso-caloric pair-fed and free-choice chow controls) experimental rats exposed to ethanol throughout gestation via maternal diet: (1) show a tuned neural response of the olfactory epithelium (as measured by optical methods) to ethanol odor; (2) the neural tuning while manifest in early postnatal rats is absent in adulthood; (3) the observed neural effect is causally related to an enhanced unconditioned sniffing response (as measured by whole-body plethysmography) to ethanol odor that is specific to this odorant; and (4) the behavioral consequence of fetal exposure also declines by adulthood.

The timing of the foregoing behavioral and physiological effects has been shown to parallel ethanol intake patterns under identical fetal exposure conditions (Youngentob et al., 2007b). That is, enhanced olfactory system responsiveness to ethanol odor is intimately related to ethanol avidity observed in early postnatal animals and the amelioration of the olfactory effects over time predicts the observed absence of an enhanced intake response in adulthood. This relationship is particularly noteworthy since a significant fraction of the enhanced ethanol intake effect in infant animals is directly attributable to the fetal exposure effect on the response to ethanol odor (Youngentob and Glendinning, submitted). Taken together, these findings suggest that in the absence of further interaction with ethanol between the fetal exposure period and adulthood the drug's experience-induced biological significance wanes (Hudson, 1999). That is, the enhancement of ethanol's chemosensory attributes and ethanol acceptance, returns to a neutral biological status by adulthood.

The exact time-course, and the underlying mechanism, by which the amelioration in olfactory system function and ethanol affinity occurs are unknown at this time. However, the finding that enhanced odor preference (e.g., Fernandez-Vidal and Molina, 2004) and ethanol acceptance (Molina et al., 2007; Spear and Molina, 2005) are consistently observed in adolescent animals suggests that alterations in olfactory function should be observable at this same developmental time-point.

The above findings highlight adolescence as a potentially significant transition point for maintaining the chemosensory effects of prior fetal ethanol exposure. Consequently, the goals of the present study were two-fold: first, we tested the hypothesis that gestational ethanol exposure results in an altered odorant-induced responsiveness to ethanol odor that persists into late adolescence; and second, that there are changes in gene expression in the olfactory bulb (OB) of adolescent rats that had been exposed to ethanol during the fetal period, but were otherwise naïve to the drug. With regard to this latter goal, there is a large body of evidence suggesting that the OB should be viewed from an information processing perspective (Wilson and Sullivan, 1994) and, more importantly, that the OB encodes that an odorant has gained associative salience (e.g., Gervais and Pager, 1983; Gervais et al., 1988; Sullivan et al., 1989; Wilson and Leon, 1988a,b). As such, we predicted changes in expression of neurotransmitter signaling components critically related to the altered physiological and behavioral responses that have been previously demonstrated in odor conditioned animals under other settings (e.g., Coopersmith and Leon, 1984; 1986; Coopersmith et al., 1986; Brennan and Keverne, 1997; McLean and Harley, 2004; McLean et al., 1999; Yuan et al., 2002, 2003; Wilson and Leon, 1988a,b).

2. Results

2.1. Behavioral analysis

Fig. 1 plots the mean ($\pm$ se) Composite Reflexive Sniffing Index values as a function of sex and maternal treatment group. With regard to this index, previous work has shown that: (1) sniffing is a complex response pattern that is known to vary with odorant stimuli; and (2) more importantly, although sniffing patterns can be deconstructed into a large number of descriptive variables (e.g., sniff frequency, sniff volume, flow-rate, etc.), knowledge about any single variable is insufficient to evaluate the meaning of the sniffing response pattern to odorant stimuli. Thus, our composite index summarizes the joint impact of fourteen variables (Table 1) (along with their corresponding weightings) derived from the airflow pattern of an animal's sniffing response as they relate to experimental manipulation (Youngentob, 2005; Youngentob et al., 1987, 2007a).

As can be seen in Fig. 1, compared to the pair-fed caloric control group (PF), both male and female rats exposed to ethanol throughout gestation (ET) show an enhanced unconditioned response to ethanol odor when tested in adolescence. ANOVA demonstrated a significant overall effect of maternal treatment on the response to ethanol odor in the adolescent animals ($F_{1,18}=5.92$; $P=0.025$). There was no evidence of an average overall sex difference ($F_{1,18}=1.60$; $P=0.22$) or a sex by maternal treatment interaction ($F_{1,18}=0.0024$; $P=0.987$).

2.2. Microarray screen

Affymetrix software (MicroArray Suite 5.0; MAS5) was initially used to perform quality control (Q/C) analysis, calculate the signal intensities from each perfect match probe on the arrays relative to the mismatch probe, and to determine whether or not the gene was present in the sample. Scale factors (used to align the median signal intensity values) were highly similar within each pooled tissue sample (i.e., male/ethanol treated, female/ethanol treated, male/control, and female/control), and ranged from 1.29 to 1.68. The 3'/5' signal ratios for several housekeeping genes (a measure of RNA quality and labeling reaction performance) were highly consistent between the 4 pooled samples, ranging from 0.96 to 1.44 for GAPDH, 1.92 to 3.83 for Hexokinase, and 1.77 to 3.88 for b-actin. Overall, of the 15,923 probes present on the RAE230A GeneChip, approximately 60% were determined by the MAS5 algorithm to be expressed in the samples.

To analyze the pooled array data in the most conservative manner possible, we used the RMA method to normalize the set of four arrays in our screen and a simple pair-wise *t*-test

to compare the two pairs of gender-matched arrays (male-ethanol/male-control, and female-ethanol/female-control). In this report, our screen focused on only those genes with the most significant (i.e., adjusted pair-wise *t*-test $P < 0.05$) changes in expression that exceeded at least 30% (1.3-fold increase or decrease) in the OB samples. Moreover, since there was no evidence for a differential behavioral effect based on sex differences, we focused only on those changes present in both genders in the microarray comparisons. In this manner, we hoped to identify genes whose expression changes could provide insight into the overall effect of maternal treatment on behavior. In total, 78 probes fulfilled these criteria (Fig. 2).

For illustrative purposes, Table 2 highlights a subset of the genes with expression changes of 40% (1.4 fold) or greater. Among the genes that show the strongest changes in expression are several involved in synaptic transmission and plasticity as well as neuronal development (including both cell fate and axon/neurite outgrowth). For example, prenatal exposure to ethanol induced a significant decrease in the expression of the GABA B1 receptor, mGluR2 receptor, Caskin, and SOX11 genes and significant increases in the expression of the Calcium/calmodulin-dependent protein kinase IV (Cam-KIV) and Potassium channel, subfamily K, member 13 transcripts.

2.3. Gene group effects screen

To explore whether prenatal exposure to ethanol differentially affected expression of genes in specific functional groups (clusters), the Gene Ontology (GO) membership of each of the 78 genes meeting our selection criterion was examined. To accomplish this, we used the NetAffx Gene Ontology Browser (Affymetrix) to cross-reference the 78 probes to the NCBI Gene Ontology database. The relative enrichment of the representation of probes in each GO cluster was determined as the ratio between the percent of the annotated component of the 78 probes for each GO category to the percent of the annotated component of all the probes on the RAE230A array. In total, the 78 probes mapped to a total of 1002 different GO clusters. These were distributed among a total of 35, 39 and 38 probes with entries in the GO Biological Process, Molecular Function, and Cellular Component categories, respectively. We next filtered the list of 1002 GO clusters to a final set of 207 with that containing 3 or more matching probes, and determined the statistical significance of the enrichment using a hypergeometric *P* value calculation. Only those clusters showing greater than a 4-fold enrichment with a *P* value less than 0.05 are shown (Table 3).

In keeping with the overall microarray screen, of the 11 GO clusters demonstrating greater than 4-fold significant enrichment, 7 were directly involved in either synaptic function or brain development. These GO clusters included Nerve-Nerve Synaptic Transmission (3 probes, $P = 0.006$), Negative Regulation of Signal Transduction (3 probes, $P = 0.0155$), and Central Nervous System Development (6 probes, $P = 0.0015$) (Table 3).

