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Prenatal Stress Alters Dendritic Morphology and Synaptic Connectivity in the Prefrontal Cortex and Hippocampus of Developing Offspring

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KEY WORDS neuroanatomy; mPFC; OFC; cerebral organization; neurogenesis

ABSTRACT The current study used stereological techniques in combination with Golg-Cox methods to examine the neuroanatomical alterations in the prefrontal cortex and hippocampus of developing offspring exposed to gestational stress. Morphological changes in dendritic branching, length, and spine density, were examined at weaning along with changes in actual numbers of neurons. Using this information we generated a gross estimation of synaptic connectivity. The results showed region-specific and sex-dependent alterations to neuroanatomy in response to prenatal stress. The two regions of the prefrontal cortex, medial prefrontal, and orbital prefrontal cortices, exhibited sexually dimorphic, opposite changes, in synaptic connectivity in response to the same experience. Both male and female offspring demonstrated a loss of neuron number and estimated synapse number in the hippocampus despite exhibiting increased spine density. The results from this study suggest that prenatal stress alters normal development and the organization of neuronal circuits in both neocortex and hippocampus early in development and thus likely influences the course of later experience-dependent synaptic changes. **Synapse 66:308–314, 2012.**

INTRODUCTION

The ability of early experiences to shape developing neuronal pathways and long-term plasticity is an area of research that is currently under intense investigation. As gestation is a critical period for many aspects of brain development (neuronal differentiation, migration, etc.) manipulations to this environment may disrupt normal patterning and organization. Despite evidence demonstrating that gestational stress may be associated with childhood behavioral disorders, depression, and anxiety (Huizink et al., 2003; O'Connor et al., 2002), very few animal studies have attempted to understand this phenomena (but see Murmu et al., 2006 and a review by Weinstock, 2005). Past research has focused primarily on understanding the behavioral correlates of prenatal stress, with very little focus on the anatomical and structural reorganization that has likely predisposed these changes in behavior. Although, many psychological disorders do not manifest until adolescence or adulthood, the underlying anatomical disturbances may be present much earlier.

The persistent nature of psychological illness implies that there are also stable changes to brain anatomy. To understand the relationship between early experiences and the possibility of long-term mental health issues, one needs to examine the structural reorganization that is beginning early in development in addition to the anatomical changes that can be seen in adulthood. At present, the majority of literature examines single aspects of dendritic morphology in adult offspring exposed to gestational stress (e.g., Michelsen et al., 2007; Muhammad and Kolb, 2011; Radley et al., 2008), with few studies addressing neuroanatomy in offspring during development (Murmu et al., 2006), and even fewer examining multiple neuroanatomical parameters in young offspring (Mychasiuk et al., 2011a).

The purpose of this study was to examine the neuroanatomical alterations in the prefrontal cortex and

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hippocampus of developing offspring exposed to gestational stress. This study was innovative because stereological techniques were used in combination with Golgi-Cox methods to investigate changes in neuronal organization that occur in response to prenatal stress. Morphological changes in dendritic branching, length, and spine density, along with changes in actual neuron number were used to generate a gross, but useful estimation of synaptic connectivity. Measures were performed on two areas of the prefrontal cortex, medial prefrontal cortex [(mPFC) as denoted Cg3 in Zilles' (1985) (this measurement excludes Cg1)], and orbital frontal cortex [(OFC) as denoted AID in Zilles' (1985) atlas (measurement excludes VLO & LO)], and hippocampus (CA1). These regions were chosen for investigation because they are intricately involved in mediating the stress response (McEwen, 2007), they have sexually dimorphic characteristics (Goldstein et al., 2001; Roof, 1993), are abundantly populated with glucocorticoid receptors (Sapolsky et al., 1984), and are believed to play a significant role in the manifestation of many psychological disorders (Kolb and Whishaw, 2008).

