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THE EFFECTS OF MODERATE PRENATAL ALCOHOL EXPOSURE ON RESTING STATE FUNCTIONAL CONNECTIVITY AND ITS RELATIONSHIP TO BEHAVIOR

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THE EFFECTS OF MODERATE PRENATAL ALCOHOL EXPOSURE ON RESTING STATE FUNCTIONAL CONNECTIVITY AND BEHAVIOR

by

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ABSTRACT

It is well established that heavy ethanol exposure during prenatal brain development leads to drastic morphological, cognitive and behavioral consequences. In contrast to heavy exposure, the effects of moderate prenatal alcohol exposure (PAE) are less severe yet continue well into adulthood. Animal models of PAE have been of immense importance to researchers for their ability to control for extraneous variables such as socio economic status, age, nutrition, stress, and co-exposure to other substances. Studies of moderate PAE have investigated several discrete brain regions, neurotransmitter systems, and animal behaviors. However, the effects of moderate PAE on resting state functional network connectivity (FNC) have not been well characterized. Moreover, the relationship between PAE, functional connectivity, and animal behavior has not been previously investigated. The present study determined whether moderate PAE alters whole brain FNC. Furthermore, the relationship between hippocampal FNC and behavior was investigated. Long-Evans rats were exposed to 5% ethanol or saccharin throughout the entire gestational period. In adulthood, rats were anesthetized (1.0-2.3% isoflurane) and BOLD signals were acquired during a 10 min echoplanar imaging

sequence in a 4.7T Bruker Biospin scanner. Following motion correction, spatial normalization and smoothing, spatial group independent component analysis (gICA) was performed using the Infomax algorithm implemented in the GIFT toolbox. A total of 17 non-artifactual components were retained for analysis of spectral power and connectivity. Components were observed in cortical, hippocampal, striatal, thalamic, and cerebellar structures. Cortical, hippocampal, and midbrain regions frequently stood out as areas that displayed more significant prenatal treatment differences. PAE animals displayed reductions in low frequency spectral power for several components. PAE animals often displayed a loss of strength in connectivity. Furthermore, analyses of social behaviors with hippocampal related connectivity showed that cortex-to-hippocampus (Cx-H) connectivity is most sensitive to alcohol exposure. PAE females and males displayed more negative correlations compared to their respective saccharin comparison groups. The results indicated that moderate fetal ethanol exposure can have long-lasting consequences on functional connectivity and that hippocampal-containing connectivity was linked to alterations in social behavior.

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1 INTRODUCTION

Fetal Alcohol Spectrum Disorders (FASDs) are a range of morphological and cognitive-behavioral outcomes that result from prenatal exposure to alcohol (Jones & Smith, 1975; Kodituwakku, 2007). The total prevalence for FASDs in the US is estimated between 1-5% (May et al., 2014) and estimates of the economic costs related to treatment, education, residential services, and lost productivity fall between $1.937 billion and $9.687 billion with a median estimate of $3.236 billion (Popova, Stade, Bekmuradov, Lange, & Rehm, 2011). On the most severe end of the FASD spectrum lies Fetal Alcohol Syndrome (FAS) (Riley, Infante, & Warren, 2011). FAS is characterized by severe gross morphological differences such as stunted growth and facial dysmorphologies (Jones & Smith, 1973, 1975). Furthermore, an array of cognitive and behavioral deficits that include attention, learning, memory, functional and social skills accompany FAS (Kodituwakku, 2007). While FAS is the most severe outcome, it is not the most common. It is estimated that 5-20% of women engage in moderate drinking (1-2 drinks per day) (Day, Cottreau, & Richardson, 1993) and that for every one child with FAS, there are five with a less severe FASD (May & Gossage, 2001). Children diagnosed with less severe FASDs typically lack the facial abnormalities characteristic of full-blown FAS and display subtle and persistent deficits in behavior and cognition especially when challenged through increased task complexity (Kodituwakku, 2007).

In addition to the large body of clinical, epidemiological and neuropsychological characterizations of FASDs, animal models have been informative to the understanding of the genetic, neuro-structural and physiological mechanisms associated with moderate alcohol exposure during prenatal development. While much attention has been placed on

the effects of moderate prenatal alcohol on discrete brain areas such as the hippocampus (Savage, Becher, de la Torre, & Sutherland, 2002), cerebellum (Servais et al., 2007), neocortex (Hamilton, Candelaria-Cook, et al., 2010), and neurotransmitter systems that include GABAergic, glutamatergic, dopaminergic and serotonergic functioning (reviewed in (Valenzuela, Morton, Diaz, & Topper, 2012; Valenzuela, Puglia, & Zucca, 2011)), whole brain functional differences have received less attention.

A number of studies have investigated the effects of prenatal alcohol exposure on the structural connectivity of rodent brains (O'Leary-Moore, Parnell, Lipinski, & Sulik, 2011). Many of these studies have focused on elucidating the effects of prenatal alcohol on white matter tractography. While structural connectivity is informative, it provides a limited understanding of how brain areas communicate or function over time. In contrast to structural connectivity, functional network connectivity (FNC) provides an additional perspective needed to understand the functional relationships among spatially distinct brain regions.

One approach to investigating functional connectivity is through functional MRI (fMRI). fMRI analysis methods allow researchers to model brain activity in spatially distinct areas across time. Some of these models are created from data gathered during resting state conditions. Resting state is an operational definition utilized to reflect experimental conditions that lack imposed stimuli, cognitive, or behaviorally salient events (Snyder & Raichle, 2012). The aim is to capture the statistical properties of endogenously (also called intrinsically or spontaneously) generated neural activity (Snyder & Raichle, 2012). In fMRI, resting state paradigms allow the acquisition of spatial and temporal fluctuations in blood-oxygen-level-dependent (BOLD) signals that

reflect synchronized patterns of neural activity and are thought to be due to neurovascular mechanisms regulating blood flow (Logothetis, Pauls, Augath, Trinath, & Oeltermann, 2001).

In the human literature, alterations in FNC have been linked to an extensive list of neurological and psychiatric disease states. Conditions such as depression (Greicius et al., 2007), anxiety (Etkin, Prater, Schatzberg, Menon, & Greicius, 2009), post-traumatic stress disorder (Bluhm, Williamson, et al., 2009), Alzheimer's (Greicius, Srivastava, Reiss, & Menon, 2004), spatial neglect (He et al., 2007), epilepsy (Zhang et al., 2009), and schizophrenia (Bluhm, Miller, et al., 2009) have all been associated with distinct changes in patterns of functional connectivity.

Furthermore, research utilizing electroencephalography (EEG) has also demonstrated reduced inter-hemispheric transfer of information in children exposed to heavy levels of pre-natal alcohol (Roebuck, Mattson, & Riley, 2002). Similarly, a recent study utilizing fMRI and a region of interest (ROI) approach showed reduced inter-hemispheric functional connectivity in children diagnosed with FASDs (Wozniak et al., 2011). These and other human studies point to the possibility that FNC is altered in several disease states including FASDs. However, the appropriate control of confounding variables such as socioeconomic status, age, nutrition, stress, co-administration of other substances, timing, duration, and amount of alcohol administered pose as significant limitations to human research. These limitations can be overcome with the use of animal models where the environment, dose, timing, and pattern of drinking are kept under strict scientific control.

Recent animal studies have characterized resting state networks (RSNs) in anesthetized monkeys (Goense & Logothetis, 2008; Vincent et al., 2007), cats (Popa, Popescu, & Pare, 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, Mirsattari, Jones, Gati, & Leung, 2010). Hutchison and colleagues found similar results in FNC utilizing two different types of anesthetic (isoflurane and a ketamine/xylazine mixture). The similar outcomes utilizing anesthetic agents with different mechanisms of action suggests resting state networks are reproducible regardless of the applied anesthetic. Furthermore, their ICA results were corroborated with an additional data analysis method, principle component analysis (PCA). In addition, their results coincided with prior seed based reports of resting state networks in rodents (Kannurpatti, Biswal, Kim, & Rosen, 2008; Lu et al., 2007; Majeed et al., 2011; Pawela et al., 2010; Zhao, Zhao, Zhou, Wu, & Hu, 2008). These research approaches, collectively suggest that functional networks can be examined in animal models.

One approach to investigating functional connectivity involves the use of seed based analyses. Seed-based analyses require the selection of a predetermined voxel or region that is believed to be involved in a network of interest. In contrast to seed based approaches, ICA does not require the selection of an a priori region of interest. ICA therefore is a data exploration technique based on computational algorithms that are created to extract functional relationships between multiple signals from various sources (Bell & Sejnowski, 1995). When applied to fMRI data, ICA has the ability to identify

distinct features of a data set that represent coherent activity. In the case of fMRI, components represent stationary sets of voxels whose activations vary together in time and are maximally distinguishable from other sets (Huettel, Song, & McCarthy, 2004; Hyvärinen, Karhunen, Oja, & NetLibrary Inc., 2001; Stone, 2004).

To explore the relationship between FASDs and functional connectivity, group ICA is applied to resting state fMRI data from rats that were exposed to moderate levels of alcohol during prenatal development. A primary aim was to explore the utility of gICA as a means to identify alterations in connectivity as a result of moderate fetal alcohol exposure. Given that moderate alcohol exposure has been shown to functionally and structurally alter several brain areas such as the hippocampus, cortex, and cerebellum (Valenzuela et al., 2012), it is reasonable to suspect that these areas would also display changes in connectivity.

While studies of FNC have linked changes in functional connectivity to sensory processing such as paw squeezes in rodent animals (Lu et al., 2007; Majeed et al., 2011), FNC has not been linked to behavior. Therefore, a secondary aim is to determine how changes in FNC are related to social behaviors gathered after the scan period.

Here, the effects of moderate levels of alcohol exposure on whole-brain functional connectivity are characterized and a link between FNC and social behaviors is established. If researchers are to develop rational treatment strategies for alcohol-related abnormalities, it is important to understand how to identify the changes in patterns of functional activity and the related cognitive and behavioral alterations of FASDs. Moreover, this research has possible implications for identifying populations that may

respond maximally to specific treatments, monitor progress, and identify additional areas of interest that have not received as much attention from alcohol researchers.

2 METHODS

2.1 Subjects

Forty-eight (24 male and 24 female) Long-Evans rats bred at the University of New Mexico Health Sciences Center Animal Research Facility (HSC-ARF; breeding protocol described below) were utilized. Subjects were pair housed in standard plastic cages with water and food available ad libitum. All breeding and experimental procedures were approved by The University of New Mexico's Institutional Animal Care and Use Committee (IACUC).