2.4. qRT-PCR validation analysis

Since the largest number of transcript changes in our screen (i.e., 16 total) was found in groups involved in either primary or secondary cellular signaling, our validation efforts focused on a subset of five of these genes that were similarly decreased in both male and female animals. More specifically, the GABA B1 receptor, mGluR2 receptor, Caskin, DRD2 receptor, and Adra1d receptor genes, as well as one reference gene (18S rRNA), were examined by qRT-PCR. We chose to include the Adra1d receptor gene, although not originally included among the genes with the most statistically robust changes in expression, because it showed a 2.4 fold decrease in expression in ethanol exposed animals and adrenergic receptor signaling is known to be critically involved in olfactory-guided behavior.

For each gene, primers were designed using Primer3 software and Affymetrix target sequences as the input. Possible primers were screened using a BLAST search to confirm the primers would specifically match the rat target sequences. The primers used are listed in Table 4. All primers were tested and optimized to prepare them for the qRT-PCR using a mixed rat brain cDNA template and standard PCR conditions and AmpliTaqGold (Applied Biosystems, Foster City, CA). The products of these reactions were resolved on ethidium-bromide stained agarose gels and each observed as single bright bands of the correct size (Table 4). DNA sequencing confirmed the identity of each amplicon.

Each PCR reaction for each gene was performed in 4 replicates for each individual animal sample. The cycles to threshold (Ct) value for each gene amplicon was then corrected for the amount of starting material by subtracting the Ct value for the reference gene amplicon (18S rRNA) from the target gene Ct value to produce a Δ Ct value. The corrected data from the runs were then averaged into a mean Δ Ct value. The Δ Ct values of the ethanol and control groups were then analyzed using multivariate analysis of variance (MANOVA) to evaluate the overall consequences of fetal ethanol exposure on gene expression in the OB. This analysis demonstrated a highly significant effect of prenatal exposure on gene expression levels ($F_{5,23}=26.6$, $P<0.0001$). Moreover, additional post-hoc analysis of the individual genes, using Scheffe's criterion for pair-wise comparisons, demonstrated a significant effect of prenatal exposure to ethanol on expression of GABA B1 ($P<0.0001$), mGluR2 ($P<0.05$), Caskin 1 ($P<0.0001$) and Adra1d ($P<0.005$) (Fig. 3A). In contrast, we found no evidence for an effect on the expression of DRD2. That is, the results seen in the pooled array data for DRD2 were not validated on individual samples when corrected for the 18S starting material.

To quantify the degree of change in gene expression relative to the foregoing results, the mean Δ Ct for each candidate gene in the ethanol treated group was subtracted from the mean Δ Ct for the same gene in the control group to generate $\Delta\Delta$ Ct values. These values were then converted to "fold changes" in the ethanol-exposed group using the formula: Fold Change= $2^{-\Delta\Delta\text{Ct}}$ (Fig. 3B). On average, gene expression was decreased for GABA B1, mGluR2, CASKIN, and Adra1d by a factor of -1.59 , -1.45 , -1.90 and -1.30 , respectively.

2.5. A shift in the expression of GABA-related genes

Our unbiased GO analysis revealed a clear enrichment of genes involved in synaptic function among those with the most robust changes in expression. This aspect, along with the results of the PCR validation motivated us to probe further into the specific subclasses of synaptic transmission genes that might be affected by early ethanol exposure. At least one of the robustly affected genes is directly and specifically involved in GABA neurotransmission (GABA B1), with others involved in glutamatergic or adrenergic transmission. To provide broader interpretability to our findings (namely, that *in utero* exposure alters bulbar gene expression) we next took an inductive approach and examined whether there was evidence of an overall shift in the expression of entire groups of probes related to GABA, glutamate, or adrenergic signaling, or groups involved in synaptic or cytoskeletal elements (using NetAffx annotation). We found evidence for a shift toward decreased expression in the expression of an entire set of 49 GABA related transcripts. This shift was evident in both the male and female ethanol-exposed samples. In contrast, histograms of the pooled data for glutamate receptor-related genes, adrenergic-related genes, synaptic-related genes, and cytoskeletal-related genes, did not show any apparent shifts in these groups (data not shown).

The GABA gene family is particularly interesting because the interneurons of the bulb all express classic GABAergic inhibition (Riback et al., 1977; Nicoll, 1971). Fig. 4 illustrates the distribution of the mean $\log_2$ ratios comparing the expression of 49 GABA-related

transcripts (determined by NetAffx annotation) in the ET animals relative to PF animals. Expression of multiple GABA-related genes was decreased in the ethanol-exposed animals. Specifically, five GABA-related genes showed decreased expression of -0.75 or less (on a $\log_2$ scale), namely: glutamate receptor, metabotropic 2 (Grm2); GABA receptor, subunit beta 1 (Gabbr1); GABA A receptor, gamma 1 (Gabrg1); GABA B receptor, 1 (Gabbr1); and G protein-coupled receptor 51 (Gpr51). In contrast, there were no increases of this magnitude observed among the GABA-related genes. Thus, there appears to be a broader decline in GABA related expression beyond that formally validated using the GABA B1 gene.

3. Discussion

Human studies point to an underlying relationship between fetal ethanol exposure and the risk for ethanol abuse in adolescence (Alati et al., 2006; Baer et al., 1998, 2003; Streissguth, 1998; Yates et al., 1998). In fact, fetal ethanol exposure in humans is, perhaps, the best predictor of later ethanol abuse during adolescence (an age-range known to be ontogenetically at-risk for high levels of ethanol intake; Spear, 2000). These observations cannot be overstated since epidemiologic studies also point to an inverse relationship between the age of first experience with ethanol drinking and the probability of developing dependence and continued abuse. However, there is a paucity of studies focusing on potential antecedent factors contributing to the progressive pattern of continued abuse. Fetal ethanol experience-induced olfactory plasticity has been implicated as an antecedent factor contributing to initial adolescent intake and continued choice behavior (Youngentob et al., 2007a,b). This suggestion notwithstanding, the previous studies by Youngentob et al., (2007a,b) demonstrated a tuned neural and unconditioned sniffing response to ethanol odor and enhanced ethanol intake in infant animals that was absent in adults: leaving open the question of whether the effects observed in infants persist into adolescence.

In the current investigation we focused on examining the relationship between fetal ethanol exposure, adolescent responsiveness to the odor of the drug and potential underlying molecular mechanisms for the odor-guided response. The behavioral findings extend upon the previous studies by providing evidence for an altered responsiveness to ethanol odor in adolescent rats that is consistent with the previously observed changes in infant animals. This observation is key since prenatal ethanol exposure has consistently been shown to enhance ethanol intake from early in postnatal development to adolescence (e.g., Chotro et al., 2007; Molina et al., 2007; Spear and Molina, 2005). Thus, the conjunction of the present results with previous findings (Youngentob et al., 2007a) lends further support to the concept that fetal ethanol-induced olfactory plasticity contributes to enhanced ethanol avidity (as a consequence of fetal exposure) by making ethanol “smell and taste better” (Youngentob and Glendinning, submitted).

In addition to the behavioral consequence of fetal ethanol experience on olfactory function, we also examined patterns of gene expression changes in the OB that would be related to the behavioral effect. Two issues regarding our results require consideration. The first issue relates to the relationship between the gene expression patterns we observed and prior work on the teratologic response of the OB to ethanol, while the second issue speaks to the link between changes in gene expression and the behavioral results, per se.