MATERIALS AND METHODS

Subjects and stressing

All experiments were carried out in accordance with the Canadian Council of Animal Care and approved by the University of Lethbridge Animal Care Committee. Nine female Long-Evans rats were paired with pregnant Long-Evans rats and housed in shoe-box cages (18 females total). All animals were maintained on 12:12 h light:dark cycle in a temperature-controlled breeding room (21°C) and were given access to food and water *ad libitum*. Prenatal stress was performed twice daily on gestational days 12–16. Pregnant dams ($n = 4$) were placed on an elevated Plexiglas® platform and exposed to bright light for 30 min. Stress sessions occurred at 9:00 A.M. and 3:00 P.M. Control dams ($n = 5$) were left undisturbed. Once pups were born, each of the mothers was housed individually with their litter. Roughly equal numbers of male and female pups were randomly selected from each litter for Stereological analysis (12 Male, 11 Female), Golgi-Cox processing (12 Male, 12 Female), or DNA analysis [DNA analysis presented in another study elsewhere, see, Mychasiuk et al., 2011b)].

Histological processing—cresyl violet

When pups reached 21 days of age (P21), they were administered an overdose of sodium pentobarbital and perfused with 0.9% saline followed by an equal volume of 4% paraformaldehyde (PFA). The brains were then removed, weighed, and preserved in 4% PFA for 1–2 days. Following preservation the brains were transferred to a solution of 30% sucrose in 4% PFA and stored for sectioning. Brains were cut at 60 μm with a cryostat, mounted on glass slides, and stained with cresyl violet (1% cresyl violet acetate in distilled water). Following staining sections were dehydrated, cleared, and cover-slipped.

Neurons were counted from six sections of AID, Cg3, and CA1 (Zilles, 1985) in the left and right hemispheres using a Zeiss Axio Imager M1. Stereo Investigator 9.03 was used for cell quantification based on the Optical Fractionator method. Software parameters were set as: Counting frame: 40 × 40 μm^2; Grid size: 225 × 225 μm^2; Optical dissector height: 15 μm; Section interval 1; Section periodicity: 4; and remained constant throughout counting. All sections were counted by a single blind analyst to maintain consistency. Regions of interest were generated using the Zilles' stereotaxis atlas (1985) and contours were copied and exported to each section to ensure consistent boundaries were maintained. Section volume was determined by the Stereo Investigator software 9.03.

Histological processing—golgi-cox

When pups reached P21 they were administered an overdose of sodium pentobarbital and intracardially perfused with 0.9% saline. The brains were removed, weighed and preserved in Golgi-Cox solution for 14 days in the dark. After 14 days the brains were transferred to 30% sucrose solution for a minimum of 3 days. At this point brains were cut on a Vibratome at 200 μm and mounted on a gelatin-coated slide. Brains were then stained according to the procedures described by Gibb and Kolb (1998).

Pyramidal cells from layer three of Cg3 and AID, and CA1 were chosen for analysis. Individual neurons from the Golgi-Cox stained brains were traced using a camera lucida mounted on a microscope. A total of 10 cells (5 per hemisphere) were traced at 250× from each brain region. The mean of cells from each hemisphere comprised the data points for statistical analysis. Neuronal investigation included basilar dendritic branch order (an estimation of dendritic complexity based on the number of branch bifurcations), basilar Sholl analysis (an estimate of dendritic length derived from the number of dendritic branches that intersect concentric circles spaced 25 μm apart), and spine density (the number of spine protrusions on a 40 μm segment of dendrite traced at 1000×). Spines were always traced from the terminal branch. Apical dendrites were excluded from analysis as a result of dendritic breakages likely associated with the age of the rats when the tissue was harvested. In our experience young tissue becomes more brittle in the Golgi-Cox processing than older tissue and tends to fracture the longer apical dendrites.