2.2 Breeding Procedures

Breeding procedures were conducted at the University of New Mexico HSC-ARF. Three-to four month-old Long-Evans rat breeders (Harlan Industries, Indianapolis, IN) were single-housed in plastic cages at 22 °C and kept on a reverse 12-h dark/12-h light schedule (lights on from 2100 to 0900 h) with Purina Breeder Block rat chow and tap water available ad libitum. Following a 1-week acclimation period, all female rats were provided with .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. Mean daily ethanol consumption was calculated for each female breeder. Females that consumed less than one standard deviation below the mean of the entire group were eliminated from the study. Average blood ethanol concentrations

reached approximately 60 mg/dL (Bird et al., 2015). Detailed maternal drinking parameters are displayed in Table 1. The remaining females were then placed with proven male breeders until pregnancy was indicated by the presence of a vaginal plug. Female rats did not engage in ethanol consumption during copulation. 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. After rat pups were born, they were culled and raised until adulthood for imaging. A total of 24 male rats (12 ethanol and 12 saccharin control) and 24 female rats (12 ethanol and 12 saccharin control) were selected.

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

2.3 MRI Data Acquisition

In adulthood (3-4 months of age), rats underwent two magnetic resonance imaging sequences in a 4.7T Bruker Biospin (Billerica, MA) magnetic resonance scanner. Rats were anesthetized with a 1.0-2.3% isoflurane/oxygen mixture and monitored for temperature and respiration rate at 40-50/min using a laptop computer running SAM PC software and a model 1025 monitoring and gating system (SA Instruments Inc., Stony Brook, NY). While anesthetized, structural MRI data was acquired during a RARE-8 T1 image sequence [FOV=3.84cmX2.88cm, matrix=256X192, TE=56ms, flip angle=180^{o}, slice thickness=1mm]. Subsequently, functional MRI data was acquired from subjects during a 10 min echoplanar imaging (EPI) sequence [FOV=3.84cmX3.84cm, matrix=64X64, TR=2000ms, TE=21.3ms, flip angle=90^{o}, slice thickness=1mm].

2.4 Image Preprocessing

Images were realigned, spatially normalized to a template (Schweinhardt, Fransson, Olson, Spenger, & Andersson, 2003) corresponding to the Paxinos and Watson atlas of the rat brain (Paxinos & Watson, 2004), and smoothed with a 0.5mm full-width-half-maximum (FWHM) Gaussian kernel. All pre-processing steps were conducted with the Statistical Parametric Mapping software ("SPM8, http://www.fil.ion.ucl.ac.uk/spm/,") toolbox running in MATLAB version R2012b ("Matlab R2012b,").

2.5 Group Independent Component Analysis (gICA)

Following preprocessing, the INFOMAX algorithm (Bell & Sejnowski, 1995) as implemented in the Group ICA of fMRI Toolbox (GIFT, http://mialab.mrn.org/software/gift/index.html) ("Medical Imaging Analysis Lab,") was selected to identify 40 individual components at the group level. Currently, there is no

optimal standard for selecting the number of components. Component selection initially began by using the minimum distance criterion (MDL) implemented in the GIFT toolbox. However, the algorithm resulted in three components and an over-reduction of the data. Subsequently 30 and 70 components were manually set in the ICA algorithm. Based on the similarity and clustering quality graphs of 70 components, forty components provided a compromise of stable non-overlapping components that did not over-reduce the data and did not increase the burden of interpretation (see Appendix A). Individual subject component maps for use in statistical analyses were then back-reconstructed from the aggregate components (Calhoun & Adali, 2012; Erhardt et al., 2011). The resulting group level components were visually inspected for artifactual features such as field inhomogeneities or homogenous signals throughout individual acquisition slices. Artifactual components were eliminated from the data set. The remaining components were sorted and labeled based on the correspondence of the peak intensity value and its anatomical and functional location according the Paxinos & Watson rat atlas (Paxinos & Watson, 2004).

2.6 Spectral Analyses (Timecourses)

Each component had an associated aggregate time course of BOLD signal fluctuations. Because the EPI sequences were obtained at rest with no discrete eliciting events, the temporal characteristics of each component and alterations in these characteristics based on prenatal treatment and sex were assessed through analyses of spectral power. Power within the 0-0.25Hz range was averaged into six equally-sized bins for each component. Separate analyses of variance (ANOVAs) were performed with the ANOVA Toolbox in Matlab. Analyses were conducted for spectral power within each

bin with prenatal treatment and sex as factors. To provide a comprehensive assessment of prenatal treatment effects on spectral power separate individual samples 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 ($p < 0.05/6$).

2.7 Functional Network Connectivity (FNC)

The FNC toolbox (FNC, http://mialab.mrn.org/software/fnc/index.html) ("Medical Imaging Analysis Lab,") was used to determine pairwise correlations between individual component timecourses for each subject. For purposes of characterization the 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 to assess the significance of individual between-component correlations. Analyses of variance (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. To provide a comprehensive assessment of prenatal treatment effects on FNC separate individual samples t-tests for the prenatal treatment factor are also reported for each sex. Due to the high number of effects evaluated and the high number of statistically significant effects obtained with uncorrected alpha values, correction for multiple comparisons is required. To address this issue, statistical significance was evaluated at $p < 0.005$ (uncorrected) and a more conservative alpha level ($p < 0.00025$) to highlight robust effects.

2.8 Behavioral Measures

After image acquisition, animals were transferred and paired housed in standard plastic changes with bedding and water and food available ad libidum. Animals were

allowed to acclimate to the Psychology Animal Research Facility (ARF) for two weeks. After acclimation, animals were allowed to explore and investigate the social behavior testing apparatus for two consecutive days during 30 minute periods (Hamilton, Magcalas, et al., 2014). The apparatus is a custom-built box of plywood with a mirrored back wall and a plexiglass front wall that allows for videotaping. At the end of the second day, the paired animals were then single-housed in separate cages for 24 hours. At the end of the 24 hour period, the animals were reunited in the social behavior testing apparatus. During this time, social behaviors were videotaped for 12 minutes. Videotapes were then transcoded to digital format for behavior quantification. The frequency, duration, and latency to first occurrence were gathered for the wrestling, crawling, anogenital sniffing, allogrooming, rearing and sniffing/digging bedding behaviors. A recent study, employing a linear discriminant analysis, revealed wrestling frequency as the behavior that best discriminates PAE animals from saccharin controls (Hamilton, Barto, et al., 2014). Thus, wrestling frequency was selected for behavior-FNC correlations.

2.9 FNC & Wrestling Behavior Correlations

Our measures of wrestling frequency were then correlated in a pairwise fashion with measures of FNC (the average r) for each possible pairwise combination of components. Of particular interest, was the relationship between wrestling behavior and hippocampal containing connectivity. Hippocampal connectivity was chosen based on its influence in general connectivity and prior work associating the hippocampus to social behaviors (Maaswinkel, Gispen, & Spruijt, 1997). Similar analyses were conducted in

male-by-sex and female-by-sex combinations. All correlations were calculated in Matlab version R2012b with custom scripts ("Matlab R2012b,").

3 RESULTS

3.1 Arterial Spin Labeling

Mean cerebral blood flow values obtained from a region of interest in the frontal cortex are not displayed. However, a two-way analysis of variance with prenatal treatment and sex as factors revealed no significant interaction [$F(1,44) = .444$, $p=0.51$] and no significant effect of prenatal treatment [$F(1,44) = .215$, $p = .645$]. There was, however, a significant effect of sex [$F(1,44) =15.8$, $p < .001$]. Males displayed a reduction in blood flow compared to females in the cortical region examined.

3.2 Whole Brain Volume

Mean (+STD) whole brain volume values obtained are not displayed. However, a two-way analysis of variance with prenatal treatment and sex as factors revealed no significant interaction [$F(1,44) = .142$, $p = .708$] and no significant effect of prenatal treatment [$F(1,44) = .279$, $p = .600$]. Consistent with normal size differences, a significant effect of sex was found [females < males; $F(1,44)$ 127.4, $p < .001$].

3.3 Components

Of the 40 components identified from gICA, 16 were excluded from further analyses due to the presence of artifactual features, as stated in the methods section. An additional 7 components were excluded on the basis of their peak value located in predominantly white matter areas. This resulted in the retention of 17 non-artifactual components for subsequent analyses (Figure 1). The retained components were distributed across the entire brain and for presentation purposes were organized into

striatal, thalamic, cortical, hippocampal, midbrain, and cerebellar groups based on the location of the component peak value. Individual components within a group were ordered from anterior to posterior, numbered within their respective grouping, and referred to in an abbreviated form indicating the region and number within that region. Overall, there were 2 striatal (ST1-ST2), 2 thalamic (T1-T2), 4 cortical (Cx1-Cx4), 5 hippocampal (H1-H7), 3 midbrain (Mb1-Mb3), and 1 cerebellar (C1) components were retained for analysis of FNC. Table 1 shows the grouping criteria, component number, and the corresponding anatomical region of the peak component value (Paxinos & Watson, 2004).

Figure 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.