Regarding the first issue, there is neuroanatomical evidence demonstrating the OB can be sensitive to ethanol exposure depending on dose and time of exposure (Sulik and Johnston, 1983; Nyquist-Battie and Gochee, 1985; Barron and Riley, 1992). Decreases in cell number resulting from exposure have been found in both the mitral/tufted cell (M/T) and granule cell layers (Bonthius et al., 1992; Bonthius and West, 1991) of the OB. M/T cells form the

primary relay for olfactory information from the olfactory sensory neurons (OSNs) to the CNS. M/T cells receive direct glutamatergic input from the OSNs (Ennis et al., 1996, 2001), and send glutamatergic projections to more central targets. The output of M/T cells is modulated both by GABAergic periglomerular cells (Kosaka et al., 1985, 1987) and granule cells (Isaacson and Strowbridge, 1998). Expression of both the mGluR2 and GABA B1 genes were decreased as a consequence of fetal exposure. M/T and granule cells also express Adra1 receptors that were found to have decreased expression in our study (indeed, the OB has the highest density of Adra1 receptors in the brain; Young and Kuhar, 1980). The activation of these receptors enhances mitral cell activation in response to weak OSN input (Hayar et al., 2001). Thus, in keeping with the prior neuroanatomical studies (Bonthius et al., 1992; Bonthius and West, 1991) the overall decreasing trend seen in our data for mGluR2, GABA B1, Adra1d, and the group of GABA-related transcripts strongly implies that fetal exposure to alcohol can exert a major impact, not only on the number of granule and M/T cells in the OB, per se, but also on neurotransmission in the remaining cells.

Notably, the foregoing suggestions are highly consistent with the pre-existing literature establishing that early ethanol exposure exerts fairly selective effects on GABAergic and glutamatergic neurons. In fact, Olney et al. (Ikonomidou et al., 2000; Olney et al., 2002) specifically proposed that "...blockade of NMDA glutamate receptors and hyperactivation of GABA(A) receptors mediates ethanol's apoptogenic action...". Although the most robust changes we observed were in the metabotropic class of GABA and glutamate receptors (and not GABA (A) or NMDA receptors), we did observe less robust decreases in at least one GABA(A) receptor subunit (gamma). Thus, while our expression data are not consistent with a widespread loss of GABAergic neurons (since many GABAergic markers did not decrease), they do support the notion of reorganization of receptor signaling mechanisms in surviving neurons, or perhaps enhanced survival of OB neurons with reduced metabotropic receptor expression.

In addition to the above, many of the robustly affected genes were involved in synaptic transmission as well as synaptic development and cell fate. For example, Caskin 1 expression was significantly decreased and this brain-specific protein appears to be important for synaptic transmission and nuclear transcription that may interact with mGluR2 (Tabuchi et al., 2002). Cytoskeletal-related transcripts such as Tropomyosin 3 gamma, Myosin IC, and Beta spectrin were also consistently altered as a consequence of fetal ethanol exposure. In neurons, these genes are involved in neurite outgrowth and maintenance of spines (e.g., Schevzov et al., 1997; Leshchyns'ka et al., 2003). Another gene with a potential role in modifying synaptic activity is Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (PGC-1alpha). PGC-1alpha is known to play a major role in stimulating mitochondrial biogenesis, and appears to be particularly enriched during development in GABAergic neurons (Cowell et al., 2007), where one study concluded that PGC-1alpha served as a "...regulator of metabolism and/or survival in GABAergic neurons during a phase of mitochondrial and synaptic changes in the developing brain..." (Cowell et al., 2007). Other studies in various tissues have also documented that PGC-1alpha (as well as mitochondria themselves) increase in number as a result of increased activity (Handschin and Spiegelman, 2006). Thus, the decrease we detected in PGC-1alpha could indicate a reduction in synaptic elements or activity in the OB as a result of an early exposure to ethanol. Finally, a distinct set of genes involved in neural cell fate decisions such as the SRY-box containing gene 11 (SOX11), which regulates nestin expression in developing neurons, SOX10, which regulates cell fate in neural crest cells, and Tangerin, a downstream target of Pax6 were decreased in expression. The consequences of a decrease in these genes are difficult to predict, however, it is possible that prenatal ethanol exposure may alter OB cell fate and/or neurogenesis. Noteworthy changes in cell fate may explain some of the alterations in the synaptic-related transcripts described above, and previous reports in the

literature regarding decreases in mitral and granule cell number following ethanol exposure (e.g., Bonthius et al., 1992).

At first glance, it may be difficult to reconcile from the foregoing discussion that fetal ethanol exposure results in an enhanced unconditioned sniffing response to ethanol odor against a landscape of untoward effects in bulbar anatomy and gene expression related to information processing in the OB. However, we suggest that these effects are not representative of the experience-induced effects in response to ethanol on the specific stimulus channels related to the processing of ethanol odor. Rather, the ethanol-related activity channels are, at a minimum, stabilized and preserved (through stimulus activation) in the face of the toxic effects of ethanol. In support of this suggestion, using an identical ethanol exposure paradigm as in the current study, Youngentob et al. (2007a) demonstrated a tuned neural response of the olfactory epithelium to ethanol odor that is accompanied by a reduced responsivity to non-exposure odorants. Notably, the neural response predicts an enhanced unconditioned sniffing response to ethanol odor (using the present behavioral paradigm) that is, not only, accompanied by enhanced ethanol acceptance (Youngentob et al., 2007b) but, indeed, a significant proportion of the fetal exposure effect on enhanced intake is directly attributable to its effect on the response to ethanol odor (Youngentob and Glendinning, submitted). Similarly, other forms of extensive odor experience enhance the behavioral sensitivity and preference for the exposure odorant and these effects occur against a landscape of untoward neural effects at the level of the OB (Laing and Panhuber, 1980; Laing et al., 1985; Panhuber and Laing, 1987; Panhuber et al., 1987; Rehn et al., 1988). Specifically, continuous odorant exposure yields shifts in the size and appearance of mitral cells in selected areas of the bulb. In other words, animals exposed to a single odorant showed small foci with enlarged mitral cells, relative to controls, and large areas with shrunken mitral cells such that the distribution pattern of these changes were specific to the exposure odorant. The detectability and preference of the specific exposure odorant was enhanced.

A note of caution must be considered in interpreting the possible causal relationship between the behavioral and gene expression results. First, the gene expression data were not collected from the same animals providing the behavioral results. This approach was purposeful by permitting the examination of the direct consequence of fetal exposure on gene expression, without the potential confounding effects of odorant testing on the expression of unknown genes. Second, we did not study the ontogeny of the observed effects on gene expression. Thus, we do not know whether they will ameliorate with time like the previously observed behavioral, neurophysiological and intake effects (Youngentob et al., 2007a,b). Finally, it is difficult to say at this time whether, and how, specific genes in the precise ethanol-related stimulus activity channels are affected by fetal exposure.

An additional potential caveat is that the behavioral data were collected at P42 whereas the OBs for the microarray studies were harvested at P48. We think this word of caution, however, is of minimal concern. Both ages are within the various reported ranges (depending on measure) of adolescence (Spear, 2000). More importantly, the lion's share of developmental changes in the OB occur during the first 2–3 weeks of postnatal life with a much slower rate of changes continuing well into maturity (Hinds and McNelly, 1977, 1981). Thus, whatever changes in bulb development (e.g., increase volume and synaptogenesis) that may have occurred between P42 and P48 are likely small at best with regard to the potential relationship between behavior and gene expression.

Taken together, therefore, our data demonstrate that prenatal exposure to ethanol via oral maternal feeding results in long-term changes in expression of a subset of genes that are temporally associated with an enhanced unconditioned sniffing response to ethanol odor.

Indeed, many of the identified genes are involved in synaptic transmission and olfactory information processing in the OB. As such, because prenatal ethanol exposure has been shown to be intimately related to alterations in ethanol odor seeking and preference behavior, and that ethanol's effect on the olfactory neural response predicts the behavioral response to ethanol odor, the specific bulbar genes we have identified may have key importance in the process of establishing ethanol preference in adolescence.

Lastly, our results also highlight the clinical view that adolescence is a critical transition point for developing patterns of ethanol abuse. That is to say, the fetal-induced chemosensory effect evident in infant animals persists, at least, into adolescence and in the absence of further ethanol experience, olfactory function returns to an ethanol "neutral" status by adulthood (Youngentob et al., 2007a).

