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Moderate Prenatal Alcohol Exposure Alters Functional Connectivity in the Adult Rat Brain

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Abstract

Background—Past studies of moderate prenatal alcohol exposure (PAE) have focused on specific brain regions, neurotransmitter systems, and behaviors. However, the effects of PAE on brain function and behavior are complex and not limited to discrete brain regions. Thus, there is a critical need to understand the global effects of moderate PAE on neural function. A primary aim of this research was to explore the functional relationships in neural activity of spatially distinct areas by applying a widely-used computational algorithm, group-independent component analysis (ICA), to resting state fMRI data from rats exposed to either an alcohol or saccharin control solution via maternal consumption during pregnancy.

Methods—Long-Evans rat dams consumed either 5% (v/v) alcohol or a saccharin control solution throughout gestation. Adult offspring from each prenatal treatment group were anesthetized for functional, structural, and perfusion magnetic resonance-based image acquisition sequences. Group ICA was applied to the functional data to extract components. To determine connectivity, component time-course correlations were computed and compared. Additionally, spectral power analyses were utilized as an additional measure of functional connectivity. Finally, blood perfusion—assessed by arterial spin labeling (ASL)—and whole brain volumetric analyses were evaluated.

Results—Analyses revealed 17 components in several brain regions such as the cortex, hippocampus, and thalamus. PAE was associated with reductions in coordinated activity between components, especially in males. PAE was also associated with reductions in low frequency spectral power, an effect that was more robust in females. Brain volumetric analyses revealed sex-

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dependent reductions in females while blood flow analyses revealed sex-dependent reductions in males.

Conclusion—Moderate PAE leads to persistent changes in functional connectivity in the absence of whole-brain volume or blood flow measures. Future studies will investigate the relationships between alterations in functional network connectivity and behavior.

Keywords

Prenatal alcohol exposure; fetal alcohol spectrum disorders; functional MRI (fMRI); brain connectivity

Introduction

Fetal alcohol spectrum disorders (FASD) include a broad range of morphological and cognitive-behavioral outcomes that result from prenatal exposure to alcohol (Kodituwakku, 2007). The most severe FASD, Fetal Alcohol Syndrome (FAS), is characterized by profound morphological consequences, facial dysmorphologies (Jones and Smith, 1973, Jones and Smith, 1975), and an array of cognitive and behavioral impairments related to attention, learning, memory, and executive function (Kodituwakku, 2007). While the prevalence of FAS is comparatively low (~0.1%), it is estimated that for every child with FAS, there are at least five with a less severe FASD (May and Gossage, 2001) that lack the facial abnormalities characteristic of FAS, but nonetheless display subtle yet persistent deficits in behavior (Kodituwakku, 2007).

In addition to the clinical, epidemiological and neuropsychological characterizations of FASD, animal models have been informative for understanding the genetic, neuro-structural and physiological mechanisms associated with prenatal alcohol exposure (PAE). While much attention has been placed on the effects of PAE on discrete brain areas such as the hippocampus (Savage et al., 2002), cerebellum (Servais et al., 2007), neocortex (Hamilton et al., 2010b), and neurotransmitter systems (Valenzuela et al., 2011), less is known about the broader alterations in brain connectivity.

Some laboratories have investigated the effects of PAE on the structural connectivity of rodent brains (O'Leary-Moore et al., 2011). These studies have focused on elucidating the effects of PAE on white matter quality. However, structural connectivity, whether altered or not, does not rule out PAE-related changes in brain function. Functional network connectivity (FNC) provides an additional perspective needed to understand the functional relationships within and among spatially distinct brain regions.

Functional connectivity can be examined with resting state functional MRI (fMRI). During resting state fMRI, blood-oxygenation-level-dependent (BOLD) signals are measured in experimental conditions that lack imposed stimuli, cognitive, or behaviorally salient events (Snyder and Raichle, 2012). BOLD signals reflect patterns of neural activity that are coupled to neurovascular mechanisms regulating blood flow (Logothetis et al., 2001), and are believed to covary with the overall level of activity in the underlying brain region. The aim of FNC analyses is to capture the statistical patterns of endogenously generated neural

activity (Snyder and Raichle, 2012) based on correlations in BOLD signals among brain regions.

Recent animal studies have characterized resting state FNC in anesthetized monkeys (Goense and Logothetis, 2008, Vincent et al., 2007), cats (Popa et al., 2009), and mice (White et al., 2011). Moreover, a recent investigation showed that applying a hypothesis free, data driven analysis technique called independent component analysis (ICA) to fMRI resting state data revealed reproducible patterns of spatio-temporal activity thought to reflect functional connectivity in a rodent model (Hutchison et al., 2010). ICA is an algorithm-dependent blind source data exploration technique adapted to extract coherent BOLD signals from various sources (Bell and Sejnowski, 1995). When applied to fMRI data, ICA identifies groups of voxels that are spatially distinct and statistically independent called components. Voxels within a given component are described as possessing highly related temporal activations (time-courses). One variant of ICA, group ICA (gICA), extracts components at the group level from aggregated subject data. Group level components are then projected to individual data sets to examine intersubject variability in component time-courses. In addition to allowing statistical comparisons between groups of subjects, gICA also reliably reveals resting state networks observed in seed-based approaches in rodents and humans (Hutchison et al., 2010, Damoiseaux et al., 2006).

In the present study, gICA was applied to resting state fMRI data obtained from adult rats that were exposed to moderate levels of alcohol during prenatal development and control rats exposed to a saccharin solution by way of maternal consumption. Our primary goals were to evaluate the effects of moderate PAE on functional connectivity, and to evaluate the utility of gICA as a means to identify alterations in component activity and connectivity as a result of moderate PAE.