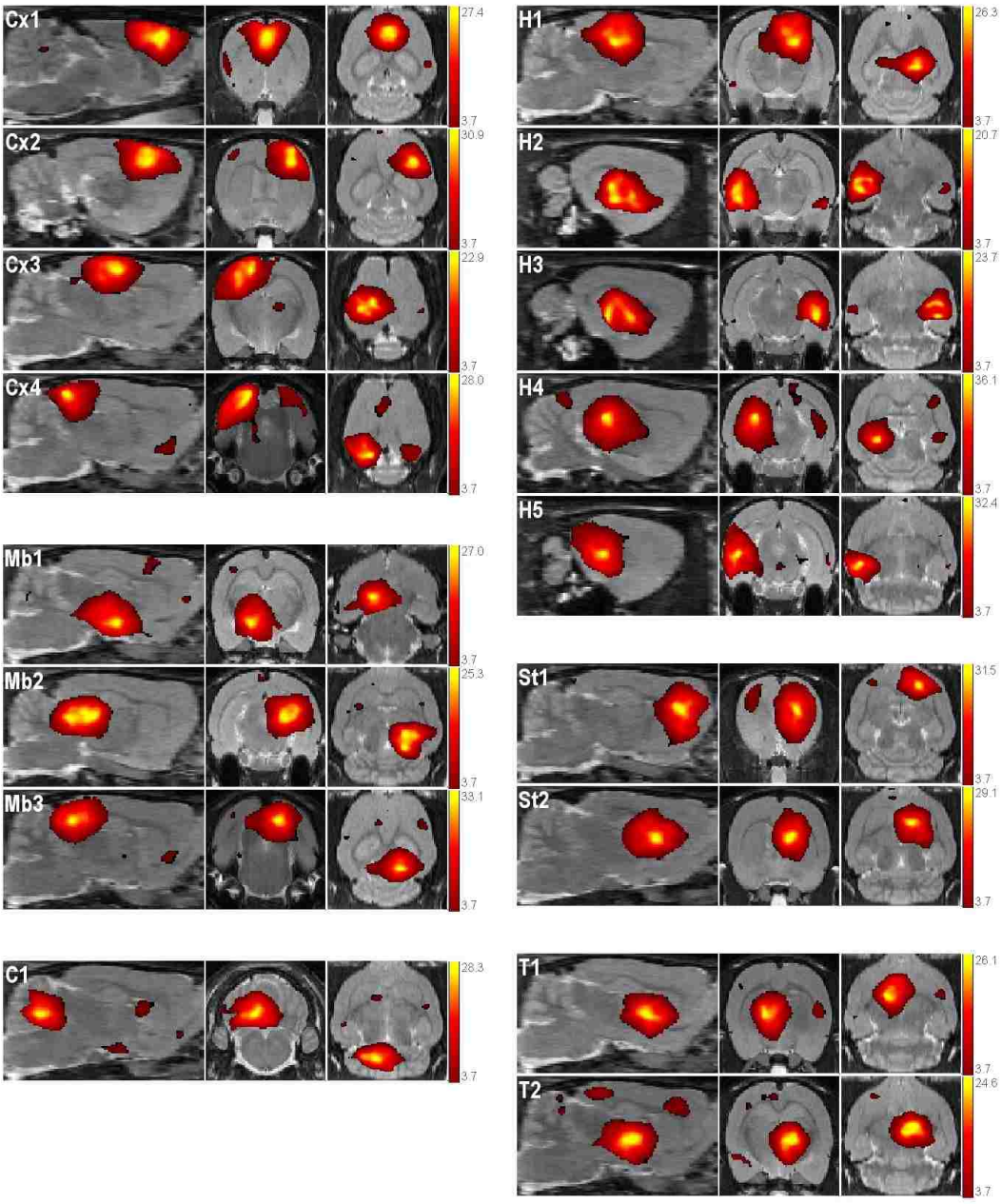


Table 2. Anatomical locations of extracted components. Components are arranged according to the Paxinos and Watson rat atlas (Paxinos & Watson, 2004) coordinates from anterior to posterior within regional grouping.

Label		AP	ML	DV	Area	
1	CX	1.5	0.1	-2.7	Cg2	cingulate cortex, area 2
2	CX	-0.1	3.5	-2.9	S1FL	primary somatosensory cortex, forelimb region
3	CX	-3.3	-1.9	-1.5	LPtA	lateral parietal association cortex
4	CX	-8.7	-2.1	-1.7	V2MM/V1M	secondary visual cortex, medo medial/primary visual cortex
1	H	-5.1	2.1	-3.3	DS	dorsal subiculum
2	H	-5.1	-5.3	-6.9	MoDG	molecular layer of the dentate gyrus
3	H	-5.9	4.9	-6.3	Or	Oriens layer of the hippocampus
4	H	-6.1	-3.1	-4.1	MoDG	molecular layer of the dentate gyrus
5	H	-6.3	-5.5	-5.5	Lmol	lacunosum moleculare layer of the hippocampus
1	Mb	-3.5	-1.9	-7.7	ZID/ZIV	zona incerta dorsal/zona incerta ventral
2	Mb	-6.3	2.5	-4.7	InG	intermediate gray layer of the superior colliculus
3	Mb	-8.3	1.7	-2.7	ECIC	external cortex of the inferior colliculus
1	ST	2.1	2.3	-4.3	Cpu	Caudate Putamen
2	ST	-0.7	1.3	-4.9	LSI	lateral septal nucleus, intermediate part
1	TH	-1.7	-1.9	-5.9	VA/VL	region where VA and VL overlap (ventral anterior thalamic nucleus/ventrolateral thalamic nucleus)
2	TH	-3.1	1.7	-6.1	Po	posterior thalamic nuclear group
1	C	-11.7	-1.5	-5.1	MedDL	medial cerebellar nucleus, dorsolateral protuberance

3.4 Spectral Analyses (Timecourses)

3.4.1 Summary

The following subsections refer to data described in Figures 2-4. The figures display the results of a two-way analysis of variance (ANOVA) and two-sample t-tests conducted on measures of spectral power from average component timecourses. Two-way ANOVAS reveal evidence for interactions and main effects that frequently implicate midbrain and cortical components. After providing a brief summary of the major findings detailed statistical tests to support the patterns of results outlined here are presented.

T-tests calculated by subtracting spectral power for saccharin animals from that of PAE animals for each level of sex (Figures 3A-B) revealed that midbrain components display the most drastic differences in females. Moreover, comparisons of prenatal treatments within female animals indicated decreases (SAC>PAE) for power in the .00-.04Hz range for all components in PAE animals. Although treatment comparisons within males did not reveal as many significant differences, there were trends that included mostly decreases (SAC>PAE) in several component regions for the .00-.20Hz ranges. Overall, these observations suggest that PAE animals displayed lower spectral power within the lower frequency bands, and that this pattern was more evident in female animals.

Separate t-tests calculated by subtracting spectral power for saccharin animals from that of PAE animals (Figure 4A) revealed a systematic decrease in PAE animals in the .00-.04Hz range for all components. This pattern of effects suggests that PAE animals lacked low frequency representations in component time courses (PAE<SAC). However,

increases in spectral power in PAE rats were most pronounced in midbrain components along the .04-.16Hz ranges.

Finally, sex differences (Figure 4B) were mixed, with a roughly even distribution of significant effects reflecting increases in females or males. However, the overall pattern of sex effects were predominantly in the direction of higher spectral power in females in the .04-.12Hz ranges, as was apparent for several components that were distributed across most brain regions.

3.4.2 ANOVA Interactions

Separate ANOVAs were conducted with sex and treatment as main factors (Figure 2B). Three significant Treatment x Sex interactions were observed in midbrain and cortical components. Midbrain components #3 and #1 were significant ($p<.05$) at .04-.08Hz (SAC>PAE) and .12-.16Hz (PAE>SAC) frequency ranges. Cortical component #2 was significant ($p<.05$) at the .12-.16Hz (SAC>PAE) frequency range. The results suggest that the spectral power of the aforementioned midbrain and cortical components is different at levels of sex depending on prenatal treatment. Because simple effects are provided for each component and spectral bin below, the simple effects needed to interpret the interactions are not presented here (see section 3.5.4). Inspection of the relevant t-tests from Figures 3A-3B strongly suggest that these interaction were due to increases in female PAE rats for the components in question, compared to decreased power in PAE males for the same components.

3.4.3 ANOVA Treatment Main Effect

The ANOVA on the main effect of Treatment (Figure 2A) resulted in a total of three significant effects in midbrain and cortical components. Two significant effects

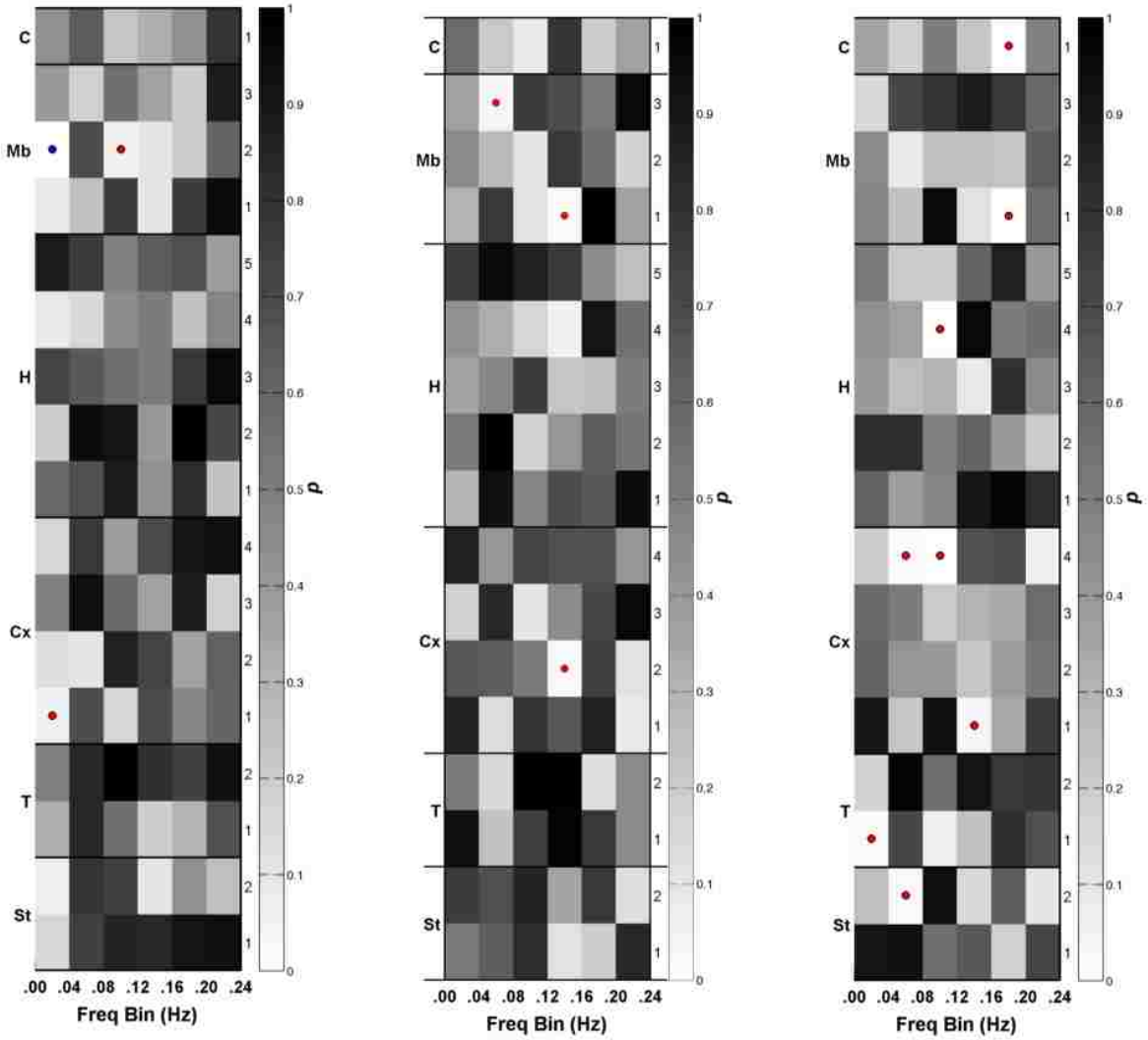
were found in midbrain component #2 (INg) at .00-.04 Hz ($p<.05/6$) and .08-.012 Hz ($p<.05$) frequency ranges. The remaining significant effect occurred in cortical component #1 at the .00-.04Hz ($p<.05$) range. These analyses reveal that for the lowest frequency range, control animals posses stronger power in midbrain and cortical components when compared to PAE animals. Although one significant main effect resulted from control animals possessing stronger power in the .08-.12Hz range, the effect was not significant upon further examination with a two sample t-test (see section 4.6).