4. Experimental procedures

4.1. Prenatal ethanol exposure

Thirty-six (18 ethanol [ET] and 18 pair-fed [PF]) progenies from nine ethanol-treated and nine pair-fed control Long-Evans dams (Harlan Sprague-Dawley, Indianapolis, IN) participated in this study. Equal numbers of male and female offspring were used.

In this study, we limited the control group to pair-fed animals for several reasons. In our previous work using the same gestational exposure paradigm as described below we found no evidence of a differential effect between the pair-fed and free choice food and water control conditions with regard to the behavioral (Youngentob et al., 2007a; Youngentob and Glendinning, submitted) and neurophysiological response to ethanol odor (Youngentob et al., 2007a) or ethanol avidity (Youngentob et al., 2007b). Moreover, we have also observed that adolescent pair-fed and free-choice control animals do not differ with respect to their orosensory responses to ethanol, quinine or sucrose (Youngentob and Glendinning, submitted). Thus, since pair-fed animals directly control for the conditional effect of an altered diet (i.e., decreased caloric intake) we limited our evaluation to this single parsimonious control group.

ET animals were exposed to ethanol *in utero* by administering ethanol to the pregnant dam in a liquid diet. Following a previously established protocol, pregnant dams were weaned (from gestational day (G) 6–10) onto an *ad libitum* liquid diet (L10251, Research Diets, New Brunswick, NJ) that provided 35% of daily calories from ethanol from G11–G20 (Miller, 1992; Youngentob et al., 2007a). This exposure paradigm provided an ethanol level (~150 mg/dl) that modeled moderate ethanol intake (Driscoll et al., 1990; Vavrousek-Jakuba et al., 1991).

Weight-matched control dams (PF) were pair-fed to the ethanol group and received an iso-caloric iso-nutritive liquid diet (L100252, Research Diets, New Brunswick, NJ) with maltose/dextrin substituted for the calories derived by ethanol.

Within 24 h of birth all litters were culled to 10 and surrogate-fostered to non-experimental dams fed chow and water during gestation.

The timing of the ethanol exposure was important in two regards. First, it permitted us to achieve the peak level of dietary exposure concentration (viz, 35% daily calories derived from ethanol) prior to the time that olfactory sensory neurons in rat fetuses (G14) begin to transduce sensory information (Gesteland et al., 1982) and just before the sensory axons have reached the olfactory bulb (G14–15) (Farbman, 1991). Second, the time period of peak exposure encompassed the period of gestation during which the organism is known to process ethanol's chemosensory cues and establish a memory for this experience that can be

potentially expressed later in life (Abate et al., 2000, 2001; Chotro and Molina, 1990, 1992; Chotro et al., 1996; Dominguez et al., 1993).

4.2. Monitoring of stimulus-induced reflexive sniffing

A total of 10 ET and 10 PF animals, with equal gender representation from each treatment group, participated in the behavioral component of the study. These animals represented one randomly selected animal of each gender from five ET and five PF litters. Animals were tested on P42 or P43. Following previously established protocols (Youngentob, 2005; Youngentob et al., 2007a), we monitored the stimulus-induced reflexive sniffing response to ethanol odor using whole-body plethysmography, in conjunction with flow-dilution olfactometry. Each experimental session consisted of testing both male and female ET animals as well as sex and age-matched PF controls. The order of animal testing was randomized.

Briefly, following a series of air only habituation trials, air and ethanol odor stimuli were presented randomly using a 6 s fixed inter-trial interval schedule. Prior to stimulus presentation, a 6 s baseline was monitored and the randomly selected stimulus presented for 6 s. Five ethanol odorant concentrations were presented in an ascending series (3.125×10^{-3} , 6.25×10^{-3} , 1.25×10^{-2} , 2.5×10^{-2} and 5×10^{-2} of vapor saturation at 20 °C, respectively) with each concentration presented for one block of trials. A total of 20 trials (10 air and 10 odorant) were presented during each block. For each presentation of a stimulus, the reflexive sniffing patterns acquired during the testing sessions were deconstructed into 14 respiratory response measures (Table 1) (Youngentob, 2005; Youngentob et al., 2007a).

As exemplified by the 14 response parameters calculated from the dissection of individual sniffing patterns, and highlighted in our previous studies, sniffing is a complex multivariate pattern of stimulus-response behavior that cannot be effectively evaluated by the examination of any one single variable (Youngentob et al., 1987, 2007a; Youngentob, 2005). That is, although direct examination of specific response parameters of sniffing are quantitatively useful if one is interested in determining the physiologic values that animals can generate while odorant sampling, knowledge about any individual parameter is not sufficient to describe the highly complex pattern of sniffing response to odors. Indeed, the relative change in individual variables, if any, (either in magnitude or direction) as a function of odorant stimulation conditions or experimental treatment are not interpretable in isolation. By contrast, this complex pattern of behavior can be effectively described and evaluated using a measure that encapsulates the derived variables into a univariate response value (Youngentob et al., 1987, 2007a; Youngentob, 2005). To accomplish this, for each animal we derived a single “Composite Reflexive Sniffing Index” that incorporated the fourteen variables of sniffing behavior in response to individual stimuli and the animal’s responses across the five concentrations of odorant tested. In this manner, the index provided a single overall behavioral measure of responsiveness to ethanol odor that could be used in our analysis of the consequence of maternal treatment. Moreover, the approach provided a means for visualizing the shifts, if any, in this complex pattern of behavior.

To reduce the high dimensionality (14 response variables) of the data to a fewer number of orthogonal dimensions without loss of response pattern information we first applied a principle components analysis (PCA). *A priori* the value of the first factor at each concentration of odorant presented was used to define an individual animal’s behavioral response to each stimulus presentation (e.g., Youngentob et al., 2007a). Next, to construct the Composite Reflexive Sniffing Index for each animal that incorporated the animal’s behavioral response across all concentrations tested the following procedures were used: with maternal treatment as the independent variable, we performed a multivariate multiple regression analysis using each individual animal’s five odorant-induced behavioral response

measures (in other words, the value from the PCA for each concentration of ethanol presented) as the dependent variables in the analysis. This provided a statistically valid estimate of the coefficient for each dependent variable. The Composite Reflexive Sniffing Index value for each animal, therefore, was the linear summation of the constant from the regression analysis plus an individual animal's PCA value at each concentration of odorant multiplied by its respective coefficient.

4.3. Comprehensive gene expression profiling

4.3.1. Tissue harvesting and RNA extraction—Tissue from a total of 8 ET and 8 PF animals, with equal gender representation from each maternal treatment group, was used in the gene expression component of the study. These samples represented one randomly selected animal of each gender from 4 ET and 4 PF litters. At P48 all animals were deeply anesthetized and then euthanized by decapitation. It should be emphasized that, relative to the animals behaviorally tested at P42–P43, those harvested at P48 are still firmly within the extended age-range of adolescence (Spear, 2000).

The OBs were removed, frozen on dry ice, and stored at -80°C . Tissues were subsequently pulverized in RNeasy lysis buffer (Qiagen, Valencia, CA) and purified using the RNeasy Mini Protocol (Qiagen, Valencia, CA). A 1 μl aliquot of each RNA sample was assessed for quality using an RNA Nanochip on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). For all samples, the 28S:18S ratios exceeded 1.5 and there was no evidence of degradation.