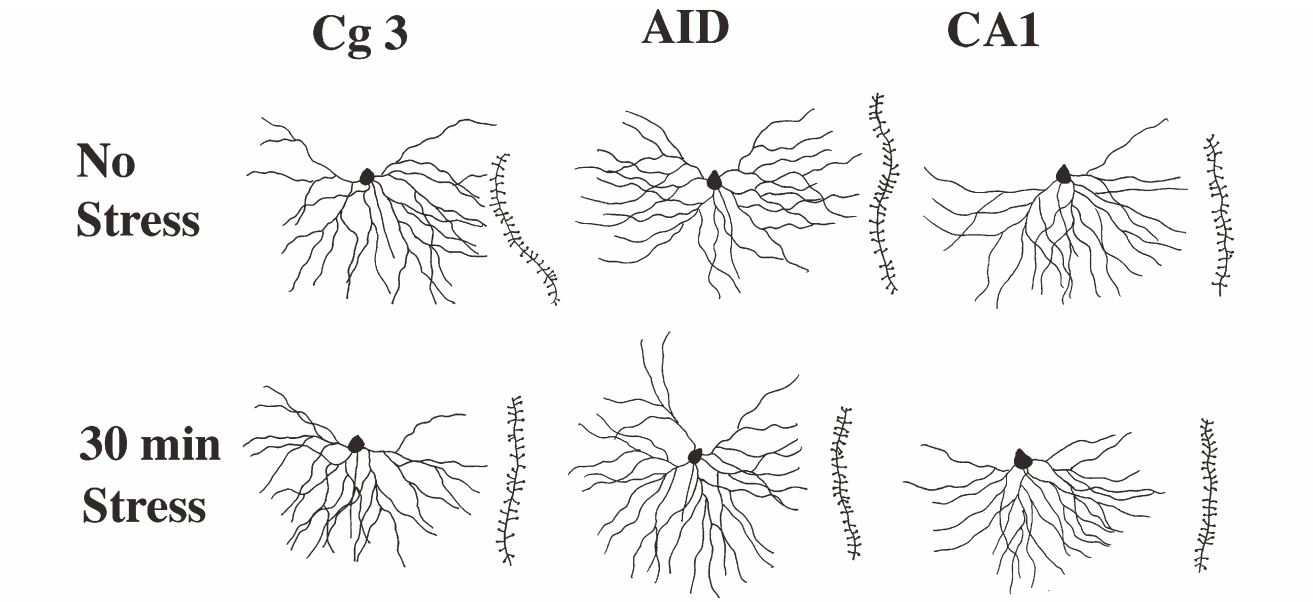

Fig. 1. Camera lucida drawing of basilar pyramidal cells and dendritic segments used for spine density calculation in the mPFC, OFC, and CA1 of male and female rats exposed to either prenatal stress or no prenatal stress. The cells depicted were selected because they were representative of group averages for our measures of dendritic form.

Estimation of excitatory synapse number

Combining the dendritic length, spine density, and neuron number data generated an estimation of the number of excitatory synapses. Standard deviations, rather than standard errors of the mean, were used in the generation of error for this analysis to avoid sample size over-representation and inflammation of *N*.

Statistical analysis

All statistical analysis was carried out using SPSS 16.0 for Mac. Analysis was conducted to ensure results could not be attributed to specific litters. Three-way ANOVA's with stress, sex, and hemisphere as factors were run for each brain area compare the neuroanatomy of prenatal stress offspring with control offspring. Hemisphere failed to show significant main effects or contribute to any interaction effects and was therefore eliminated as a factor and two-way ANOVA's with stress and sex as factors were run. The results from the two-way ANOVAs are presented. *Post hoc* analysis was not computed for any of the results. Significance is reported for main effect results demonstrating $P < 0.01$. All graphical illustrations represent comparisons made between prenatally stressed offspring and control offspring.