Methods

Subjects, Breeding Procedures, & Prenatal Alcohol Exposure

All breeding and experimental procedures were approved by The University of New Mexico's Institutional Animal Care and Use Committee (IACUC). Breeding procedures were conducted at the University of New Mexico HSC-Animal Resource Facility. Three-to four month-old Long-Evans rat breeders (Harlan Industries, Indianapolis, IN) were single-housed in plastic cages kept at 22°C and on a reverse 12-h dark/12-h light schedule (lights on from 2100 to 0900 h). Prenatal exposure procedures were previously described in (Hamilton et al., 2014). Following a 1-week acclimation period, all female rats were provided with 0.066% saccharin in tap water solution for 4 hours each day from 1000 to 1400 h. During days 1 and 2, the saccharin solution contained 0% ethanol, on days 3 and 4, the saccharin solution contained 2.5% ethanol (v/v). During day 5 and thereafter, the saccharin solution contained 5% ethanol (v/v). Daily 4-h consumption of ethanol was monitored for at least 2 weeks and mean daily ethanol consumption was calculated for each female breeder. Females that consumed one standard deviation above or below the mean of the entire group were eliminated from the study. The remaining females were placed with proven male breeders until pregnancy, as indicated by the presence of a vaginal plug, was confirmed. Female rats did not consume ethanol during the breeding period. Once pregnant,

females were placed in either a saccharin-control or 5% ethanol treatment condition. Prenatal treatment groups were matched so that the mean pre-pregnancy ethanol consumption by each group was similar. Beginning on gestational day 1 and thereafter, rat dams were provided with saccharin water solution or 5% ethanol solution from 1000 to 1400 h. Rat chow and water were available *ad libitum* during both the drinking and non-drinking portions of the breeding paradigm. Rat pups were culled to litters of ten pups, weaned at 24 days of age, and then same-sex group-housed (either two males or three females per cage) until the imaging procedures were performed at three to four months of age. A total of 24 male rats (12 each ethanol (PAE) and saccharin (SAC)) and 24 female rats (12 each ethanol and saccharin) were used for this study. To reduce potential litter effects, no more than 1-2 animals per litter were included in this study. All animals were housed with a same-sex cage-mate.

MRI Data Acquisition

Rats were anesthetized with a 1.0-2.3% isoflurane/ 95% oxygen mixture that was modulated in-session to maintain respiratory rate at 40-50/min and underwent two magnetic resonance imaging sequences in a 4.7 T Bruker Biospin (Billerica, MA) MRI scanner. Temperature and respiration were monitored using a computer running SAM PC software and a model 1025 monitoring system (SA Instruments Inc., Stony Brook, NY). While anesthetized, structural MRI data were acquired with a RARE-8 T1 image sequence [FOV=3.84cm×2.88cm, matrix=256×192, TE=56ms, flip angle=180°, slice thickness=1mm]. Functional MRI data were acquired from subjects during a 10 min echo-planar imaging (EPI) sequence [FOV=3.84cm×3.84cm, matrix=64×64, TR=2000ms, TE=21.3ms, flip angle=90°, slice thickness=1mm]. An additional FAIR-RARE arterial spin labeling (ASL) sequence [FOV=3.84cm×3.84cm, matrix=128×128, TE=46ms, flip angle=180°, slice thickness=1mm] was utilized to measure blood flow.

Blood Flow and Whole-Brain Volume Analyses

Blood flow values (ml/100g/min) were computed using the Bruker BioSpin Paravision 5.1 software and ASL analysis macro in one coronal slice of frontal cortex, approximately +3.00 mm from bregma in Paxinos & Watson space (Paxinos and Watson, 2004). The resulting values were analyzed using a two factor analysis of variance (ANOVA) with prenatal treatment and sex as factors.

Whole-brain volume estimates, per animal, were gathered from 19-21 T1-weighted MRI images in ImageJ 1.45s (Wayne Rasband, National Institutes of Health, USA). A region of interest was drawn around coronal sections of cortical tissue using the free-hand selection tool. Area values were calculated with the Measure function and converted to volume by multiplying area by slice thickness (1mm). The sum of slice volumes provided whole brain volume estimates. Slices included the first anterior section showing cortical tissue and continued until the last slice that displayed cerebellar tissue.

Functional Image Preprocessing

Functional images were realigned, spatially normalized to a template (Schweinhart et al., 2003) corresponding to the Paxinos & Watson atlas (Paxinos and Watson, 2004), and

smoothed with a 0.5 mm full-width-half-maximum (FWHM) Gaussian kernel. All pre-processing steps were conducted in Statistical Parametric Mapping 8 (SPM8; Wellcome Department of Cognitive Neurology, London, UK) and MATLAB software (Mathworks, Inc., Natick, MA) version R2012b.

Group Independent Component Analysis (gICA)

Following preprocessing, the Infomax algorithm (Bell and Sejnowski, 1995) implemented in the Group ICA of fMRI Toolbox (GIFT, <http://mialab.mrn.org/software/gift/index.html>) was selected to extract 40 group-level components. Group-level, aggregate, components were then projected onto individual subject datasets for use in statistical analyses, a process known as backwards-reconstruction (Calhoun and Adali, 2012, Erhardt et al., 2011). Components with artifactual features were removed from further analyses while the remaining components were sorted and labeled based on the location of the peak intensity value in Paxinos & Watson space (Paxinos and Watson, 2004). Criteria for artifactual features consisted of the presence of signal loss or field inhomogeneities upon component visual inspection and physiologic noise upon visual time-course inspection. Average component time-courses were utilized in subsequent analyses.

Functional Network Connectivity (FNC)

Individual components were associated with an average time-course (BOLD-signal fluctuations over time) and the FNC toolbox (FNC, <http://mialab.mrn.org/software/fnc>) was used to determine pairwise correlations between individual component time-courses for each subject. Average correlations for the overall sample and separately for each combination of prenatal treatment and sex were calculated and subjected to one-sample t-tests for statistical significance. The number of possible pairwise correlations was determined by calculating

$\frac{C * (C - 1)}{2}$ where $C = \text{the number of components}$. In this study, a total of 136 pairwise correlations per each combination of prenatal treatment and sex were calculated. Statistical thresholds were determined *a priori* at $p < 0.0005$ and $p < 0.000025$ to enhance contrast among an expected high number of statistically significant effects. However, a post hoc Bonferroni correction—based on the number of final components—yielded a critical value of $p < 0.00037$ ($.05/136$). Thus the lower threshold (0.000025) was a more conservative statistical threshold than provided by the Bonferroni correction.

ANOVAs were performed with the ANOVA Toolbox in Matlab. Separate two-way ANOVAs were performed for each correlation with prenatal treatment and sex as factors. ANOVA alpha values were chosen *a priori* at the traditional $p < 0.05$ and a more conservative $p < 0.005$ to create contrast of statistically significant effects.