4.3.2. Microarray screening of RNA samples—We systematically recorded the total amount of RNA yield from each OB of each animal, which serves as an indirect measure of total cellularity in an encapsulated tissue such as the OB. We did not observe any significant differences in the total RNA yields between any of the groups regardless of gender or treatment ($P>0.3$ for all comparisons). Despite the lack of differences in total RNA yields, to further control for possibly different amounts of cells contributing to the microarray screen, we carefully matched the molarity of RNA used from each animal in each of the groups. Specifically, equal quantities of total RNA (1.3 mg per animal) were combined into a single sample for each gender and treatment group (4 pooled samples total; 3.9 mg total RNA each). In this manner, all rats in each pool were equally represented in the data. The cRNA amplification and labeling reactions for these experiments were performed according to the GeneChip[®] Expression Analysis Technical Manual (Affymetrix publication 701021 Rev. 3). During this procedure, we further equalized the subsequent reaction products at two points in the labeling protocol (following cDNA synthesis and purification and following cRNA synthesis and purification). The pooled RNA samples were profiled using the Affymetrix Rat RAE230A GeneChip[®] (Affymetrix, Santa Clara, CA). This array contains 15,923 probes, including the vast majority of annotated sequences in the rat genome. The GeneChips were hybridized at 45°C for 16 h with constant rotation (60 rpm), then washed and stained on the Fluidics Station (Affymetrix) according to the EukGE-WS2v4 protocol. After washing and staining, fluorescent images were scanned at 2 mm resolution, using the Gene Array Scanner. Affymetrix software (MicroArray Suite 5.0; MAS5) was initially used to perform quality control (Q/C) analysis, calculate the signal intensities from each perfect match probe on the arrays relative to the mismatch probe, and to determine whether or not the gene was present in the sample. After obtaining these Q/C metrics, we used the RMA method to normalize the set of four arrays in our screen (GeneTraffic software: Stratagene, La Jolla, CA). The two pairs of gender-matched arrays (male-ethanol/male-control, and female-ethanol/female-control) were compared using a simple pair-wise *t*-test with *P* value adjustment using variance inflation. To explore whether the differentially expressed genes (with at least a 1.3-fold change in expression) belonged to specific functional groups

(clusters), the Gene Ontology membership of each of the 78 genes meeting our selection criterion was examined and compared to that which would have been expected by chance using a hyper-geometric *P* value test (equivalent to a one-sided Fisher's exact test). The Gene Ontology memberships of each probe were obtained using the NetAffx Gene Ontology Browser (Affymetrix).

4.3.3. Real-time quantitative RT-PCR (qRT-PCR)—Specific genes of interest identified from the initial microarray screen were validated using qRT-PCR in individual animal samples. Matching amounts of total RNA (250 ng from each individual rat's OB) were used in a reverse transcription (RT) reaction (Superscript II protocol: Invitrogen, Carlsbad, CA) to synthesize the template for PCR. This reaction was diluted to a final volume of 200 μ l. A Master Mix was used for qRT-PCR that included ABsolute SYBR Green ROX Mix (12.5 μ l), primer mix (0.4 μ l), RNase-free H₂O (11.1 μ l), and cDNA template (1 μ l). 1 μ l of each reaction was used in quadruplicate PCR reactions to assess the levels of expression of each gene of interest (see below) in the individual rat OB samples. The SYBR Green dye in the mixture specifically labeled double-stranded DNA amplicons were the products of the PCR reactions. Once bound, the SYBR Green emitted a fluorescent signal. As the amount of double-stranded product increased after each PCR cycle, the fluorescent signal increased. The number of cycles for each target gene to reach the threshold for detection (Ct) (set at half the maximal fluorescence achieved) was determined for each experimental and pair-fed control animal.

Acknowledgments

Supported by NIH-NIAAA Grant AA014871 (S.L.Y.)

References

- Abate P, Pepino MY, Dominguez HD, Spear NE, Molina JC. Fetal associative learning mediated through maternal alcohol intoxication. *Alcohol: Clin Exp Res.* 2000; 24:39–47. [PubMed: 10656191]
- Abate P, Spear NE, Molina JC. Fetal and infantile alcohol-mediated associative learning in the rat. *Alcohol: Clin Exp Res.* 2001; 25:989–998. [PubMed: 11505023]
- Abel EL, York JL. Absence of effect of prenatal ethanol on adult emotionality and ethanol consumption in rats. *J Stud Alcohol.* 1979; 40:547–553. [PubMed: 491661]
- Alati R, AlMamum A, Williams GMO, Callaghan M, Najman JM, Bor W. In utero alcohol exposure and prediction of alcohol disorders in early adulthood: a birth cohort study. *Arch Gen Psych.* 2006; 63:1009–1016.
- Arias C, Chotro MG. Increased preference for ethanol in the infant rat after prenatal ethanol exposure, expressed on intake and taste reactivity tests. *Alcoholism: Clin Exp Res.* 2005; 29:337–346.
- Baer JS, Sampson PD, Barr HM, Conner PD, Streissguth AP. A 21-year longitudinal analysis of the effects of prenatal alcohol exposure on young adult drinking. *Arch Gen Psych.* 2003; 60:377–386.
- Baer JS, Barr HM, Bookstein FL, Sampson PD, Streissguth AP. Prenatal alcohol exposure and family history of alcoholism in the etiology of adolescent alcohol problems. *J Stud Alcohol.* 1998; 59:533–543. [PubMed: 9718105]
- Barron S, Riley EP. The effects of prenatal alcohol exposure on behavioral and neuroanatomical components of olfaction. *Neurotoxicol Teratol.* 1992; 14:291–297. [PubMed: 1522834]
- Bond NW, Di Giusto EL. Effects of prenatal alcohol consumption on open-field behaviour and alcohol preference in rats. *Psychopharmacology.* 1976; 46:163–165.
- Bonthius DJ, West JR. Acute and long-term neuronal deficits in the rat olfactory bulb following alcohol exposure during the brain growth spurt. *Neurotoxicol Teratol.* 1991; 13:611–619. [PubMed: 1779948]

- Bonthius DJ, Bonthius NE, Napper RM, West JR. Early postnatal alcohol exposure acutely and permanently reduces the number of granule cells and mitral cells in the rat olfactory bulb: a stereological study. *J Comp Neurol.* 1992; 324:557–566. [PubMed: 1430337]
- Brennan PA, Keverne EB. Neural mechanisms of mammalian olfactory learning. *Prog Neurobiol.* 1997; 51:457–481. [PubMed: 9106902]
- Chotro MG, Arias C. Prenatal exposure to ethanol increases ethanol consumption: a conditioned response? *Alcohol.* 2003; 30:19–28. [PubMed: 12878271]
- Chotro MG, Molina JC. Acute ethanol contamination of the amniotic fluid during gestational day 21: postnatal changes in alcohol responsiveness in rats. *Dev Psychobiol.* 1990; 23:535–547. [PubMed: 2272409]
- Chotro MG, Molina JC. Bradycardiac responses elicited by alcohol odor in rat neonates: Influence of in utero experience with ethanol. *Psychopharmacology (Berl).* 1992; 106:491–496. [PubMed: 1579622]
- Chotro MG, Kraebel KS, McKinzie DL, Molina JC, Spear N. Prenatal and postnatal ethanol exposure influences preweanling rats' behavioral and autonomic responding to ethanol odor. *Alcohol.* 1996; 13:377–385. [PubMed: 8836327]
- Chotro MG, Arias C, Laviola G. Increased ethanol intake after prenatal ethanol exposure: studies with animals. *Neurosci Biobehav Rev.* 2007; 31:181–191. [PubMed: 17010438]
- Coopersmith R, Leon M. Enhanced neural response to familiar olfactory cues. *Science.* 1984; 225:849–851. [PubMed: 6474157]
- Coopersmith R, Leon M. Enhanced neural response by adult rats to odors experienced early in life. *Brain Res.* 1986; 371:400–403. [PubMed: 3697770]
- Coopersmith R, Henderson SR, Leon M. Odor specificity of the enhanced neural response following early odor experience in rats. *Brain Res.* 1986; 392:191–197. [PubMed: 3708377]
- Cowell RM, Blake KR, Russell JW. Localization of the transcriptional coactivator PGC-1 α to GABAergic neurons during maturation of the rat brain. *J Comp Neurol.* 2007; 502:1–18. [PubMed: 17335037]
- Dominguez HD, Chotro MG, Molina JC. Alcohol in the amniotic fluid prior to cesarean delivery: effects of subsequent exposure to the drug's odor upon alcohol responsiveness. *Behav Neural Biol.* 1993; 60:129–138. [PubMed: 8117238]
- Driscoll CD, Streissguth AP, Riley EP. Prenatal alcohol exposure: comparability of effects in humans and animal models. *Neurotoxicol Teratol.* 1990; 12:231–237. [PubMed: 2196422]
- Ennis M, Zimmer LA, Shipley MT. Olfactory nerve stimulation activates rat mitral cells via NMDA and non-NMDA receptors in vitro. *Neuroreport.* 1996; 7:989–992. [PubMed: 8804037]
- Ennis M, Zhou FM, Ciombor KJ, Aroniadou-Anderjaska V, Hayar A, Borrelli E, Zimmer LA, Margolis F, Shipley MT. Dopamine D2 receptor-mediated presynaptic inhibition of olfactory nerve terminals. *J Neurophysiol.* 2001; 86:2173–2182. [PubMed: 11698509]
- Faas AE, Sponton ED, Moya PR, Molina JC. Differential responsiveness to alcohol odor in human neonates: effects of maternal consumption during gestation. *Alcohol.* 2000; 22:7–17. [PubMed: 11109023]
- Farbman, AI. Developmental neurobiology of the olfactory system. In: Getchell, TV., editor. *Smell and Taste in Health and Disease.* Raven Press; New York: 1991. p. 19-33.
- Fernandez-Vidal JM, Molina JC. Socially mediated alcohol preferences in adolescent rats following interactions with an intoxicated peer. *Pharmacol Biochem Behav.* 2004; 79:229–241. [PubMed: 15501298]
- Gervais R, Pager J. Olfactory bulb excitability selectively modified in behaving rats after local 6-hydroxydopamine treatment. *Behav Brain Res.* 1983; 9:165–179. [PubMed: 6309193]
- Gervais R, Holley A, Keverne B. The importance of central noradrenergic influences on the olfactory bulb in the processing of learned olfactory cues. *Chem Senses.* 1988; 13:1–12.
- Gesteland RC, Yancey RA, Farbman AI. Development of olfactory receptor neuron selectivity in the rat fetus. *Neuroscience.* 1982; 7:3127–3136. [PubMed: 7162629]
- Handschin C, Spiegelman BM. Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocrine Rev.* 2006; 27:728–735. [PubMed: 17018837]