3.4.4 ANOVA Sex Main Effect

The ANOVA on the main effect of Sex (Figure 2C) results in eight significant effects in most component regions; all reached $p<.05$ significance level. The cerebellar component was significant at the .16-.20Hz range (males>females). Midbrain component #1 was significant at the .16-.20Hz range (males>females). Hippocampal component #4 was significant at the .08-.12 Hz range (females>males. Cortical component #4 was significant at the .04-.08Hz (females>males) and .08-.12Hz ranges (females>males). Cortical component #1 was significant at the .12-.16Hz range (males>females). Thalamic component #1 was significant at the .00-.04 Hz range (males>females). Striatal component #2 was significant in the .04-.08Hz range (females>males). Upon further examination, males displayed stronger frequency representations in the .12-.16Hz range whereas females displayed stronger representations in the .04-.12Hz ranges (see section 4.6).

Figure 2. P-value matrices for the omnibus ANOVA on spectral power. Statistical significance for the main effect pre-natal treatment (A), interaction (B), and main effect of sex (C) are indicated by circles; red= p<.05; blue= p<.05/6). Lighter squares indicate smaller p values. Numbers indicate region component number.

A) Treatment **B) Interaction** **C) Sex**



3.4.5 Female and Male Treatment T-Tests

Females PAE-SAC

Examination of prenatal treatment within each sex (Figure 3) revealed a marked pattern of results. A total of five significant treatment effects were observed within females (Figure 3A). The majority of these differences occurred in midbrain components suggesting that midbrain components in females may be more sensitive to the effects of PAE. All midbrain components were significant at the $p<.05$ level. Midbrain component #3 was significant at the .04-.08 Hz range (PAE> SAC), midbrain component #2 was significant at the .00-.04Hz (SAC> PAE) and .08-.12Hz (PAE> SAC) ranges, and midbrain component #1 was significant at the .12-.16Hz range (PAE> SAC). Three of the four midbrain effects resulted from saccharin females expressing weaker frequency representations in the .04-.16Hz ranges. The remaining significant midbrain component resulted from saccharin females possessing stronger low frequency (.00-.04Hz) representations. Cerebellar component #1 was significant at the $p<.05$ level and occurred in the .08-.12Hz range and resulted in saccharin animals possessing weaker mid frequency spectral power.

In addition to the significant effects, a stark pattern in spectral power representation emerges in the lowest (.00-.04Hz) range. Control saccharin females consistently displayed stronger low frequency representation that was diminished in the PAE females.

Males PAE-SAC

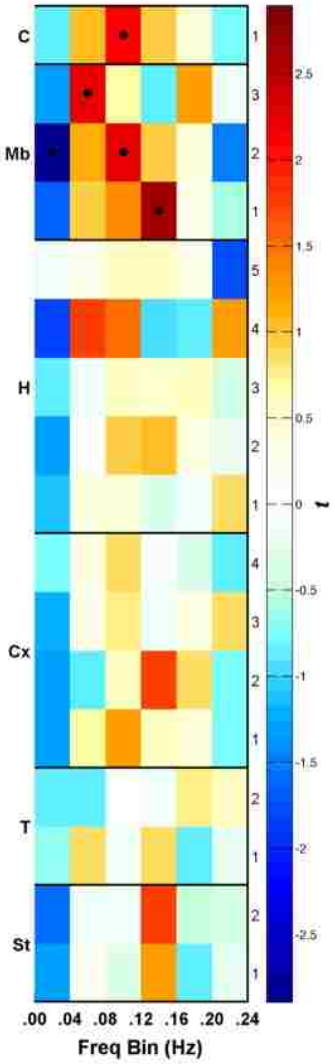
Male data are displayed in figure 3B. Midbrain component #2 was the only significant difference (SAC>PAE ; $p<.05$) at the .00-.04Hz observed in males.

Interestingly, this is the same effect displayed in the female analyses above. In line with analyses of female spectral power, male saccharin animals displayed a pattern of possessing stronger low frequency representations for several components across all regions. However, in contrast to females, male saccharin animals displayed more frequent instances of possessing stronger representation for mid (.08-.12Hz) and some higher ranged (.12-.20Hz) frequencies.

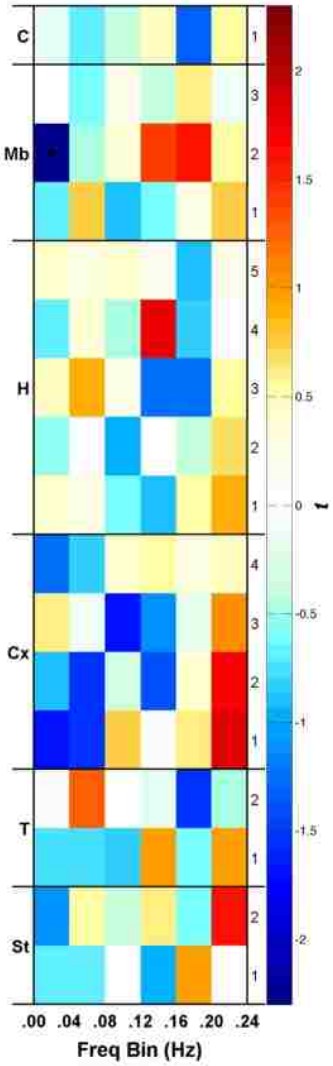
Figure 3. T-value matrices for spectral power.

Two-sample t-tests (subtracting SAC from PAE animals) within sex; A) Females, B) Males. 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.

A) Treatment t-test females (PAE-SAC)



B) Treatment t-test males (PAE-SAC)



3.4.6 Sex and Treatment T-Tests

3.4.6.1 PAE-SAC

Two significant differences were observed when comparing all animals according to prenatal treatment by two sample t-test subtracting saccharin from PAE power spectra (Figure 4A). Midbrain component #2 was significantly different ($p<.05/6$) at the .00-.04Hz range (SAC>PAE). Cortical component #1 was significantly different ($p<.05$) at the .00-.04Hz range (SAC>PAE).

Furthermore, saccharin animals displayed a clear pattern of greater low-frequency spectral power (.00-.04Hz) for most components in most regions. This observation suggests a loss of low-frequency spectral power in PAE animals. Although not as strong or generalized, a similar pattern is observed in the .04-.20Hz ranges.

3.4.6.2 Male-Female

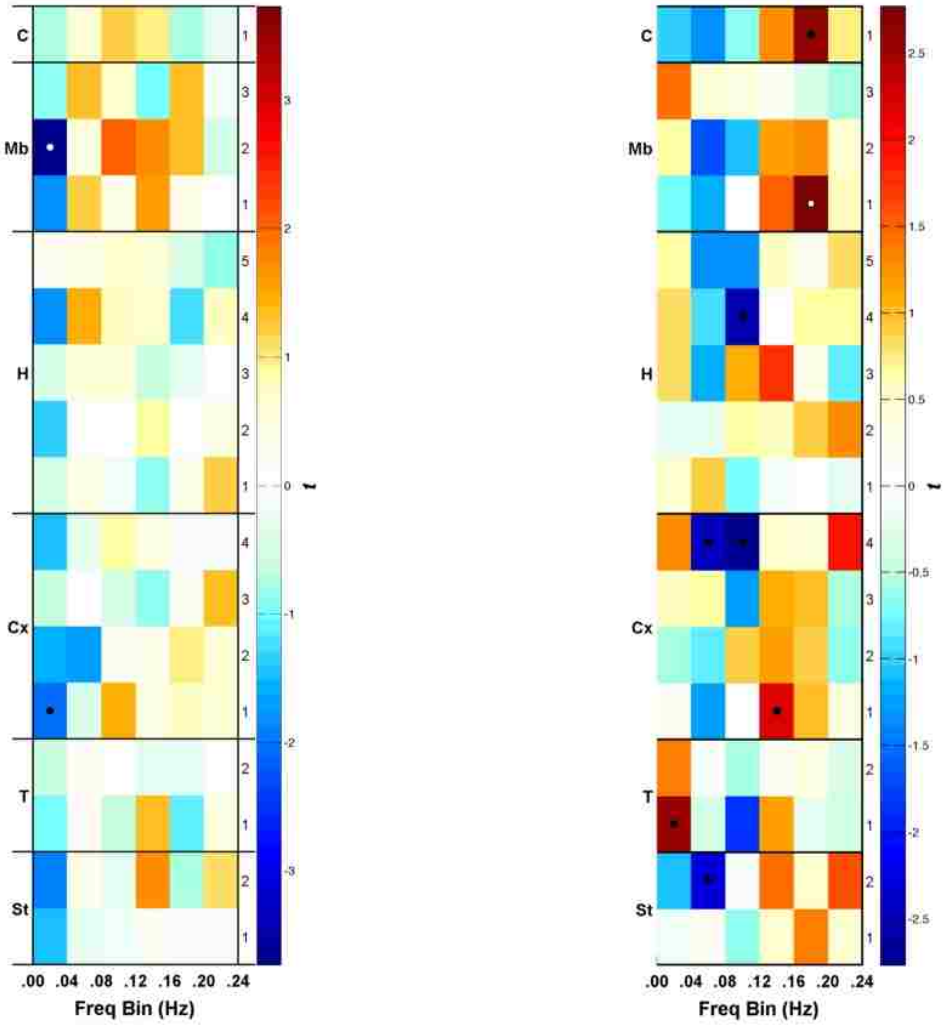
Two sample t-tests (subtracting female from male) spectral power (Figure 4B) revealed 8 significant sex differences. The cerebellar component was significantly different ($p<.05$) in the .16-.20Hz range (males>females). Midbrain component #1 was significantly different ($p<.05/6$) in the .16-.20Hz range (males>females). Hippocampal component #4 was significantly different ($p<.05$) in the .08-.12Hz range (females>males). Cortical component #4 was significantly different at the .04-.08Hz and .08-.12Hz ranges; both ranges were significant at the $p<.05$ level (females>males). Thalamic component #1 was significantly different ($p<.05$) at the .00-.04Hz ranges (males>females). Striatal component #2 was significant ($p<.05$) at the .04-.08Hz range (females>males).

All significant effects where females displayed greater spectral power occurred in the .04-.12Hz ranges. Although not significant, additional components in several regions display a similar pattern of females possessing greater spectral power for the same frequency ranges. This pattern was not observed in any of the other frequency ranges.

Figure 4. Sex and treatment t-value matrices on spectral power.