Spectral Analyses (Time-courses)

Aggregate component time-course of BOLD signal fluctuations were assessed for spectral power. Power within the 0-0.25Hz range was averaged into six equally-sized bins for each component. Separate ANOVAs were performed with the ANOVA Toolbox in Matlab within each bin and with prenatal treatment and sex as factors. To provide a comprehensive assessment of prenatal treatment effects on spectral power, separate individual sample t-tests

for the prenatal treatment factor are also reported for each sex. Statistical significance was assessed for uncorrected ($p < 0.05$) and corrected for the number of bins ($p < (0.05/6)$ or $p < 0.0083$).

Results

Prenatal Ethanol Exposure Paradigm

Table 1 summarizes maternal consumption of ethanol and gestational data of rat dams and pups. Daily four-hour ethanol consumption in g/kg of body weight were consistent before breeding and throughout gestation. Maternal serum blood ethanol concentrations averaged 60.8 ($\pm$ 5.8) mg/dL 45 minutes after the introduction of the drinking tubes. Maternal weight gain between saccharin and PAE animals was comparable. Litter size across both prenatal treatment groups was consistent at 12.2-12.5 live fetal pups/litter. Pup birth weight in grams was also comparable. Maternal and pup data suggest that this paradigm for moderate PAE induces negligible effects on maternal weight gain, litter size, and pup birth weight.

Arterial Spin Labeling & Whole Brain Volume

The following section describes the results of from the Arterial Spin Labeling analysis which measured average blood perfusion in the frontal cortex and estimates of whole brain volume. Average blood perfusion measures in ml/100g/mi were analyzed in a two-way ANOVA with prenatal treatment and sex as between subjects factors and revealed no significant interaction [$F(1,44) = .444$, $p=0.51$] or significant main effect of prenatal treatment [$F(1,44) = .215$, $p = 0.645$; data not shown]. A significant effect of sex was observed [males < females; $F(1,44) = 15.8$, $p < 0.001$].

Whole brain volume was estimated by quantifying volume (per slice) of multiple T1 weighted MR images and analyzed in a two-way ANOVA with prenatal treatment and sex as factors and revealed no significant interaction [$F(1,44) = .142$, $p=0.708$] or main effect of prenatal treatment [$F(1,44) = .279$, $p=0.600$; data not shown]. There was, however, a significant effect of sex [females < males; $F(1,44) = 127.4$, $p<0.001$].

Group ICA Components

Upon visual inspection, 16 of the 40 extracted components were excluded due to the presence of artifactual features. An additional 7 components localized to white matter pathways were excluded. A total of 17 non-artifactual components were retained for subsequent analyses (Figure 1). Components were distributed across the entire brain and organized into striatal, thalamic, cortical, hippocampal, midbrain, and cerebellar groups based on anatomical location of component peak values. Individual components within a group were ordered from anterior to posterior, numbered within their grouping, and referred to in an abbreviated form indicating the region and number. Overall, 2 striatal (St1-St2), 2 thalamic (T1-T2), 4 cortical (Cx1-Cx4), 5 hippocampal (H1-H5), 3 midbrain (Mb1-Mb3), and 1 cerebellar (C1) components were retained. Table 2 displays regional groupings, component labels, and the corresponding anatomical region of the peak component values in anatomical coordinates (Paxinos and Watson, 2004).

Connectivity (average correlation between component time-courses)

A two-way omnibus ANOVA conducted on measures of connectivity yielded twelve significant Prenatal Treatment X Sex interactions (Figure 2A), which included component relationships among the following areas: St-T, St-Cx, T-H, T-Mb, Cx-Cx, Cx-H, Cx-Mb, Cx-C, H-Mb, H-C, and Mb-C. Of these, one was significant at the $p < 0.005$ threshold while the remaining correlations were significant at $p < 0.05$.

The ANOVA revealed prenatal treatment main effects for several between-component correlations, including St-Cx, St-H, St-C, Cx-Cx, Cx-Mb, Cx-Mb and Mb-C relationships (Figure 2B). Of the ten significant prenatal treatment effects, eight reached statistical significance at the $p < 0.05$ level while the remaining two differences reached the $p < 0.005$ significance level.

Planned two-sample t-tests on the overall treatment FNC (PAE - SAC), revealed differences involving cortical, midbrain, and cerebellar component correlations (Figure 3A). A total of nine significant effects between prenatal treatment groups were detected: two of the nine effects reached $p < 0.05$ threshold and resulted in greater connectivity for PAE animals between St-Cx, St-H, Cx-Cx, and Cx-C co-activations. An additional effect reached $p < 0.005$ threshold and displayed stronger PAE connectivity between St-C components. Five effects reached the $p < 0.05$ threshold, but displayed weaker connectivity in PAE animals for Cx-Cx, Cx-Mb, St-H, and Mb-C co-activations. The remaining effect reached $p < 0.005$ and displayed weaker connectivity between Cx-Mb components in PAE animals.

Although exploring sex differences in FNC, independent of the effects of moderate PAE, was not a primary goal of the present study, sex effects from the omnibus ANOVA are included here for completeness. Ten significant sex main effects were observed (Figure 2C). Effects were found in St-St, St-T, St-Cx, T-H, Cx-H, Cx-Mb, Cx-C, H-H, and H-Mb correlations. Nine of the ten differences reached $p < 0.05$ threshold while the remaining significant difference reached the $p < 0.005$ threshold.

FNC data for saccharin females are displayed in Figure 4A. The majority of significant correlations were found for H-Mb, Cx-H, and Cx-Mb relationships. A total of 30 significant relationships were observed; 15 at $p < 0.0005$ and 15 at $p < 0.00025$. The majority of significant correlations were in the positive direction (20) compared to the negative direction (10). PAE females displayed a total of 21 significant relationships (Figure 4B); 13 at $p < 0.0005$ and 8 at $p < 0.00025$. The correlations that were most frequently significant occurred between Cx-H, Cx-Mb, H-H, and H-Mb couplings. Slightly more positive (11) relationships rather than negative (10) were also observed. *Thus, PAE* females displayed a reduction in significant correlations when compared to saccharin females. Interestingly, PAE females also did not show the H-H correlations that were observed in saccharin females. Further, whereas saccharin females displayed a higher ratio of positive to negative correlations, positive and negative relationships were observed roughly equally in PAE females.