- Hayar A, Heyward PM, Heinbockel T, Shipley MT, Ennis M. Direct excitation of mitral cells via activation of alpha1-noradrenergic receptors in rat olfactory bulb slices. *J Neurophysiol.* 2001; 86:2173–2182. [PubMed: 11698509]
- Hinds JW, McNelly NA. Aging of the rat olfactory bulb: growth and atrophy of constituent layers and changes in size and number of mitral cells. *J Comp Neurol.* 1977; 171:345–368. [PubMed: 833360]
- Hinds JW, McNelly NA. Aging in the rat olfactory system: correlation of changes in the olfactory epithelium and olfactory bulb. *J Comp Neurol.* 1981; 203:441–453. [PubMed: 7320235]
- Hudson R. From molecule to mind: the role of experience in shaping olfactory function. *J Comp Physiol A.* 1999; 185:297–304. [PubMed: 10555266]
- Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovskaya V, Hörster F, Tenkova T, Dikranian K, Olney JW. Ethanol-Induced Apoptotic Neurodegeneration and Fetal Alcohol Syndrome. *Science.* 2000; 287:1056–1060. [PubMed: 10669420]
- Isaacson JS, Strowbridge BW. Olfactory reciprocal synapses: Dendritic signaling in the CNS. *Neuron.* 1998; 20:749–761. [PubMed: 9581766]
- Kosaka T, Hataguchi Y, Hama K, Nagatsu I, Wu JY. Coexistence of immunoreactivities for glutamate decarboxylase and tyrosine hydroxylase in some neurons in the periglomerular region of the rat main olfactory bulb: possible coexistence of gamma-aminobutyric acid (GABA) and dopamine. *Brain Res.* 1985; 343:166–171. [PubMed: 2864104]
- Kosaka T, Kosaka K, Heizmann CW, Nagatsu I, Wu JY, Yanaihara N, Hama K. An aspect of organization of the GABAergic system in the rat main olfactory bulb: laminar distribution of immunohistochemistry defined subpopulations of GABAergic neurons. *Brain Res.* 1987; 411:373–378. [PubMed: 2886184]
- Laing DG, Panhuber H. Olfactory sensitivity of rats reared in an odorous or deodorized environment. *Physiol Behav.* 1980; 25:555–558. [PubMed: 7208652]
- Laing DG, Panhuber H, Pittman EA, Willcox ME, Eagleson GK. Prolonged exposure to an odor or deodorized air alters the size of mitral cells in the olfactory bulb. *Brain Res.* 1985; 336:81–87. [PubMed: 4005578]
- Leshchynska I, Sytnyk V, Morrow JS, Schachner M. Neural cell adhesion molecule (NCAM) association with PKCbeta2 via beta1 spectrin is implicated in NCAM-mediated neurite outgrowth. *J Cell Biol.* 2003; 161:625–639. [PubMed: 12743109]
- McGivern RF, Clancy AN, Mousa S, Couri D, Noble EP. Prenatal alcohol exposure alters enkephalin levels, without affecting ethanol preference. *Life Sci.* 1984; 34:585–589. [PubMed: 6694536]
- McLean JH, Harley CW. Olfactory learning in the rat pup: a model that may permit visualization of a mammalian memory trace. *Neuroreport.* 2004; 15:1691–1697. [PubMed: 15257129]
- McLean JH, Harley CW, Darby-King A, Yuan Q. pCREB in the neonate rat olfactory bulb is selectively and transiently increased by odor preference-conditioned training. *Learn Mem.* 1999; 6:608–618. [PubMed: 10641765]
- Mennella JA. The transfer of alcohol to human milk: sensory implications and effects on mother–infant interaction. In: Hannigan, JH.; Spear, LP.; Spear, NE.; Goodlett, CR., editors. *Alcohol: Effects in Brain and Development.* Erlbaum; Mahwah, NJ: 1999. p. 177–198.
- Mennella JA. Regulation of milk intake after exposure to alcohol in mothers' milk. *Alcoholism: Clin Exp Res.* 2001; 25:590–593.
- Mennella JA, Beauchamp GK. Infants' exploration of scented toys: effects of prior experiences. *Chem Senses.* 1998; 23:11–17. [PubMed: 9530965]
- Miller MW. Circadian rhythm of cell proliferation in the telencephalic ventricular zone: effect of in utero exposure to ethanol. *Brain Res.* 1992; 595:17–24. [PubMed: 1467954]
- Molina, JC.; Chotro, MG.; Dominguez, HD. Fetal alcohol learning resulting from alcohol contamination of the prenatal environment. In: Lecanuet, J.; Fifer, WP.; Krasnegor, NA.; Smotherman, WP., editors. *Fetal Development: A Psychobiological Perspective.* Erlbaum; Hillsdale, NJ: 1995. p. 419–438.
- Molina, JC.; Dominguez, HD.; Lopez, MF.; Pepino, MY.; Faas, AE. The role of fetal and infantile experience with alcohol in later recognition and acceptance patterns of the drug. In: Hannigan,