Two sample t-values from A) SAC animals subtracted from PAE (PAE - SAC); B) Females subtracted from Males (Males - Females). Statistical significance is indicated by a circle for a two-sample t-test (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.

A) Treatment t-test PAE - SAC **B) Sex t-test Males - Females**



3.5 Connectivity (Average correlation between component timecourses)

3.5.1. Orientation to Results Presentation and Summary

The following section presents the data and statistical comparisons conducted on measures of FNC. Due to the richness of the dataset, an orientation to the organization of FNC results is appropriate. First, qualitative descriptions of FNC matrices are presented for the overall (combined PAE and SAC groups), PAE, and SAC FNC matrices. The descriptions are then followed by qualitative comparisons of the treatment groups. Second, statistical analyses (ANOVAs) are presented beginning with the interaction and main effect of prenatal treatment. Because a major research aim of this study was to investigate the effect of prenatal treatment on functional connectivity, the ANOVA main effect of treatment is elaborated upon with simple effects and additional two sample t-tests for evaluating sex differences across treatment. Finally, this section ends with the ANOVA main effect of sex. All ANOVA sections begin with a qualitative description of the FNC data matrices followed by the appropriate statistical analyses. The reader is encouraged to examine Figures 5-8 which display the results of FNC analyses, two-way ANOVAs, and follow-up two sample T-tests. In addition to the FNC matrices, Table 2 summarized the connectivity results displayed in Figure 5A into a frequency table format.

The general results of the connectivity analyses indicated that PAE animals displayed a loss of strength in connectivity. Furthermore, cortical, hippocampal, and midbrain regions frequently stood out as areas that displayed more significant prenatal treatment differences in ANOVAs and two sample t-tests. PAE females displayed reductions in connectivity compared to saccharin females. Saccharin males displayed more significant relationships than PAE males. Saccharin males displayed fewer

significant relationships when compared to control females. Similarly, PAE males displayed fewer significant relationships when compared to PAE females.

3.5.2 Qualitative Description of Overall FNC and Prenatal Treatment FNC

3.5.2.1 Combined Overall FNC

The overall FNC data are displayed in Figure 5A and summarized in Table 2. Overall connectivity measures suggest cortex, hippocampus, and midbrain relationships most frequently meet statistical significance. A total of 136 pairwise correlations were calculated to examine the overall functional connectivity of components. Significant correlations were found between most regional groupings. Exceptions are in T-C correlations and St-St which had no significant correlations. A total of 82 out of 136 correlations reached one of two significance levels. A large portion (73/82) of the significant correlations are at the $p<.000025$ level. The correlations between Cx-H, H-Mb, and Cx-Mb components displayed a higher number of significant relationships compared to other groupings. There were slightly more positive relationships (42) than negative relationships (40).

3.5.2.2 SAC FNC

Data for saccharin animals are displayed in Figure 5B, a total of 26 out of 136 relationships reach statistical significance. The majority of significant relationships (20/26) reach $p<.000025$ significance; the remaining 6 were at $p<.00005$. The relationships between Cx-H, Cx-Mb, and H-Mb components were most frequently observed. Slightly more positive (14) relationships rather than negative (12) were observed.

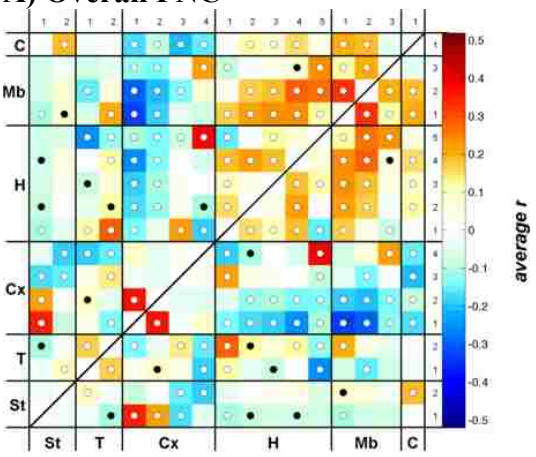
3.5.2.3 PAE FNC

In PAE animals, a total of 16 out of 136 relationships reached statistical significance (Figure 5C). Four of the 16 significant correlations reached $p<.000025$ level; the remaining 12 were significant at $p<.0005$. The pairwise relationships between Cx-H and Cx-Mb most frequently displayed statistical significance. Slightly more significant positive (9) relationships than negative (7) relationships were observed.

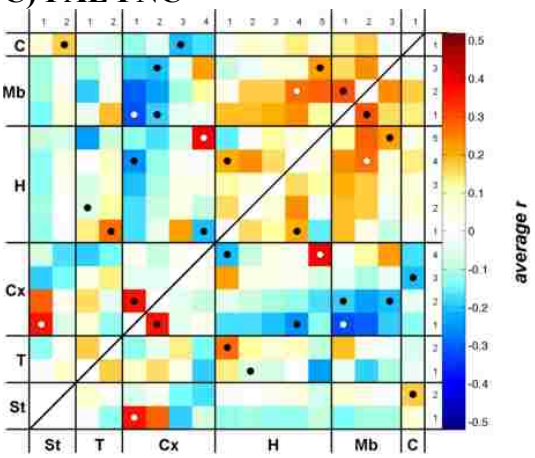
Figure 5. R-value matrices for component co-activations

Color scales indicate the direction of the 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) Overall FNC



C) PAE FNC



B) SAC FNC

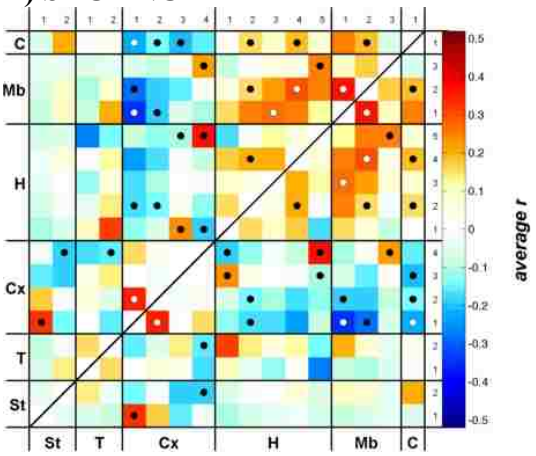


Table 3. Frequency table of overall connectivity measures.

Regions	Total Correlations	Closed Circle p<.0005	Open Circle p<.000025	Positive Closed p<.0005	Negative Closed p<.0005	Positive Open p<.000025	Negative Open p<.000025
St-St	1	0	0	0	0	0	0
St-T	4	1	1	0	1	1	0
St-Cx	8	0	5	0	0	2	3
St-H	10	2	2	0	2	0	2
St-Mb	6	1	1	1	0	0	1
St-C	2	0	1	0	0	0	1
T-T	1	0	1	0	0	1	0
T-Cx	8	1	4	1	0	1	3
T-H	10	1	6	1	0	3	3
T-Mb	6	0	2	0	0	1	1
T-C	2	0	0	0	0	0	0
Cx-Cx	6	0	1	0	0	1	0
Cx-H	20	1	13	0	1	4	9
Cx-Mb	12	0	8	0	0	1	7
Cx-C	4	0	4	0	0	0	4
H-H	10	0	5	0	0	4	1
H-Mb	15	2	9	2	0	8	1
H-C	5	0	3	0	0	2	1
Mb-MB	3	0	3	0	0	3	0
Mb-C	3	0	2	0	0	2	0

3.5.2.4 Qualitative Comparison of PAE FNC vs SAC FNC

Qualitative comparisons of treatments conditions showed several striking differences. First, PAE animals displayed a reduction in the number of significant relationships when compared to saccharin animals. PAE animals displayed 16 significant correlations whereas saccharin animals displayed 26 significant correlations. A reduction in statistically significant correlations between components may be indicative of a loss of coordinated activity in PAE animals. Both prenatal treatment conditions however display several significant relationships between Cx-H, Cx-Mb, and H-Mb components. In addition, more statistically significant positive relationships rather than negative relationships were found in PAE and saccharin animals. While PAE animals displayed a loss of significant relationships, the areas and pattern of the remaining correlations were similar to saccharin animals.

3.5.3 FNC ANOVA Prenatal Treatment x Sex Interaction

The results indicated the ANOVA interactions were especially pronounced for a select number of component relationships including cortical, midbrain, and hippocampal components (Figure 6A). Interactions were found in St-T, St-Cx, T-H, T-Mb, Cx-Cx, Cx-H, Cx-Mb, Cx-C, H-Mb, H-C, and Mb-C. A total of twelve significant interactions were observed from the ANOVA with prenatal treatment and sex as factors. Most significant interactions (11) reached $p<.05$ threshold while the remaining interaction was significant at the $p<.005$ threshold. Further detailed analyses that provide a clearer picture of effects are found below (section 3.5.4).

3.5.4 ANOVA FNC Treatment Main Effect and Simple Effects

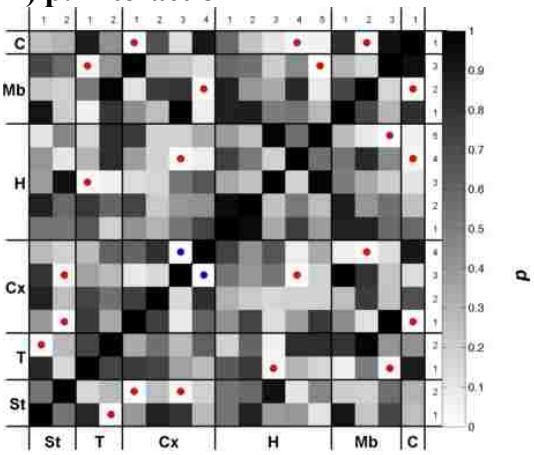
The main effect of treatment resulted in differences between St-Cx, St-H, St-C, Cx-Cx, Cx-Mb, Cx-Mb and Mb-C components (Figure 6B). A total of ten significant differences between treatment groups were detected when collapsing across sex. Of the ten differences, eight reached statistical significance at the $p<.05$ level while the remaining two differences reached the $p<.005$ significance level.

Planned two sample t-tests on the overall treatment FNC matrices, subtracting saccharin from PAE animals, also revealed differences in mostly cortical, midbrain, and cerebellar component correlations (Figure 8A). A total of 9 significant differences between groups were detected. Differences were found in St-Cx (PAE>SAC; $p<.05$), St-H (PAE>SAC; $p<.05$), St-C (PAE>SAC; $p<.005$), Cx-Cx (PAE<SAC; $p<.05$), Cx-Mb (PAE<SAC; $p<.05$), Cx-C (PAE>SAC; $p<.05$) and Mb-C (PAE<SAC; $p<.05$).