Direct comparisons of saccharin and PAE female FNC data using two-sample t-tests (Figure 3B) *revealed* a total of eight significant differences; (7 at $p < 0.05$, 1 at $p < 0.005$) that

support these characterizations. The majority of significant differences in female prenatal two-sample t-tests resulted from higher correlations in PAE females compared to saccharin females. Three of the eight differences were due to saccharin females possessing greater FNC measures for St-H, Cx-Mb, and H-Mb couplings. The remaining significant differences were found between St-C (PAE > SAC), T-H (PAE > SAC), Cx-Cx (PAE > SAC) and T-Mb (PAE > SAC) correlations.

A total of 26 significant relationships were found in male saccharin animals (20 at $p < 0.0005$ and 6 at $p < 0.000025$), the majority of which were observed between Cx-H, Cx-Mb, and H-Mb components (Figure 4C). Slightly more positive (14) significant correlations were observed than negative (12) correlations. FNC data from PAE males are shown in Figure 4D. PAE males displayed a total of 16 significant relationships (12 at $p < 0.0005$ and 4 at $p < 0.000025$). The correlations that were most frequently significant in PAE males occurred between Cx-H and Cx-Mb components, and more positive relationships (10) rather than negative (6) were observed. Thus, as with PAE females, PAE males displayed a reduction in the number of significant relationships when compared to saccharin controls. Saccharin males also displayed more significant relationships in H-Mb correlations. However, the areas with the most significant co-activations were the same in both groups (cortical, midbrain, and hippocampal regions).

Direct comparisons of saccharin and PAE male FNC data using planned two-sample t-tests are represented in Figure 3C. A total of thirteen significant differences (11 at $p < 0.05$, 2 at $p < 0.005$) were observed. Six of the thirteen differences were due to higher correlations in saccharin males in T-Mb, Cx-Cx, Cx-Mb, and H-C couplings. These observations suggest a loss of coordinated activity in PAE males. The remaining seven differences were due to higher correlations in PAE animals when compared to saccharin controls indicative of abnormal functional connectivity. This pattern was displayed in St-Cx, Cx-C, Cx-H, and Cx-Mb couplings.

Figure 5 displays a schematic representation of component locations and changes in connectivity, in 2-dimensional space, for PAE females (Figure 5A) and PAE males (Figure 5B). Refer to Table 2 for anatomical component locations. Only components that reached statistically significant changes from two-sample t-tests in connectivity within sex conditions are shown (Fig. 3B-C). PAE females displayed increases in connectivity between thalamic, hippocampal, midbrain, striatal, cortical, and cerebellar components. Specifically, PAE females displayed increases in connectivity between T1-H3, T2-H3, T1-Mb1, St1-C1, and Cx3-Cx4 couplings. When compared to controls, PAE females displayed decreases in functional connectivity among cortical, hippocampal, midbrain, and stratal components; decreases between Cx1-H5, H5-Mb3, and St1-H4 couplings were observed. PAE males displayed increases in connectivity among cortical, cerebellar, hippocampal, midbrain, and striatal components. More specifically, increases in functional connectivity were observed between Cx2-C1, Cx3-C1, Cx3-H4, Cx4-H4, Cx4-Mb1, Cx4-Mb2, and Cx1-St2 couplings. When compared to controls, PAE males displayed decreases in connectivity between Cx1-Cx4, Cx1-Mb3, Cx2-Mb3, Cx3-Cx4, T1-Mb3, and H4-C1 couplings. In sum, PAE was associated with distinct changes in connectivity when compared within sex. Of particular note, cortical, cerebellar, and hippocampal components in PAE males were implicated in

several changes in connectivity whereas PAE females displayed more changes in thalamic, hippocampal, and striatal components.

Spectral Analyses (Component time-course frequencies)

The omnibus two-way ANOVA on measures of spectral power was conducted with sex and treatment as factors (Figure 6). Three significant Prenatal Treatment x Sex interactions were observed in Mb and Cx components (Figure 6A). These included interactions for Mb3 and Mb1 at the 0.04-0.08Hz (SAC > PAE) and 0.12-0.16Hz (PAE > SAC) frequency ranges. The interaction for component Cx2 was significant at the 0.12-0.16Hz (SAC > PAE) frequency range. Inspection of the relevant t-tests from Figures 7B-7C strongly suggest statistical interactions were due to increases in spectral power female PAE rats for the components in question, compared to decreased power in PAE males for the same components.

There were three prenatal treatment main effects in Mb and Cx components (Figure 6B). Two significant effects were found in Mb2 at 0-0.04 Hz ($p < 0.05/6$) and 0.08-0.12 Hz ($p < 0.05$) frequency ranges (see Figure 7A for two-sample t-tests that represent the direction of these effects). The remaining significant effect occurred in Cx1 (SAC > PAE) at the 0-0.04Hz ($p < 0.05$) range. These analyses reveal that for the lowest frequency range, control animals displayed stronger power in Mb and Cx components when compared to PAE animals. Furthermore, SAC animals displayed a clear pattern of greater low-range spectral power (0.00-0.04Hz) for most components in most regions (see Figure 7A). This observation suggests a loss of low-range spectral power in PAE animals. Although not as widespread and robust, a similar pattern was observed in the 0.04-0.20Hz ranges. Although one significant ANOVA main effect resulted from control animals possessing stronger power in the 0.08-0.12Hz range, the effect was not significant upon further examination with a two-sample t-test (see Figure 7A).

ANOVAs examining sex main effects (Figure 6C) resulted in eight significant effects distributed across most component regions. Three significant effects were found in Cx4 and Cx1 components in the 0.04-0.08 Hz, 0.08-0.12 Hz, and 0.12-0.16 Hz ranges. All statistical significant main effects of sex were observed at the $p < 0.05$ level. Additional statistically significant main effects of sex were observed for St2 in the 0.04-0.08 Hz, T1 in 0.00-0.04Hz, H4 in 0.08-0.12Hz, Mb1 in 0.16-0.20Hz, and C1 in the 0.16-0.20Hz range.