- JH.; Spear, LP.; Spear, NE.; Goodlett, CR., editors. Alcohol: Effects on Brain and Development. Erlbaum; Mahwah, NJ: 1999. p. 199-228.
- Molina JC, Spear NE, Spear LP, Mennella JA, Lewis MJ. Alcohol and development: beyond fetal alcohol syndrome. *Dev Psychobiol.* 2007; 49:227–242. [PubMed: 17380525]
- Nicoll RA. Pharmacological evidence for GABA as the transmitter in granule cell inhibition in the olfactory bulb. *Brain Res.* 1971; 35:137–149. [PubMed: 4332422]
- Nyquist-Battie C, Gochee A. Alterations in the development of the mouse after ethanol exposure. *Int J Dev Neurosci.* 1985; 3:211–222.
- Olney JW, Wozniak DF, Jevtovic-Todorovic V, Farber NB, Bittigau P, Ikonomidou C. Glutamate and GABA receptor dysfunction in the fetal alcohol syndrome. *Neurotoxicol Res.* 2002; 4:315–325.
- Panhuber H, Laing DG. The size of mitral cells is altered when rats are exposed to an odor from their day of birth. *Brain Res.* 1987; 431:133–140. [PubMed: 3620982]
- Panhuber H, Mackay-Sim A, Laing DG. Prolonged odor exposure causes severe cell shrinkage in the adult rat olfactory bulb. *Brain Res.* 1987; 428:307–311. [PubMed: 3828836]
- Phillips D, Stainbrook G. Effects of early alcohol exposure upon adult learning abilities and taste preferences. *Physiol Psychol.* 1976; 4:473–475.
- Rehn B, Panhuber H, Laing DG, Breipohl W. Spine density on olfactory granule cell dendrites is reduced in rats reared in a restricted olfactory environment. *Brain Res.* 1988; 468:143–147. [PubMed: 2454148]
- Riback CE, Vaughn JE, Saito K, Barber R, Roberts E. Glutamate decarboxylase localization in neurons of the olfactory bulb. *Brain Res.* 1977; 126:1–18. [PubMed: 856413]
- Schevzov G, Gunning P, Jeffrey PL, Temm-Grove C, Helfman DM, Lin JJ, Weinberger RP. Tropomyosin localization reveals distinct populations of microfilaments in neurites and growth cones. *Mol Cell Neurosci.* 1997; 8:439–454. [PubMed: 9143561]
- Spear LP. The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev.* 2000; 24:417–463. [PubMed: 10817843]
- Spear, NE.; Molina, JC. Consequences of early exposure to alcohol: how animal studies reveal later patterns of use and abuse in humans. In: Carroll, M.; Overmier, B., editors. *Animal Research and Human Health*. APA; Washington DC: 2001. p. 85-99.
- Spear NE, Molina JC. Fetal or infantile exposure to ethanol promotes ethanol ingestion in adolescence and adulthood. *Alcoholism: Clin Exp Res.* 2005; 29:909–929.
- Streissguth, A. Teratogenic and genetic influences on adolescent and adult alcohol use and abuse. Symposium presented at the annual meeting of the Research Society on Alcoholism; Hilton Head, SC. 1998.
- Sulik KK, Johnston MC. Sequence of developmental alterations following acute ethanol exposure in mice: craniofacial features of the fetal alcohol syndrome. *Am J Anat.* 1983; 166:257–269. [PubMed: 6846205]
- Sullivan RM, Wilson DA, Leon M. Norepinephrine and learning-induced plasticity in infant rat olfactory system. *J Neurosci.* 1989; 9:3998–4006. [PubMed: 2585063]
- Tabuchi K, Biederer T, Butz S, Sudhof T. CASK participates in alternative tripartite complexes in which mint 1 competes for binding with caskin 1, a novel CASK-binding protein. *J Neurosci.* 2002; 22:4264–4273. [PubMed: 12040031]
- Vavrousek-Jakuba EM, Baker RA, Shoemaker WJ. Effect of ethanol on maternal and offspring characteristics: comparison of three liquid diet formulations fed during gestation [erratum appears in *Alcoholism: Clin Exp Res* 1992;16, 479]. *Alcoholism: Clin Exp Res.* 1991; 15:129–135.
- Wilson DA, Leon M. Spatial patterns of olfactory bulb single-unit responses to learned olfactory cues in young rats. *J Neurophysiol.* 1988a; 59:1770–1782. [PubMed: 3404204]
- Wilson DA, Leon M. Noradrenergic modulation of olfactory bulb excitability in the postnatal rat. *Dev Brain Res.* 1988b; 42:69–75.
- Wilson DA, Sullivan RM. Neurobiology of associative learning in the neonate: early olfactory learning. *Behav Neural Biol.* 1994; 61:1–18. [PubMed: 7907468]

- Yates WR, Cadoret RJ, Troughton EP, Stewart M, Giunta TS. Effect of fetal alcohol exposure on adult symptoms of nicotine, alcohol, and drug dependence. *Alcoholism: Clin Exp Res.* 1998; 22:914–920.
- Young WS, Kuhar MJ. Noradrenergic alpha 1 and alpha 2 receptors: light microscopic autoradiographic localization. *Proc Natl Acad Sci U S A.* 1980; 77:1696–1700. [PubMed: 6246501]
- Youngentob SL. A method for the rapid automated assessment of olfactory function. *Chem Senses.* 2005; 30:219–229. [PubMed: 15741600]
- Youngentob SL, Mozell MM, Sheehe PR, Hornung DE. A quantitative analysis of sniffing strategies in rats performing odor detection tasks. *Physiol Behav.* 1987; 41:59–69. [PubMed: 3685154]
- Youngentob SL, Kent PF, Sheehe PR, Molina JC, Spear NE, Youngentob LM. Experience-induced fetal plasticity: the effect of gestational ethanol exposure on the behavioral and neurophysiologic olfactory response to ethanol odor in early postnatal and adult rats. *Behav Neurosci.* 2007a; 121:1293–1305. [PubMed: 18085882]
- Youngentob SL, Molina JC, Kent PF, Spear NE, Youngentob LM. The effect of gestational ethanol exposure on voluntary ethanol intake in early postnatal and adult rats. *Behav Neurosci.* 2007b; 121:1306–1315. [PubMed: 18085883]
- Yuan Q, Harley CW, McLean JH, Knopfel T. Optical imaging of odor preference memory in the rat olfactory bulb. *J Neurophysiol.* 2002; 87:3156–3159. [PubMed: 12037216]
- Yuan Q, Harley CW, McLean JH. Mitral cell beta1 and 5-HT2A receptor colocalization and cAMP coregulation: a new model of norepinephrine-induced learning in the olfactory bulb. *Learn Mem.* 2003; 10:5–15. [PubMed: 12551959]

Fig. 1. Composite reflexive sniffing indexes values as a function of sex and maternal treatment. The values represent the mean composite index values $\pm$ the standard error. ET: fetal-ethanol-exposed rats, PF: pair-fed rats. See text for details.

Fig. 2.

Significant changes in expression seen in the OB of young adult rats prenatally exposed to ethanol. “Volcano” plot displays the relationship between the corrected *P* value and the mean log₂ ratio of the change seen in the OB of 8 ethanol-treated rats compared to 8 pair-fed control rats. The total RNA from the OB of four rats of each gender in each condition was pooled into a single sample for the screen, and the two pairs of pooled samples were compared using a pair-wise *t*-test, with variance inflation to identify the most robustly affected genes (enclosed in the boxed in areas) in this simple screen.

Fig. 3.

Confirmation of microarray changes by individual animal qRT-PCR analysis. (A) Five genes of interest relative to one reference gene (18S rRNA) were measured in the individual RNA samples that had been previously combined for the microarray screen. Individual gene comparisons between control and fetal ethanol exposed animals were based on Scheffe's criterion. Data are expressed as the mean ΔC_t value $\pm$ SE. PF=pair-fed control group; ET=fetal ethanol exposed group. (B) The data highlights the mean fold-change in expression for each gene in the array and PCR data.

Fig. 4.

GABA genes are affected by prenatal ethanol exposure. Fig. 4 plots the mean log₂ ratios for 49 transcripts involved in GABA signaling or metabolism. The distribution clearly illustrates the presence of genes with strongly decreased expression, and the absence of GABA related genes with strong increases in expression.