RESULTS

Quantitative analysis confirms our hypothesis that prenatal stress affects the neuroanatomical development of rat offspring. For evidence that this prenatal stress paradigm altered gross brain weight, behavior, and the epigenome of offspring refer to Mychasiuk et al. (2011b). See Figure 1 for an illustrative representation of basilar cells and spines from AID, Cg3, and CA1 of male and female offspring. Table I summarizes the results from all anatomical measures in the three brain regions of interest. Figures 2–6 display the main findings for each anatomical measure and demonstrate that prenatal stress does not produce a clear pattern of neuroanatomical change in all developing offspring.

TABLE I. Summary of two-way ANOVA evaluations of the significance between prenatal stress and sex for the 15 independent parameters measured in this study

Parameter	*N**	Male	Female
AID dendritic branching	42	N	N
AID dendritic length	42	↓	↓
AID spine density	42	↑	↑
AID neuron count	42	↓	↑
AID synapse estimation		↓	↑
Cg3 dendritic branching	42	N	N
Cg3 dendritic length	42	N	N
Cg3 spine density	42	↑	↑
Cg3 neuron count	38	N	N
Cg3 synapse estimation		↑	↓
CA1 dendritic branching	42	N	↑
CA1 dendritic length	42	↑	↑
CA1 spine density	42	↑	↑
CA1 neuron count	38	↓	↓
CA1 synapse estimation		↓	↓
% Significant		67 %	73 %

↑ significant increase ($P < 0.01$); ↓ significant decrease ($P < 0.01$).
N, nonsignificant main effect (all comparisons were made between prenatally stressed offspring and control offspring).
*N** represents hemispheres analyzed and varies due to staining quality. Cell counts were preformed on Cresyl violet-stained brains whereas dendritic analyses were performed on Golgi-Cox stained brains.

AID

Both male and female offspring of stressed moms exhibited a decrease in dendritic length, an increase

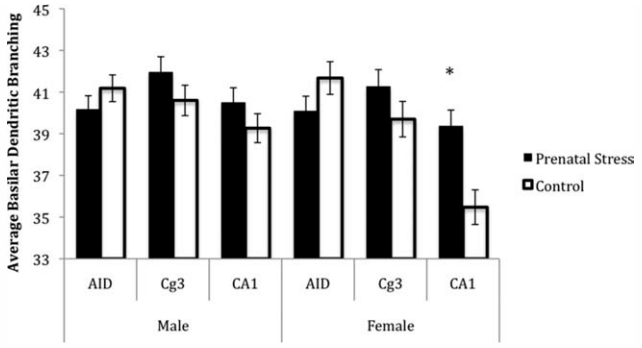

Fig. 2. Average basilar dendritic branching of neurons in three brain regions for male and female offspring at the time of sacrifice (P21) (*$P < 0.01$). Comparisons are made between prenatally stressed and no-stress control offspring.

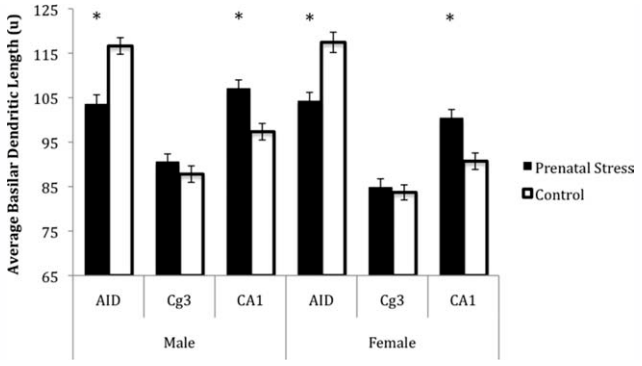

Fig. 3. Average basilar dendritic length of neurons in three brain areas for male and female offspring at the time of sacrifice (P21) (*$P < 0.01$). Comparisons are made between prenatally stressed and no-stress control offspring.

in spine density, and no change in dendritic branching in AID. Male and female offspring did; however, show differences in neuron count: male offspring exhibited a significant reduction in the number of neurons in AID whereas female offspring exhibited a significant increase in the number of neurons relative to same-sex controls. The ANOVA of neuron number in AID was the only analysis that exhibited a significant interaction effect, $F(2, 40) = 9.74$, $P < 0.01$. Despite showing an increase in spine density, male offspring demonstrated an overall drop in excitatory synapse number. Conversely, female offspring demonstrated an increase in spine density and an increase in excitatory synapses.