Examination of prenatal treatment within each sex (Figures 7B-C) revealed a marked pattern of results of spectral power representation. A total of five significant treatment effects were observed in females (Figure 7B). The majority of these differences occurred in Mb components. There were effects for Mb3 at the 0.04-0.08 Hz range (PAE > SAC), for Mb2 at the 0-0.04Hz (SAC > PAE) and 0.08-0.12Hz (PAE > SAC) ranges, and Mb1 at the 0.12-0.16Hz range (PAE > SAC). Three of the four Mb effects resulted from saccharin females expressing weaker frequency representations in the 0.04-0.16Hz ranges. The remaining significant Mb component resulted from saccharin females possessing stronger low frequency (0-0.04Hz) representations. The prenatal treatment comparison for component C1 was significant at the $p < 0.05$ level in the 0.08-0.12Hz range and was due to saccharin animals possessing weaker mid-range spectral power. In addition to the significant effects, a stark pattern in spectral power representation emerges in the lowest (0-0.04Hz)

range. Control saccharin females consistently displayed stronger low-range frequency representation that was diminished in the PAE females.

Male data are displayed in Figure 7C. Prenatal treatment effects were only observed for component Mb2 (SAC>PAE; $p < 0.05$) at the 0-0.04Hz range in males. Similar to female analyses of spectral power, male SAC animals displayed a pattern of possessing stronger low-range representations for several components across all regions. However, in contrast to females, male SAC animals displayed more frequent instances of possessing stronger representations for mid (0.08-0.12Hz) and some higher ranged (0.12-0.20Hz) frequencies.

Discussion

The characterization of moderate levels of prenatal alcohol exposure on whole-brain FNC yielded clear alterations in functional connectivity across a broad range of brain regions. PAE animals consistently displayed losses of coordinated activity in cortical, hippocampal, midbrain and cerebellar component couplings. Cortical component Cx4 (primary & secondary visual cortex) displayed reductions with other cortical components in PAE animals. Treatment-related reductions in connectivity were also observed in Mb3 (inferior colliculi). Finally, C1 (cerebellum) displayed an overall pattern of alcohol-related alterations in connectivity in PAE animals (decreases with midbrain, but increases with striatal and cortical components; Figure 3). Further examinations revealed sex-dependent alterations in connectivity (Figure 5). For example, PAE males displayed more treatment-related alterations in connectivity when compared to PAE females. Furthermore, PAE males displayed more reductions in connectivity between cortical, midbrain, and thalamic components (Figure 5) compared to SAC males. PAE males also displayed increases in connectivity between cerebellar, striatal, hippocampal, midbrain and cortical components.

In contrast to males, females displayed increases in connectivity between striatal, cerebellar, thalamic, and hippocampal components. Moreover, a number of sex effects were observed in analyses of spectral power. Prenatal treatment-related differences in spectral power were consistently observed in cortical, hippocampal and midbrain components. In addition to statistically significant PAE-related reductions in spectral power for cortical and midbrain components, a striking and consistent pattern of reduced spectral power in the 0-0.04Hz range was observed in all component regions in PAE animals. Females displayed higher measures of spectral power for the 0.04-0.08Hz and 0.08-0.12Hz ranges. These effects were localized to several components in cerebellar, hippocampal, midbrain, striatal, and cortical regions. Low-frequency fluctuations are characteristic features of resting-state paradigms and are thought to reflect the intrinsic connectivity of the brain and may be altered in psychiatric and neurological conditions that include depression, anxiety, Alzheimer's disease, autism, and schizophrenia (Kannurpatti et al., 2008, Biswal et al., 1995, Fox and Greicius, 2010).

Functional connectivity changes in PAE animals add to the small, but growing number of studies showing abnormal resting state FNC in children and adults with FASD (Wozniak et al., 2013, Wozniak et al., 2011, Santhanam et al., 2011). Components showing alterations after PAE have been linked to deficits in visual responding tasks (visual cortex), working

memory (parietal cortex), and auditory processing (inferior colliculi) in neuroimaging studies of children with FASD (Coffman et al., 2013, Astley et al., 2009a, Astley et al., 2009b, Stephen et al., 2012).

Previous animal research has also linked the hippocampus and cerebellum to the behavioral and cognitive impairments of PAE in a wide range of animal models (Varaschin et al., 2010, Hamilton et al., 2010b, Hamilton et al., 2010a, Marquardt et al., 2014) and in human populations (Hamilton et al., 2003). *In vivo* and *in vitro* electrophysiological experiments targeting hippocampal cell function revealed prenatal alcohol-related reductions in glutamate release and disruptions in long-term potentiation (LTP) mechanisms in rodent models (Savage et al., 1998, Varaschin et al., 2010, Sutherland et al., 1997). *In vitro* electrophysiological experiments in a rodent model have also targeted cerebellar cell function and revealed prenatal alcohol-related reductions in glutamate release and alterations in long-term potentiation (LTP) (Servais et al., 2007). Additional research on prenatal alcohol effects in the cerebellum revealed deficits in motor coordination and cellular morphological alterations (Schneider et al., 2011, Gonzalez-Burgos and Alejandro-Gomez, 2005) that may contribute to reductions in functional connectivity. Collectively, the results suggest that moderate levels of PAE produced abnormal FNC and frequency representations in component time-courses that are not due to alterations in cortical blood flow nor brain volume. Although volumetric differences between males and females were detected, these differences reflect normal and expected sexually dimorphic differences and are minimized in the spatial normalization procedure.

Limitations & Future Directions

One limitation of this work concerns the use of general anesthesia to limit movement and related artifacts during scanning. Functional neuroimaging of awake animals requires immobilization using restraining-devices, which even after acclimation may introduce potential confounds related to differential activation of stress responses or fear-related neural circuitry in PAE and control rats. Here, we utilized general anesthesia to overcome these confounds at the expense of limiting our analyses to connectivity patterns in the anesthetized state. However, caution in generalizing the present results is highly encouraged. Previous evidence suggests that anesthetic agents and anesthetic depth can influence functional connectivity pattern outcomes in mice and rats (Grandjean et al., 2014, Liu et al., 2013). Of particular importance from the aforementioned reports, are findings that indicate higher levels of anesthesia (>1.5-1.8%) can lead to reductions in coherence and alterations of frequency representations. Moreover, not all resting state networks are equally influenced by anesthesia depth. Comparable, although weaker, connectivity patterns under isoflurane anesthesia are observed for neural circuits underpinning sensorimotor functions, but not cognitive and emotional processes (Liang et al., 2015). As a result, a programmatic line of research comparing connectivity between different anesthetic agents and doses, analysis methods, and comparing resting to awake states in PAE animals will be needed to advance the utility of these approaches for discriminating animals with PAE.