Table 1

List of fourteen reflexive sniffing variables

1	Sniffing frequency (Hz)
2	Average volume of an expiratory sniff
3	Average volume of an inspiratory sniff
4	Number of expiratory sniffs
5	Number of inspiratory sniffs
6	Average duration of an expiratory sniff
7	Average duration of an inspiratory sniff
8	Average flow rate of an expiratory sniff
9	Average flow rate of an inspiratory sniff
10	Total expiratory volume
11	Total inspiratory volume
12	Average peak flow rate of an expiratory sniff
13	Average peak flow rate of an inspiratory sniff
14	Total apneic period (ms)

Table 2

Genes with significant changes in expression in the OH of adult rats exposed to prenatal ethanol

Affymetrix ID	Gene name	Mean fold change	Raw <i>P</i> value	Adjusted <i>P</i> value
<i>Increased expression</i>				
1387617_at;	Tropomyosin 3, gamma	3.88	0.008	0.014
1377451_at;	EST; sim to <i>M. musculus</i> La ribonucleoprotein domain family member 4	2.58	0.018	0.025
1390722_at;	EST; sim to <i>M. musculus</i> nuclear casein kinase and cyclin-dependent kinase substrate	12.33	0.007	0.016
1390740_at;	Heterogeneous nuclear ribonucleoprotein A1 (Helix-destabilizing protein)	2.29	0.025	0.034
1375707_at;	58K membrane-associated protein	2.15	0.019	0.028
1375598_at;	Calcium/calmodulin-dependent protein kinase IV	1.95	0.002	0.013
1392928_at;	EST; <i>R. norvegicus</i> similar to RIKEN cDNA 2810055F11	1.86	0.009	0.021
1390659_at;	CD44 antigen	1.85	0.035	0.047
1370728_at;	Interleukin 13 receptor, alpha 1	1.78	0.015	0.028
1374453_at;	Similar to plexin 1	1.76	0.036	0.049
1390469_at;	Nurim (nuclear envelope membrane protein)	1.74	0.020	0.033
1377529_at;	CAMP-regulated guanine nucleotide exchange factor 11	1.71	0.026	0.040
1368801_at;	CXXC finger4	1.66	0.027	0.042
1368013_at;	DNA-damage-inducible transcript 4-like	1.62	0.028	0.044
1374259_at;	EST; sim to <i>H. sapiens</i> pleiomorphic adenoma gene 1	1.61	0.016	0.032
1369172_at;	Phosphodiesterase 1A, calmodulin-dependent	1.55	0.002	0.019
1374786_at;	Cytotoxic granule-associated RNA banding protein 1	1.55	0.029	0.046
1388591_at;	DNA cross-link repair 1B, PS02 homolog (<i>S. cerevisiae</i>)	1.54	0.006	0.024
1398290_at;	Potassium channel, subfamily K. member 13	1.52	0.026	0.044
1378945_at;	Aconitase 2, mitochondrial	1.50	0.006	0.025
1368186_at;	Solute carrier family 34 (sodium phosphate), member 2	1.42	0.008	0.029
<i>Decreased expression</i>				
1370998_at;	Glutamate receptor, metabotropic 2	-2.86	0.017	0.024
1387275_at;	SRY-box containing gene 11	-2.54	0.020	0.028
1370089_at;	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	-2.53	0.038	0.046
1368708_a_at;	Dopamine receptor 2	-2.22	0.029	0.039
1390249_at;	Similar to DKFZP434H132 protein	-2.16	0.012	0.022
1368439_at;	SRY-box containing gene 10	-1.86	0.007	0.019
1369295_at;	Progastricsin (pepsinogen C)	-1.81	0.025	0.037
1388030_a_at;	Gamma-aminobutyric acid (GABA) B receptor 1	-1.73	0.035	0.049
1374191_at;	Rhomboid family 1 (<i>Drosophila</i>)	-1.69	0.022	0.036
1368425_at;	Cask interacting protein 1	-1.68	0.033	0.047
1377433_at;	Zinc finger protein 580	-1.63	0.021	0.036
1389598_at;	Ceroid-lipofuscinosis, neuronal 6	-1.63	0.019	0.035
1368421_at;	Protein tyrosine phosphatase, non-receptor type 5	-1.62	0.026	0.042
1376156_at;	Glycosyltransferase (ago6l gene)	-1.58	0.026	0.043

Affymetrix ID	Gene name	Mean fold change	Raw <i>P</i> value	Adjusted <i>P</i> value
1387800_at;	Fas death domain-associated protein	-1.57	0.029	0.046
1367998_at;	Secretory leukocyte peptidase inhibitor	-1.56	0.001	0.018
1370473_a_at;	Protein tyrosine phosphatase, non receptor type 23	-1.56	0.006	0.023
1376564_at;	Spectrin beta 4	-1.55	0.000	0.018
1376845_at;	Putative ISG12(b) protein	-1.54	0.003	0.020
1373561_at;	Chondroitin polymerizing factor	-1.53	0.011	0.029
1372063_at;	Similar to novel protein of unknown function (DUF423) family member	-1.52	0.005	0.023
1388917_at;	Myosin IC	-1.52	0.029	0.047
1389071_at;	Tangerin	-1.48	0.028	0.048
1375996_at;	Septin 1	-1.46	0.028	0.047
1390253_at;	Ankyrin repeat domain 9	-1.46	0.015	0.035
1387720_at;	Calsyntenin 2	-1.44	0.029	0.050
1389209_at;	Similar to K11G12.6.p (<i>Caenorhabditis elegans</i>)	-1.43	0.016	0.037
1373414_at;	Brain specific membrane-anchored protein precursor	-1.42	0.007	0.029
1367886_at;	Proprotein convertase subtilisin/kexin type 7	-1.40	0.009	0.032
a1368534_at;	Adrenergic receptor, alpha 1d	-2.42	0.072	0.205

^a Adra1d is included because it was decreased in both male and female ethanol-exposed OB samples and was chosen for real time PCR.

Gene Ontology (GO) groups containing disproportionate enrichment with significantly changed genes

Table 3

GO category	GO ID	GO cluster	Array probes	List probes	Array percent	List percent	Fold enrichment	Hypergeometric P value
Biological	7270	Nerve-nerve synaptic transmission	95	3	1.1%	8.6%	7.8	0.0060
Cellular	16604	Nuclear body	102	3	1.1%	7.9%	6.9	0.0082
Biological	14070	Response to organic cyclic substance	124	3	1.4%	8.6%	6.0	0.0120
Biological	9968	Negative regulation of signal transduction	137	3	1.6%	8.6%	5.4	0.0155
Molecular	3712	Transcription cofactor activity	149	3	1.5%	7.7%	5.0	0.0189
Biological	7417	Central nervous system development	326	6	3.8%	17.1%	4.5	0.0015
Biological	50804	Regulation of synaptic transmission	163	3	1.9%	8.6%	4.5	0.0238
Cellular	44456	Synapse part	209	4	2.3%	10.5%	4.5	0.0097
Biological	51969	Regulation of transmission of nerve impulse	167	3	1.9%	8.6%	4.4	0.0252
Cellular	15629	Actin cytoskeleton	226	4	2.5%	10.5%	4.2	0.0124
Cellular	30424	Axon	170	3	1.9%	7.9%	4.2	0.0294

Table 4

Gene primers chosen for real time qRT-PCR validation

Primer	Length	Tm	% GC	Sequence	Product size (bp)
GABA B1					
Left	21	57.8	47.6	ccgtggaciatgagatcgaa	150
Right	22	57.9	45.0	cagggtcaataaagactggag	
mGluR2					
Left	22	55.8	50.0	ggagatgaccagtgctactgt	188
Right	22	56.9	50.0	ggacaactactgggtaaggag	
DR92					
Left	22	57.0	45.5	tgatccccctactctccaat	131
Right	21	57.3	47.6	caagtaccacatccagcttc	
Caskin 1					
Left	22	57.0	50.0	atactgactggctctctcctgt	165
Right	23	57.4	47.8	ccgtgtccacacactaccitagt	
Adrald					
Left	20	55.1	50.0	ctactcttactgtccacctg	147
Right	19	54.8	45.0	gttaggagtgtgggaaga	