Cg3

There were no significant changes in dendritic branching, length, or neuron count for either male or female offspring from stressed moms. Nonetheless, there were stress-related differences in spine density in Cg3 as male offspring demonstrated an increase in spine density and a similar increase in excitatory synapse number, whereas female offspring demonstrated

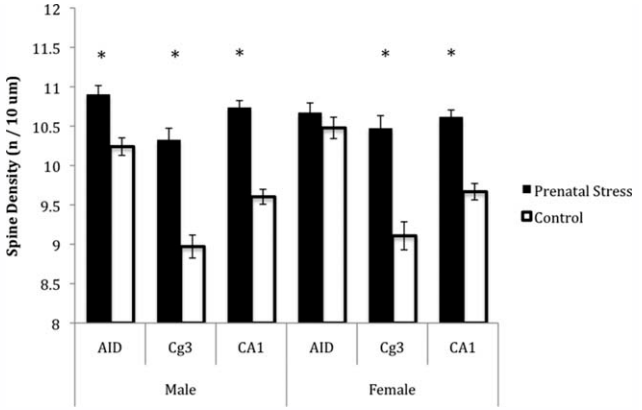

Fig. 4. Average density of excitatory synapses on basilar dendrites of neurons in three brain regions for male and female offspring at the time of sacrifice (P21) (*$P < 0.01$). Comparisons made between prenatally stressed and no-stress offspring.

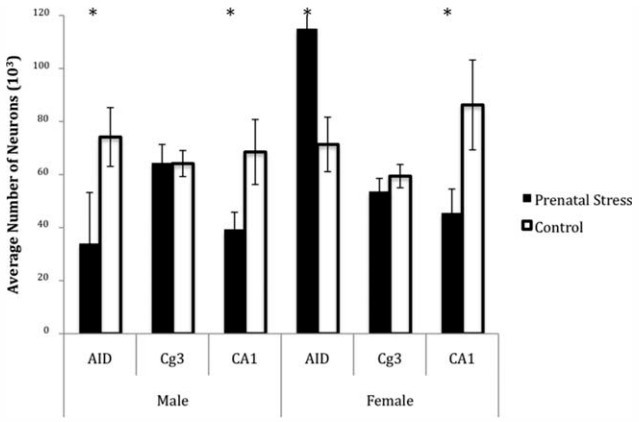

Fig. 5. Average number of neurons in three brain regions for male and female offspring at the time of sacrifice (P21) (*$P < 0.01$). Comparisons made between prenatally stressed and no-stress offspring.

an increase in spine density but a significant reduction in excitatory synapse number.

CA1

The changes identified in CA1 were similar for male and female offspring. Stressed animals from both sexes exhibited an increase in dendritic length and spine density along with a decrease in neuron count and excitatory synapse number. Female offspring however, also showed increased dendritic branching that was not found in male offspring.

Sex differences

The neuroanatomy of male and female offspring demonstrated roughly equal susceptibility to the influence of prenatal stress (male; 10/15 measures, female; 11/15 measures). It is important to note however, on 27% of the measures (4/15) the dendritic and neuronal changes in the two sexes were different (Table I).