It is also important to note that rats in both prenatal treatment conditions engaged in pre-pregnancy drinking, which was implemented to more closely model the human drinking

condition, insofar as mothers are unlikely to begin drinking during pregnancy. Because pre-pregnancy drinking was not manipulated, potential interactions between PAE epigenetic factors cannot be addressed in the current design. Importantly, rat dams did not drink during the breeding period, which minimizes potential for acute epigenetic consequences. We do note that earlier studies from our laboratory that did not utilize pre-pregnancy drinking have yielded similar effects on behavior, learning, memory, and synaptic plasticity to those of the present model (Sutherland et al., 1997, Varaschin et al., 2010), however, the effects of pre-pregnancy drinking on functional connectivity assessed by fMRI have not been systematically examined and represent a topic for future research.

An additional and important topic for future investigations is to explore the relationship between resting state FNC and behavior as this may inform whether or not alterations in FNC can be used to blindly discriminate between PAE and control animals or predict behaviors associated with PAE. This avenue of research may provide important insights that may lead to the development of novel diagnostic methods for individuals with FASD, especially those who lack the hallmark facial dysmorphologies, yet display subtle deficits in cognition and behavior. Furthermore, such approaches may also be informative and pragmatic in evaluating potential pharmacological treatments or behavioral interventions for FASD that can become fruitful areas of research.

Conclusion

To our knowledge, this study represents the first report in examining the consequences of prenatal alcohol exposure on functional connectivity in a rodent model. This work represents a critical first step in determining how functional connectivity is altered after PAE and how these changes may inform diagnosis, treatment, or predictions of aberrant behavior. Alterations in prenatal treatment condition and sex were observed for both connectivity and measures of spectral power in components localized to diverse regions. Differences were particularly focused in cortical, midbrain, and hippocampal components, suggesting that moderate PAE broadly alters functional connectivity well into adulthood in a sex-dependent manner.

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References

- Astley SJ, Aylward EH, Olson HC, Kerns K, Brooks A, Coggins TE, Davies J, Dorn S, Gendler B, Jirikowic T, Kraegel P, Maravilla K, Richards T. Functional magnetic resonance imaging outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. *J Neurodev Disord.* 2009a; 1:61–80. [PubMed: 21547622]
- Astley SJ, Aylward EH, Olson HC, Kerns K, Brooks A, Coggins TE, Davies J, Dorn S, Gendler B, Jirikowic T, Kraegel P, Maravilla K, Richards T. Magnetic resonance imaging outcomes from a comprehensive magnetic resonance study of children with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res.* 2009b; 33:1671–89. [PubMed: 19572986]

- Bell AJ, Sejnowski TJ. An Information Maximization Approach to Blind Separation and Blind Deconvolution. *Neural Computation*. 1995; 7:1129–1159. [PubMed: 7584893]
- Biswal B, Yetkin FZ, Haughton VM, Hyde JS. Functional connectivity in the motor cortex of resting human brain using echo-planar MRI. *Magn Reson Med*. 1995; 34:537–41. [PubMed: 8524021]
- Calhoun VD, Adali T. Analysis of complex-valued functional magnetic resonance imaging data: are we just going through a “phase”? *Bulletin of the Polish Academy of Sciences-Technical Sciences*. 2012; 60:371–387.
- Coffman BA, Kodituwakku P, Kodituwakku EL, romero L, Sharadamma NM, Stone D, Stephen JM. Primary visual response (M100) delays in adolescents with FASD as measured with MEG. *Hum Brain Mapp*. 2013; 34:2852–62. [PubMed: 22674650]
- Damoiseaux JS, Rombouts SA, Barkhof F, Scheltens P, Stam CJ, Smith SM, Beckmann CF. Consistent resting-state networks across healthy subjects. *Proc Natl Acad Sci U S A*. 2006; 103:13848–53. [PubMed: 16945915]
- Erhardt EB, Rachakonda S, Bedrick EJ, Allen EA, Adali T, Calhoun VD. Comparison of Multi-Subject ICA Methods for Analysis of fMRI Data. *Human Brain Mapping*. 2011; 32:2075–2095. [PubMed: 21162045]
- Fox MD, Greicius M. Clinical applications of resting state functional connectivity. *Front Syst Neurosci*. 2010; 4:19. [PubMed: 20592951]
- Goense JB, Logothetis NK. Neurophysiology of the BOLD fMRI signal in awake monkeys. *Curr Biol*. 2008; 18:631–40. [PubMed: 18439825]
- Gonzalez-Burgos I, Alejandre-Gomez M. Cerebellar granule cell and Bergmann glial cell maturation in the rat is disrupted by pre- and post-natal exposure to moderate levels of ethanol. *Int J Dev Neurosci*. 2005; 23:383–8. [PubMed: 15927762]
- Grandjean J, Schroeter A, Batata I, Rudin M. Optimization of anesthesia protocol for resting-state fMRI in mice based on differential effects of anesthetics on functional connectivity patterns. *Neuroimage*, 102 Pt. 2014; 2:838–47.
- Hamilton DA, Akers KG, Rice JP, Johnson TE, Candelaria-Cook FT, Maes LI, Rosenberg M, Valenzuela CF, Savage DD. Prenatal exposure to moderate levels of ethanol alters social behavior in adult rats: relationship to structural plasticity and immediate early gene expression in frontal cortex. *Behav Brain Res*. 2010a; 207:290–304. [PubMed: 19852984]
- Hamilton DA, Candelaria-Cook FT, Akers KG, Rice JP, Maes LI, Rosenberg M, Valenzuela CF, Savage DD. Patterns of social-experience-related c-fos and Arc expression in the frontal cortices of rats exposed to saccharin or moderate levels of ethanol during prenatal brain development. *Behav Brain Res*. 2010b; 214:66–74. [PubMed: 20570698]
- Hamilton DA, Kodituwakku P, Sutherland RJ, Savage DD. Children with Fetal Alcohol Syndrome are impaired at place learning but not cued-navigation in a virtual Morris water task. *Behav Brain Res*. 2003; 143:85–94. [PubMed: 12842299]
- Hamilton DA, Magcalas CM, Barto D, Bird CW, Rodriguez CI, Fink BC, Pellis SM, Davies S, Savage DD. Moderate Prenatal Alcohol Exposure and Quantification of Social Behavior in Adult Rats. *Journal of Visualized Experiments*. 2014; 94
- Hutchison RM, Mirsattari SM, Jones CK, Gati JS, Leung LS. Functional networks in the anesthetized rat brain revealed by independent component analysis of resting-state FMRI. *J Neurophysiol*. 2010; 103:3398–406. [PubMed: 20410359]
- Jones KL, Smith DW. Recognition of the fetal alcohol syndrome in early infancy. *Lancet*. 1973; 302:999–1001. [PubMed: 4127281]
- Jones KL, Smith DW. The fetal alcohol syndrome. *Teratology*. 1975; 12:1–10. [PubMed: 1162620]
- Kannurpatti SS, Biswal BB, Kim YR, Rosen BR. Spatio-temporal characteristics of low-frequency BOLD signal fluctuations in isoflurane-anesthetized rat brain. *Neuroimage*. 2008; 40:1738–47. [PubMed: 18339559]
- Kodituwakku PW. Defining the behavioral phenotype in children with fetal alcohol spectrum disorders: a review. *Neurosci Biobehav Rev*. 2007; 31:192–201. [PubMed: 16930704]
- Liang Z, Liu X, Zhang N. Dynamic resting state functional connectivity in awake and anesthetized rodents. *Neuroimage*. 2015; 104:89–99. [PubMed: 25315787]