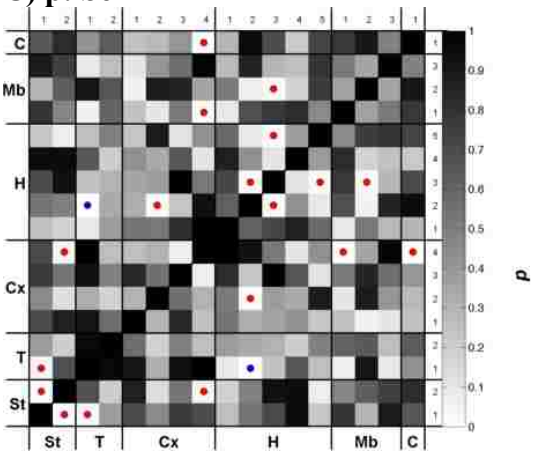
Figure 6. P-value matrices for ANOVA on FNC measures

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.

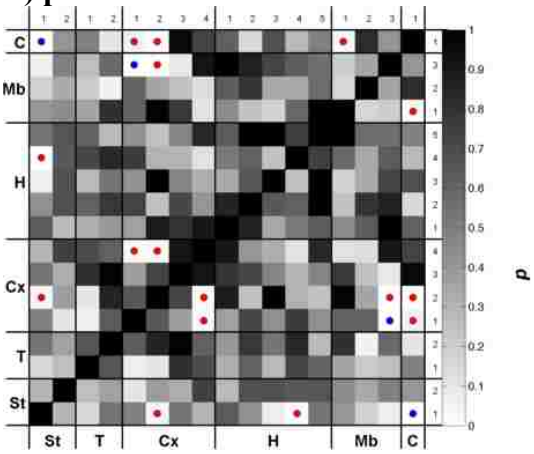
A) p: Interaction



C) p: Sex



B) p: Prenatal Treatment



Comparisons of Females Across Levels of Treatment

3.5.4.2 SAC Females FNC

The data for saccharin females are displayed in Figure 7A. The majority of significant correlations were found between H-Mb, Cx-H, and Cx-Mb components. A total of 30 significant relationships were observed; 15 at $p<.0005$ and 15 at $p<.000025$. The majority of significant correlations were in the positive (20) direction compared to the negative (10) direction.

3.5.4.3 PAE Females FNC

PAE females displayed a total of 21 significant relationships (Figure 7B); 13 at $p<.0005$ and 8 at $p<.000025$. The correlations that were most frequently significant occurred between Cx-H, Cx-Mb, H-H, and H-Mb components. Slightly more positive (11) relationships rather than negative (10) were also observed.

3.5.4.4 Qualitative Comparison of Sac Females FNC vs PAE Females FNC

PAE females displayed a reduction in significant correlations when compared to saccharin females. An interesting pattern that emerges when comparing female groups is that PAE females did not show the H-H correlations that were found in saccharin females. There was also an equal contribution of positive and negative relationships in PAE females whereas saccharin females displayed a higher ratio of positive to negative correlations.

3.5.4.5 PAE-SAC Females T-Tests

The majority of significant differences in female prenatal two sample t-tests resulted from higher r values in PAE females compared to saccharin females (Figure 8B). A total of eight significant differences were observed in two sample t-tests subtracting

SAC from PAE females; (7 at p<.05, 1 at p<.005). Three of the eight differences were due to SAC animals possessing greater FNC measures for St-H (SAC>PAE), Cx-Mb (SAC>PAE), and H-Mb (SAC>PAE) components. The remaining significant differences were found between St-C (PAE>SAC), T-Hx2 (PAE>SAC), Cx-Cx (PAE>SAC) and T-Mb (PAE>SAC) relationships.

Comparisons of Males Across Levels of Treatment

3.5.4.6 SAC Males FNC

The majority of statistically significant correlations in saccharin males were found between Cx-H, Cx-Mb, and H-Mb components (Figure 7C). A total of 26 significant relationships were found in male saccharin animals; 20 at p<.0005 and 6 at p<.000025. Slightly more positive (14) significant correlations were observed than negative (12) correlations.

3.5.4.7 PAE Males FNC

The correlations that were most frequently significant in PAE males occurred between Cx-H and Cx-Mb components (Figure 7D). PAE males displayed a total of 16 significant relationships; 12 at p<.0005 and 4 at p<.000025. More positive relationships (10) rather than negative (6) were observed.

3.5.4.8 Qualitative Comparison of Sac Males FNC vs PAE Males FNC

PAE males displayed a reduction in the number of significant relationships when compared to saccharin males. Saccharin males also displayed more significant relationships in H-Mb correlations. However, the areas with the most significant correlations were the same in both male groups (cortical, midbrain, and hippocampal regions).

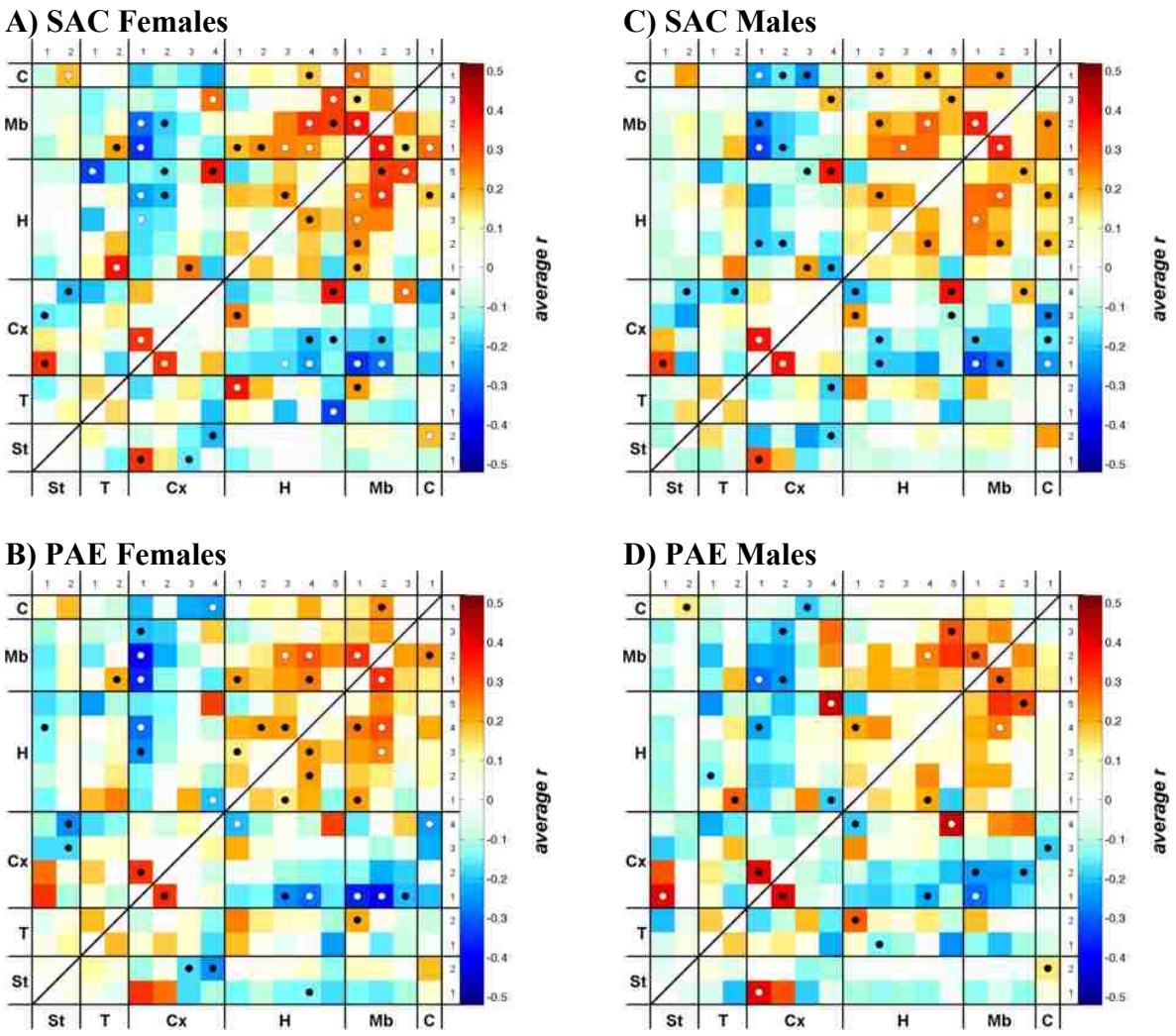
3.5.4.9 PAE-SAC Males T-Tests

Initial male treatment ANOVAs were further investigated with planned paired two-sample t-tests displayed in Figure 8C. A total of thirteen significant differences (11 at $p<.05$, 2 at $p<.005$) were observed. Six of the thirteen differences were due to higher correlation values in SAC animals in T-Mb (SAC>PAE), Cx-Cx (SAC>PAE), Cx-Mb (SAC>PAE), and H-C (SAC>PAE) relationships. One interpretation of these results is that there was a loss of coordinated activity in PAE animals that was not present in saccharin animals.

The remaining seven differences were due to higher correlation values in PAE animals when compared to saccharin. This pattern was observed in St-Cx (PAE>SAC), Cx-C (PAE>SAC), Cx-H (PAE>SAC), and Cx-Mb (PAE>SAC) relationships. The trend indicated stronger correlations in the PAE males. One interpretation is that the coordination of activity in the negative direction was lost suggesting that PAE animals possessed weaker functional connectivity.

Figure 7. R-value matrices separated by sex and treatment.

R-values between component co-activations for A) SAC Females; B) PAE Females; C) SAC Males; D) PAE Males. 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



3.5.5 ANOVA Sex Main Effect

When collapsing across the prenatal treatment factor, ten significant effects due to sex were observed (Figure 6C). Differences 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 $p<.05$ threshold while the remaining significant difference reached the $p<.005$ threshold. Thus the number of observable differences was similar between sex and prenatal treatment factors.

Qualitative Comparison of Sac Females FNC vs SAC Males FNC

Similar to our PAE comparison of males and females, saccharin females displayed more significant relationships compared to males. Additionally, more positive relationships were observed in females compared to males. Interestingly, the same areas that displayed the majority of significant relationships in Cx-H, Cx-Mb, and H-Mb connectivity were observed.