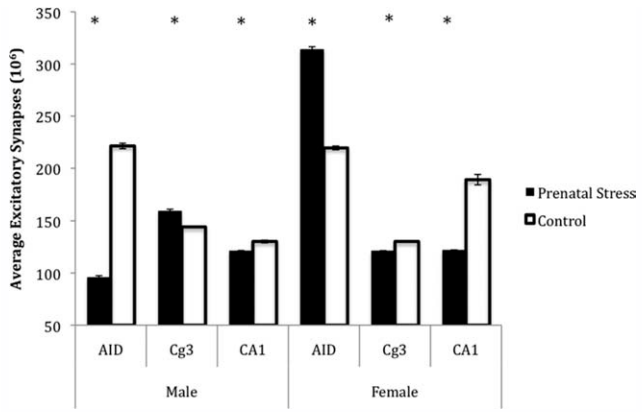

Fig. 6. Estimation of the average number of excitatory synapses in the three brain regions obtained from data regarding neuron number, dendritic length, and spine density for male and female offspring at the time of sacrifice (P21). Comparisons made between prenatally stressed offspring and no-stress offspring.

DISCUSSION

Our study demonstrates that prenatal stress significantly alters synaptic communication by changing the dendritic morphology and actual neuronal volume in OFC, mPFC, and CA1 of developing rat offspring. Furthermore, the neuroanatomical changes associated with prenatal stress that we identified were region-specific and sex-dependent. Male offspring exhibited an overall decrease in synaptic connectivity in the OFC and CA1, but increased synaptic expression in mPFC. Conversely, female offspring exposed to prenatal stress demonstrated an overall decrease in synaptic communication in the mPFC and CA1, but an increase in OFC. Interestingly, similar to previous studies, which have reported opposite structural responses of mPFC and OFC to drug exposure (Robinson and Kolb, 2004) and chronic adult stress (Liston et al., 2006), our offspring also exhibit opposing structural changes in the mPFC and OFC (males: ↓ OFC, ↑ mPFC, females: ↑ OFC, ↓ mPFC).

Male offspring

Male offspring demonstrated significant changes in 10 of the 15 neuroanatomical parameters measured. Prenatal stress appears to influence neurogenesis and/or apoptosis in the developing male brain. The number of neurons was decreased in the OFC and CA1, but was unaffected in the mPFC. To our knowledge, there has been no prior stereological examination in the prefrontal cortex of prenatally stressed offspring, however Lemaire et al. (2000) also found decreased neuron volume in the hippocampus of prenatally stressed rodents. Although, spine density was increased in the OFC, mPFC, and CA1, basilar dendritic branching was not affected in any of the brain regions examined. Murmu et al. (2006) also failed to see changes in basilar dendritic branching in the prefrontal cortex of prenatally stressed male rodents. The literature regarding spine density, however, appears to be less conclusive. Similar to our results, Martinez-Tellez et al. (2009) found increased spine densities in CA1 of prepubertal male rats exposed to prenatal stress. Contradictory to our results, Murmu et al. (2006) found decreased spine density on basilar dendrites in the OFC and no change in spine density in mPFC of prenatally stressed male offspring. Although, the offspring used in Murmu's experiment were sacrificed at the same age as ours, the differential findings may have resulted from differences in the stressing procedure and stress intensity (Mychasiuk et al., 2011b). Our experiments utilized the elevated platform paradigm at consistent time points for five consecutive days, whereas Murmu et al. (2006) used an unpredictable stress paradigm consisting of restraint stress, crowded living space, and forced swim stress for six consecutive days, but later in the pregnancy (P15-20, vs. our P12-P16). These differences in stress protocol may contribute to the variations found in the neuroanatomical results.