- Liu X, Zhu XH, Zhang Y, Chen W. The change of functional connectivity specificity in rats under various anesthesia levels and its neural origin. *Brain Topogr.* 2013; 26:363–77. [PubMed: 23208517]
- Logothetis NK, Pauls J, Augath M, Trinath T, Oeltermann A. Neurophysiological investigation of the basis of the fMRI signal. *Nature.* 2001; 412:150–7. [PubMed: 11449264]
- Marquardt K, Sigdel R, Caldwell K, Brigman JL. Prenatal ethanol exposure impairs executive function in mice into adulthood. *Alcohol Clin Exp Res.* 2014; 38:2962–8. [PubMed: 25581651]
- May PA, Gossage JP. Estimating the prevalence of fetal alcohol syndrome. A summary. *Alcohol Res Health.* 2001; 25:159–67. [PubMed: 11810953]
- O'leary-Moore SK, Parnell SE, Lipinski RJ, Sulik KK. Magnetic resonance-based imaging in animal models of fetal alcohol spectrum disorder. *Neuropsychol Rev.* 2011; 21:167–85. [PubMed: 21445552]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates - The New Coronal Set.* Academic Press; 2004.
- Popa D, Popescu AT, Pare D. Contrasting activity profile of two distributed cortical networks as a function of attentional demands. *J Neurosci.* 2009; 29:1191–201. [PubMed: 19176827]
- Santhanam P, Coles CD, Li Z, Li L, Lynch ME, Hu X. Default mode network dysfunction in adults with prenatal alcohol exposure. *Psychiatry Res.* 2011; 194:354–62. [PubMed: 22079659]
- Savage DD, Becher M, De La Torre AJ, Sutherland RJ. Dose-dependent effects of prenatal ethanol exposure on synaptic plasticity and learning in mature offspring. *Alcohol Clin Exp Res.* 2002; 26:1752–8. [PubMed: 12436066]
- Savage DD, Cruz LL, Duran LM, Paxton LL. Prenatal ethanol exposure diminishes activity-dependent potentiation of amino acid neurotransmitter release in adult rat offspring. *Alcohol Clin Exp Res.* 1998; 22:1771–7. [PubMed: 9835293]
- Schneider ML, Moore CF, Adkins MM. The effects of prenatal alcohol exposure on behavior: rodent and primate studies. *Neuropsychol Rev.* 2011; 21:186–203. [PubMed: 21499982]
- Schweinhardt P, Fransson P, Olson L, Spenger C, Andersson JL. A template for spatial normalisation of MR images of the rat brain. *J Neurosci Methods.* 2003; 129:105–13. [PubMed: 14511814]
- Servais L, Hourez R, Bearzatto B, Gall D, Schiffmann SN, Cheron G. Purkinje cell dysfunction and alteration of long-term synaptic plasticity in fetal alcohol syndrome. *Proc Natl Acad Sci U S A.* 2007; 104:9858–63. [PubMed: 17535929]
- Snyder AZ, Raichle ME. A brief history of the resting state: the Washington University perspective. *Neuroimage.* 2012; 62:902–10. [PubMed: 22266172]
- Stephen JM, Kodituwakku PW, Kodituwakku EL, Romero L, Peters AM, Sharadamma NM, Caprihan A, Coffman BA. Delays in auditory processing identified in preschool children with FASD. *Alcohol Clin Exp Res.* 2012; 36:1720–7. [PubMed: 22458372]
- Sutherland RJ, McDonald RJ, Savage DD. Prenatal exposure to moderate levels of ethanol can have long-lasting effects on hippocampal synaptic plasticity in adult offspring. *Hippocampus.* 1997; 7:232–8. [PubMed: 9136052]
- Valenzuela CF, Puglia MP, Zucca S. Focus on: neurotransmitter systems. *Alcohol Res Health.* 2011; 34:106–20. [PubMed: 23580048]
- Varaschin RK, Akers KG, Rosenberg MJ, Hamilton DA, Savage DD. Effects of the cognition-enhancing agent ABT-239 on fetal ethanol-induced deficits in dentate gyrus synaptic plasticity. *J Pharmacol Exp Ther.* 2010; 334:191–8. [PubMed: 20308329]
- Vincent JL, Patel GH, Fox MD, Snyder AZ, Baker JT, Van Essen DC, Zempel JM, Snyder LH, Corbetta M, Raichle ME. Intrinsic functional architecture in the anaesthetized monkey brain. *Nature.* 2007; 447:83–6. [PubMed: 17476267]
- White BR, Bauer AQ, Snyder AZ, Schlaggar BL, Lee JM, Culver JP. Imaging of functional connectivity in the mouse brain. *PLoS One.* 2011; 6:e16322. [PubMed: 21283729]
- Wozniak JR, Mueller BA, Bell CJ, Muetzel RL, Hoecker HL, Boys CJ, Lim KO. Global functional connectivity abnormalities in children with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res.* 2013; 37:748–56. [PubMed: 23240997]