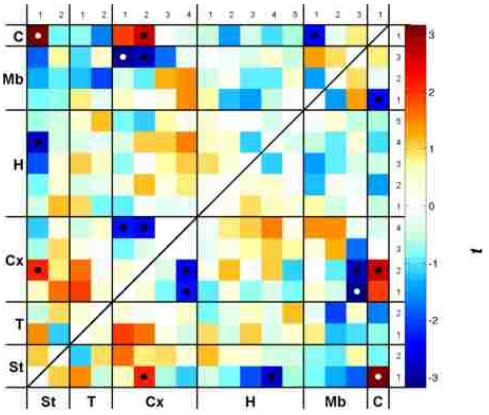
Qualitative Comparison of PAE Females FNC vs PAE Males FNC

PAE males displayed a reduction in the number of significant correlations when compared to PAE females. Although there were reductions in the PAE males, both groups tended to display more positive than negative relationships that reached statistical significance thresholds. Both male and female PAE groups also displayed the most significant correlations in the Cx-H and Cx-Mb groupings, although females displayed more changes in H-H and H-Mb components.

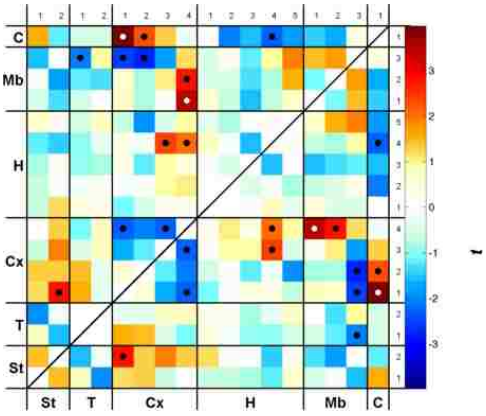
Figure 8. T-value matrices for two sample t-tests on condition and sex.

Matrices represent t-values determined by subtracting saccharin from PAE animals (A); females condition (B) and males condition (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.

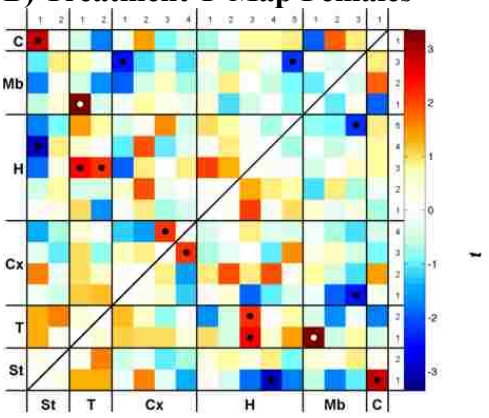
A) Treatment T-Map Overall PAE – SAC



C) Treatment T-Map Males



B) Treatment T-Map Females



3.6 Behavior Correlations

3.6.1 Saccharin Animals Wrestling Frequency

Overall saccharin animals displayed more negative correlations between measures of FNC and frequency of wrestling behavior (Figure 9A). Table 3 summarizes the data for measures of hippocampal FNC and behavior and is displayed in Figure 9. St-H and H-C relationships displayed a slightly increased number of positive relationships while the ratio of negative and positive relationships between T-H components was one. Cx-H, H-H, and H-Mb relationships showed a larger number of negative rather than positive correlations with behavior. All areas showed very few neutral relationships between FNC and behavior. Only two relationships met statistical significance; those were observed in St-H (positive and significant at the $p<.0005$ level) and Cx-H (negative and significant at the $p<.0005$ level).

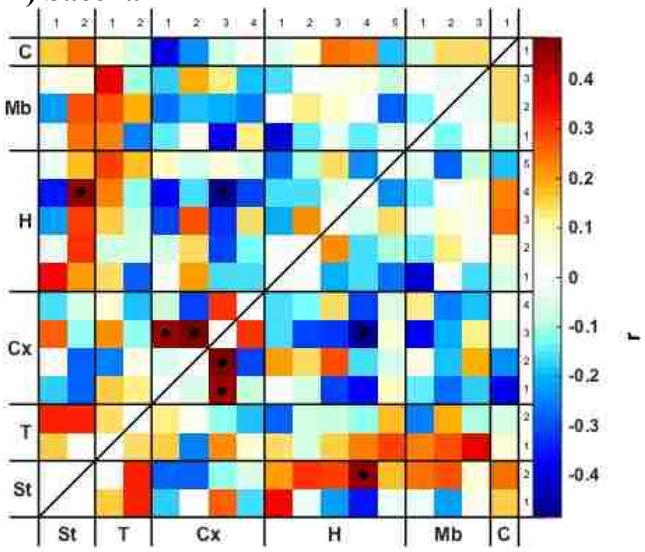
3.6.2 PAE Animals FNC and Wrestling Frequency

Far more negative relationships were found for H-C and St-H PAE FNC when compared to saccharin animals. Interestingly, PAE correlations between cortical component #4 and H, Mb, and C components were mostly positive and significant. This pattern was not observed in the SAC animals, as these correlations were mostly negative and non-significant. The opposite pattern was observed for striatal component #2; PAE animals displayed mostly positive and significant correlations whereas SAC animals displayed mostly negative correlations. More positive relationships were observed for H-Mb FNC. While these differences stood out, overall PAE and SAC animals displayed more negative, rather than positive relationships between measures of FNC and wrestling behavior (Figure 9B). PAE animals showed similar ratios of positive to negative

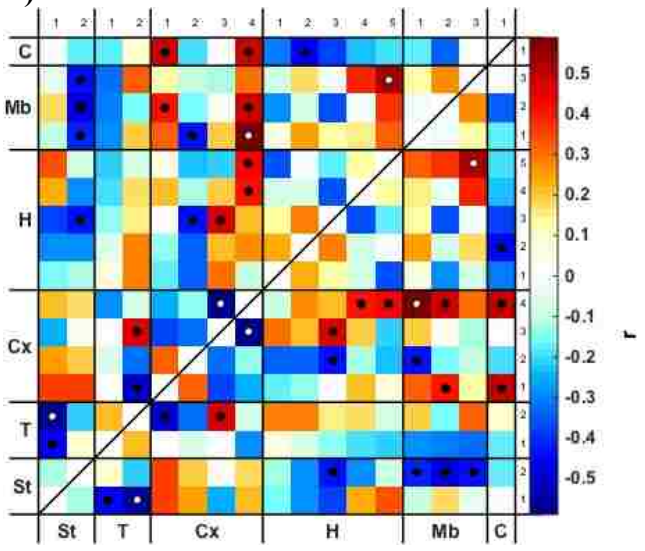
correlations among T-H, Cx-H, and H-H relationships. A lower number of neutral relationships were found in PAE animals when compared to saccharin animals.

Figure 9. R-value matrices between FNC and measures of wrestling behavior frequency. Colored scales indicate strength and direction of r-values (blue=negative; red=positive) for Saccharin (A) and PAE groups (B). Statistical significance is indicated by a circle for a one-sample t-test closed circles=p<.0005; open =p<.000025. Numbered labels indicate component number within regional grouping.

A) Saccharin



B) PAE



3.6.3 Males and Female Analyses

Figure 10 displays the matrices of the correlation values between FNC and measures of wrestling frequency. Because hippocampal regions are of importance to social (Maaswinkel et al., 1997) and cognitive behaviors and have been previously associated with PAE (Varaschin, Akers, Rosenberg, Hamilton, & Savage, 2010), the correlations between hippocampal FNC and wrestling frequency were selected for further analyses. The results are displayed in Table 4 and elaborated upon in this section.

Sex differences across prenatal treatment, are displayed in Figure 10. Both saccharin and PAE females displayed more statistically significant correlations when compared to saccharin and PAE males, respectively. In contrast, PAE females displayed more statistically significant relationships than saccharin females. Furthermore, these correlations were often stronger (in both positive and negative directions) than those found in saccharin females. Saccharin males displayed more frequent and stronger negative correlations when compared to PAE males.

Saccharin males displayed a clear pattern of more component connectivity with negative relationships with measures of wrestling behavior when compared to PAE males (Table 4). PAE males showed more differences in H-Mb when compared to control males. A notable switch in the direction of correlations was observed and was not apparent in the remaining hippocampal containing FNC correlations with behavior.

Comparisons of SAC females (Figure 10A) to PAE females (Figure 10B) revealed a very similar pattern of results. Both have the exact same ratio of negative to neutral to positive relationships, although from a different set of contributions from

individual combinations of connectivity between components. Furthermore, both saccharin and PAE females displayed more positive relationships compared to males.

Figure 10. R-value matrices for behavior and FNC broken down by sex and treatment. Colored scales indicate r-values between FNC and measures of wrestling frequency (blue=negative; red=positive) for saccharin females (A), saccharin males (B), PAE females (B), and PAE males (C). Statistical significance is indicated by a circle for a one-sample t-test circles=p<.0005; open =p<.000025. Numbered labels indicate component number within regional grouping.