Research has demonstrated that offspring exposed to different intensities of prenatal stress exhibit very different behavioral outcomes and brain DNA methylation patterns (Mychasiuk et al., 2011b). When examining plastic changes from a broad perspective, an experiment that utilized the same stressing procedure as ours but examined neuroanatomy in adult offspring found a similar pattern of results in prefrontal cortex (Muhammad et al., 2011). Comparing results from the two experiments, it can be suggested that although the brain may use different mechanistic responses at different ages, the overall plastic response to prenatal stress is the same. For example, Muhammad et al. (2011) found decreased dendritic branching, decreased dendritic length, and no change in spine density of basilar neurons in the OFC of adult males exposed to prenatal stress. The overall combination of these changes likely would have resulted in decreased synaptic connectivity in the OFC induced by the prenatal stress. Although, our P21 male offspring exhibited no change in dendritic branching, decreased dendritic length, and increased spine density in the OFC, the overall plastic response was calculated to be decreased synaptic connectivity, just as with the adult rats. These results demonstrate the importance of measuring multiple components of the neuroanatomical architecture when examining the brain's response to experiences.

Female offspring

Female offspring exposed to stress during gestation exhibited changes on 11 of the 15 neuroanatomical parameters measured. Prenatal stress appears to

have had strikingly contradictory effects on synaptic connectivity in the prefrontal cortex of male and female offspring. Female offspring exhibited increased neuron numbers and a subsequent amplification of synaptic connectivity in the OFC, which is opposite to male offspring who experienced a reduction in neuron numbers and decreased synaptic connectivity in the same region of the prefrontal cortex. Furthermore, female offspring demonstrated decreased synaptic connectivity in the mPFC, a brain region that exhibited increased synapse estimates in male offspring. The majority of previous prenatal stress (Martinez-Tellez et al., 2009; Michelsen et al., 2007) and chronic stress (Brown et al., 2005; Radley et al., 2008; Watanabe et al., 1992) research has been conducted on male offspring so there is very little research to compare our results to. However, Garrett and Wellman (2009) found sex-dependent responses to chronic stress in adulthood in apical dendrites of the mPFC, and Murmu et al. (2006) found sex-specific alterations to dendritic morphology in OFC and the anterior cingulate following prenatal stress.

The prefrontal cortex of the male and female brain appears to show a significantly different response to the same experience. The divergent alterations to prefrontal neuroanatomy could result from significant disruption to sex-dependent rates of cortical brain maturation (Kolb and Whishaw, 2008), following exposure to maternal glucocorticoids. It is also possible that differences in estrogen receptor localization (Yokosuka et al., 1997), along with variations in masculinizing sex hormones (Wu et al., 2009) or sex differences in neurotrophic factors such as FGF-2 (Molteni et al., 2001) in the prefrontal cortex of the male and female brain, contribute to their distinct neuroanatomical responses to the same prenatal experience. Finally, sex-dependent differences in epigenetic susceptibility (Dunn et al., 2011; McCarthy et al., 2009) may exist in the epigenome of the prefrontal cortex that permit differential modifications to dendritic morphology and neurogenesis.

The sex-dependent differential response to prenatal stress was not present in the hippocampus of offspring. Female and male offspring demonstrated surprisingly similar changes in region CA1. Both offspring demonstrated increased dendritic length, increased spine density, decreased neuronal numbers, and an overall reduction in synaptic connectivity. The only change that was different for male and female offspring following exposure to prenatal stress was in dendritic branching. Female offspring experienced an increase in cell complexity, where as, there was no change in CA1 dendritic branching in male offspring. The dramatic decrease in synaptic connectivity in the hippocampus is consistent with previous stress research (Martinez-Tellez et al., 2009; McKittrick et al., 2000; Watanabe et al., 1992) and is thought to serve as a compensatory mechanism to decrease glucocorticoid activation of the HPA axis.

On the basis of the substantial differences in neuroanatomical changes we identified in male and female offspring of dams exposed to stress during gestation, it is tempting to conclude that prenatal stressors could contribute to the sexually-dimorphic incidence of many behavioral disorders such as ADHD, schizophrenia, and depression. The connection between such disorders and the alterations in neuronal networks is, of course, still a mystery as is the relationship between intervention programs and synaptic reorganization. However, in view of the sexually-dimorphic synaptic changes it would seem likely that treatment programs for behavioral disorders may require sex-dependent therapies.

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