Wozniak JR, Mueller BA, Muetzel RL, Bell CJ, Hoecker HL, Nelson ML, Chang PN, Lim KO. Inter-hemispheric functional connectivity disruption in children with prenatal alcohol exposure. *Alcohol Clin Exp Res*. 2011; 35:849–61. [PubMed: 21303384]

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Fig. 1. Sagittal, coronal, and transverse images of component t-maps
Peak component t-value (t threshold = 3.7), (see Table 1) were used to group components into 6 regions: 4 cortical, 3 midbrain, 5 hippocampal, 2 striatal, 2 thalamic, and 1 cerebellar. Within regional groups, components are arranged from anterior to posterior. Coordinates relative to bregma are provided in Table 1.

Fig. 2. P-value matrices for ANOVA on FNC measures

P-values derived from a 2x2 ANOVA with sex and prenatal treatment as factors. Scale indicates p-values (0-1; light-dark). P-values marked by a circle indicate statistical significance for the statistical interaction (A), main effect of prenatal treatment (B), and main effect of sex (C); Red at $p < .05$; Blue at $p < .005$. Number labels indicate component number within regional grouping.

Fig. 3. T-value matrices for two sample t-tests on condition and sex

Matrices represent t-values determined by subtracting all saccharin from all PAE animals (PAE-SAC) (A); females (B) and males (C). Colored scales indicate t-value (blue=negative; red=positive). Statistical significance is indicated by a circle (closed circle $p < .05$; open circle $p < .005$). Number labels indicate component number within regional grouping.

Fig. 4. R-value matrices separated by sex and treatment

R-values between component co-activations for SAC Females (A); PAE Females (B); SAC Males (C); PAE Males (D). Color scales indicate the direction and strength of correlation values (blue negative; red positive). Mean correlations marked by a circle indicate significance from a one-sample t-test (closed = $p < .0005$; open = $p < .000025$). Numbered labels indicate component number within regional grouping.

A) PAE females compared to SAC females

B) PAE males compared to SAC males

Fig. 5. Connectivity changes in PAE animals relative to their respective, treatment condition and sex appropriate controls
Circles represent approximate component location in 2-dimensional space. Lines represent statistically significant changes in connectivity revealed by two sample t-tests conducted on the pairwise correlation coefficients between components (Figure 4B-4C). Solid red lines correspond to an increase in connectivity whereas dotted blue lines correspond to decreases in connectivity. Component labels and corresponding anatomical coordinates are listed in Table 2.

Fig. 6. P-value matrices for the omnibus ANOVA on spectral power

Statistical significance for the interaction (A), main effect pre-natal treatment (B), and main effect of sex (C) are indicated by circles; red= $p < 0.05$; blue= $p < 0.05/6$. Lighter squares indicate smaller p values. Numbers indicate region component number.

Fig. 7. T-value matrices for spectral power

Two-sample t-tests (subtracting SAC from PAE animals) within sex; Females (A), Males (B). Statistical significance is indicated by a circle (closed at $p < .05$; open at $p < .05/6$). Negative t-values (red) indicate $PAE > SAC$; positive t-values (blue) indicate $SAC > PAE$. Numbers indicate regional component number.

Table 1
Maternal consumption and gestational data of rat dams

Maternal consumption rates for saccharin, serum ethanol concentration, increase in body weight, litter size, and pup birth weights are shown for (SAC) and alcohol exposed (PAE) rat dams. Data are mean (SEM) and group sample size.

	SAC	PAE (5% Ethanol)
Daily four-hour ethanol consumption (grams EtOH consumed/kg body weight/day)	NA	2.04(0.08) n=32
Daily four-hour ethanol consumption: week 1	NA	1.73(0.09) n=32
Daily four-hour ethanol consumption: week 2	NA	2.07(0.09) n=32
Daily four-hour ethanol consumption: week 3	NA	2.04(0.08) n=32
Maternal serum EtOH concentration (mg EtOH/dL serum, 45 minutes into drinking)	NA	60.8(5.8) n=62
Maternal weight gain during pregnancy (grams increase in body weight)	107(4) n=32	104 (5) n=32
Litter size (number of live fetal pups/litter)	12.5(0.15) n=32	12.2(0.29) n=32
Pups birth weight (grams)	6.17(0.13) n=41	6.13(.11) n=42

Table 2

Anatomical locations of extracted component peak values

Components are arranged according to the Paxinos and Watson rat atlas (Paxinos and Watson, 2004) coordinates from anterior to posterior within regional grouping.

Label	AP	ML	DV	Area
1 CX	1.5	0.1	-2.7	Cg2
2 CX	-0.1	3.5	-2.9	S1FL
3 CX	-3.3	-1.9	-1.5	LPTA
4 CX	-8.7	-2.1	-1.7	V2MM/VIM
1 H	-5.1	2.1	-3.3	DS
2 H	-5.1	-5.3	-6.9	MoDG
3 H	-5.9	4.9	-6.3	Or
4 H	-6.1	-3.1	-4.1	MoDG
5 H	-6.3	-5.5	-5.5	Lmol
1 Mb	-3.5	-1.9	-7.7	ZID/ZIV
2 Mb	-6.3	2.5	-4.7	InG
3 Mb	-8.3	1.7	-2.7	ECIC
1 ST	2.1	2.3	-4.3	Cpu
2 ST	-0.7	1.3	-4.9	LSI
1 TH	-1.7	-1.9	-5.9	VA/VL
2 TH	-3.1	1.7	-6.1	Po
1 C	-11.7	-1.5	-5.1	MedDL