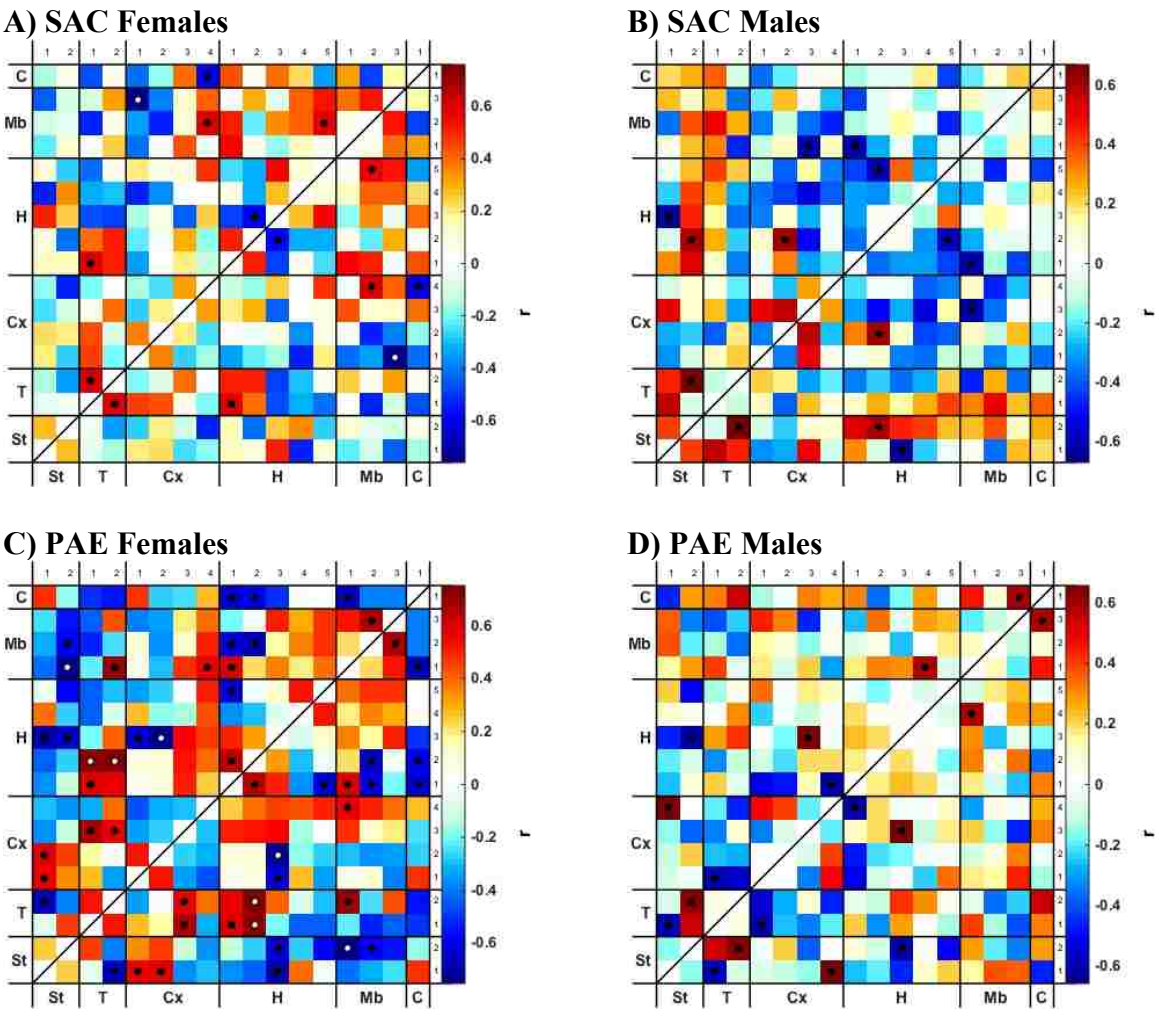


Table 4. Wrestling Frequency Correlations

Displays the number of positive, neutral, and negative correlations and the number of statistically significant relationships in superscript for hippocampal containing component group couplings and measures of wrestling behavior according to overall treatment condition.

	SAC			PAE		
	sex-collapsed			sex-collapsed		
	-	/	+	-	/	+
St-H	4	0	$6^{(1)}$	$8^{(1)}$	0	2
T-H	5	0	5	5	0	5
Cx-H	$13^{(1)}$	1	6	$9^{(1)}$	1	$10^{(3)}$
H-H	7	1	2	5	1	4
H-Mb	9	2	4	6	0	$9^{(1)}$
H-C	2	0	3	$5^{(1)}$	0	0

Table 5. Wrestling frequency correlations by sex and treatment condition

Demonstrates the number of positive, neutral, and negative correlations. The number of statistically significant relationships in superscript for hippocampal containing component group couplings and measures of wrestling behavior broken down by sex and prenatal treatment condition.

	SAC Male			PAE Male			SAC Female			PAE Female		
	-	/	+	-	/	+	-	/	+	-	/	+
St-H	$4^{(1)}$	0	$6^{(1)}$	$3^{(1)}$	2	5	3	0	7	$3^{(2)}$	0	7
T-H	4	0	6	$4^{(1)}$	2	4	5	1	$4^{(1)}$	6	0	$4^{(3)}$
Cx-H	14	1	$5^{(1)}$	$12^{(1)}$	1	$7^{(1)}$	$5^{(1)}$	0	5	$9^{(2)}$	3	8
H-H	$8^{(1)}$	0	2	6	1	3	4	1	$10^{(1)}$	$5^{(1)}$	0	$5^{(1)}$
H-Mb	$12^{(1)}$	2	1	5	1	$9^{(1)}$	4	1	$10^{(1)}$	$4^{(2)}$	1	$10^{(1)}$
H-C	4	0	1	3	0	2	1	1	3	0	2	$3^{(2)}$

.

4. DISCUSSION

The characterization of moderate levels of alcohol exposure on whole-brain FNC yielded clear reductions in functional connectivity across a range of brain regions compared to unexposed saccharin controls. Furthermore, spectral power within the lowest frequency ranges, for all components, was dramatically reduced in PAE animals. Finally, a relationship between hippocampal FNC and social behavior, which has been shown to dissociate PAE and control animals, was established, demonstrating the utility of resting state gICA as a means to identify functional differences that are related to behavior.

One of the most pronounced outcomes of this research is observed in PAE animals. These animals consistently displayed a loss of coordinated activity in measures of component cross correlations. PAE animals displayed fewer statistically significant correlations and displayed more connectivity in the negative direction. Both patterns of results indicate a loss of strength in effective connectivity.(Coffman et al., 2013)

Furthermore, alterations in FNC measures due to prenatal treatment exposure were consistently observed in cortical, hippocampal, midbrain and cerebellar components. One cortical component that often displayed alterations in connectivity, especially with behavior was localized to primary and secondary visual cortex (Cx4). Previous research that utilized a clinical population of adolescents and young adults diagnosed with a FASD observed alterations in primary visual responding in saccade task requiring a shift of attention and gaze to relevant stimuli (Coffman et al., 2013). The results of this study suggest that PAE animals displayed reductions in connectivity with primary and secondary visual cortices. Thus, the results parallel sensory deficits observed in clinical populations. Furthermore, components Mb2 and Mb3, which represent the

superior and inferior colliculi, respectively, often displayed treatment related differences in connectivity. The superior and inferior colliculi area well known to be involved in the processing of visual and auditory information, respectively (Doubell, Skaliora, Baron, & King, 2003). However, connectivity midbrain components tended to be increased in PAE animals suggesting an ethanol related alteration in connectivity with auditory and visual processing. Previous work that employed a human clinical population also found deficits in auditory processing in preschool children diagnosed with a FASD (Stephen et al., 2012) suggesting further inquiries into visual and auditory processing are warranted.

Hippocampal and additional cortical areas also showed frequent changes in connectivity in FNC analyses. These findings supports previous research that observed deficits in hippocampal (Varaschin et al., 2010) and neocortical (Hamilton, Akers, et al., 2010; Hamilton, Candelaria-Cook, et al., 2010) functioning after moderate prenatal alcohol exposure.

Finally, the cerebellar component displayed an overall pattern of alcohol related reductions in connectivity in PAE animals and especially in PAE males. In vitro electrophysiological experiments targeting the cerebellar cell function revealed prenatal alcohol related reductions in glutamate release and disruptions in long-term potentiation (LTP) mechanisms (Servais et al., 2007) in a rodent model. Additional research on the prenatal alcohol effects on the cerebellum revealed deficits in motor coordination and cellular morphological alterations (Gonzalez-Burgos & Alejandre-Gomez, 2005; Schneider, Moore, & Adkins, 2011) that may contribute to reductions in functional connectivity.

Similarly, alterations in spectral power were consistently observed in cortical, hippocampal and midbrain components. There were robust differences in spectral power between treatment groups in these regions compared to others. In addition to statistically significant PAE-related reductions in spectral power for cortical and midbrain components, a consistent pattern of reduced spectral power in the .00-.04Hz range was observed in all component regions in PAE animals. Low range 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. (Biswal, Yetkin, Haughton, & Hyde, 1995; Fox & Greicius, 2010; Kannurpatti et al., 2008).

In addition to treatment differences, a number of sex effects were observed in analyses of spectral power and FNC. Females displayed higher measures of spectral power for the .04-.08Hz and .08-.12Hz ranges. These effects were localized to several components in cerebellar, hippocampal, midbrain, striatal, and cortical regions. Females also displayed more statistically significant relationships in measures of FNC for both prenatal treatment conditions. Furthermore, saccharin females displayed stronger positive correlations when compared to males. Sex effects on FNC were most often observed in cortical, hippocampal, and midbrain components. In sum, sex effects were associated with similar component regions for spectral power and FNC as prenatal treatment condition effects.

Collectively, the FNC and spectral power results suggest that moderate levels of prenatal alcohol produced alterations in connectivity and component timecourses. While it is possible that these differences are due, in part, to cerebral blood flow, the results

from that analysis of arterial spin labeling revealed no significant differences between treatment conditions or sexes, suggesting FNC and spectral power alterations are not due to differences in blood perfusion. Additionally, whole brain volumetric analyses revealed no significant differences between prenatal treatment conditions within their appropriate sex comparisons. Although, there were differences between male and female animals, these differences reflect normal and expected sexually dimorphic differences. Furthermore, volumetric differences are dampened in the preprocessing stages of imaging data.

Importantly, alterations in hippocampal connectivity and spectral power were linked to alterations in social behavior. Saccharin animals displayed more negative rather than positive correlations with wrestling behavior, a measure that has been shown to discriminate PAE and saccharin rats (Hamilton, Barto, et al., 2014), especially in cortical, hippocampal, and midbrain relationships. There was a similar pattern of more negative correlations with measures of wrestling, although not as strong, observed in PAE animals. When examining males, both saccharin and PAE males displayed a higher number of negative correlations with behavior and hippocampal connectivity. In contrast, females displayed more positive relationships compared to males. This finding suggests alterations in resting state activity can be linked with changes in behavior after image acquisition has taken place and in spite of reports of cognitive and behavioral dysfunction after induction with anesthesia (Zurek et al., 2014).

One limitation to this study concerns the component selection method. There is no current standard approach of selecting components. Forty components were chosen based

on multiple instantiations of the ICA algorithm utilizing 30, 40, and 70 components. Based on a stability index of 70 components (see appendix A), 40 components were chosen to offer a wider scope of the data with out overcomplicating the data analysis. Additionally, prior work with resting state animal models successfully utilized 40 ICA components to investigate connectivity and low frequency fluctuations (Hutchison et al., 2010). An additional limitation concerns the use of isoflurane anesthesia which, by its pharmacological action, influences GABAergic neurotransmission (Zurek et al., 2014). However, reports from the human literature and animal literature suggest that resting state networks are reliably observed under anesthesia (Vincent et al., 2007).

Future investigations may take on two approaches. First, corroborating results using additional approaches such as a spatially constrained ICA or seed based approach will be helpful in establishing how moderate PAE affects functional connectivity. Hutchison and colleagues (Hutchison et al., 2010) corroborated their results using ICA and PCA; thus the utilization of other network connectivity measures may help target additional research. Furthermore, corroborations from multisite electrophysiological recordings would provide strong evidence for changes in functional connectivity as a result of moderate prenatal alcohol exposure. In addition, further investigations utilizing white matter tractography or diffusion tensor imaging, could help reconcile the findings in functional connectivity with measures of structural connectivity.

In conclusion, this study characterized the effects of moderate levels of prenatal alcohol on resting state FNC. 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 component, suggesting that moderate PAE broadly alters connectivity. In addition, changes in connectivity were further linked to changes in behavior alterations measured well after image acquisition.

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APPENDIX A

Appendix A. Stability index of 70 components.

When 70 components are chosen from the GIFT algorithm, 40 components provides the least number of components with the highest level of stability.