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ALTERED BRAIN MORPHOLOGY AND FUNCTIONAL CONNECTIVITY REFLECT A VULNERABLE AFFECTIVE STATE AFTER CUMULATIVE MULTIGENERATIONAL STRESS IN RATS

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Abstract—Prenatal stress is a risk factor for abnormal neuroanatomical, cognitive, behavioral and mental health outcomes with potentially transgenerational consequences. Females in general seem more resilient to the effects of prenatal stress than males. Here, we examined if repeated stress across generations may diminish stress resiliency and cumulatively enhance the susceptibility for adverse health outcomes in females. Pregnant female rats of three successive generations were exposed to stress from gestational days 12–18 to generate multigenerational prenatal stress (MPS) in the maternal lineage. Stress response was measured by plasma corticosterone levels and open-field exploration in each generation. Neuromorphological consequences of MPS were investigated in the F3 generation using *in vivo* manganese-enhanced magnetic resonance imaging (MEMRI), T_2-relaxometry, and cytoarchitectonics in relation to candidate gene expression involved in brain plasticity and mental health. Each additional generation of prenatal stress incrementally elevated hypothalamic–pituitary–adrenal axis activation, anxiety-like and aversive behaviors in adult female offspring. Elevated stress responses in the MPS F3 generation were accompanied by reduced neural density in prefrontal cortex, hippocampus and whole brain along with altered brain activation patterns in *in vivo* MEMRI. MPS increased ephrin receptor A5 (*Epha5*), neuronal growth regulator (*Negr1*) and synaptosomal-associated protein 25 (*Snap25*) gene expression and reduced fibroblast growth factor 12 (*Fgf12*) in prefrontal cortex. These genes regulate neuronal maturation, arborization and synaptic plasticity and may explain altered brain cytoarchitectonics and connectivity. These findings emphasize that recurrent stress across generations may cumulatively increase stress vulnerability and the risk of adverse health outcomes through perinatal programing in females.

Key words: multigenerational prenatal stress, manganese-enhanced magnetic resonance imaging, T_2-relaxometry, gene expression, stress resilience, HPA axis.

INTRODUCTION

Approximately one third of the world's population is afflicted by mental illness, such as anxiety and depression, which are commonly related to stress. Individual predisposition to higher stress sensitivity and thus greater risk to mental illness may be programed by the early environment. The perinatal period is particularly vulnerable to the programing of stress response and mental health (Ruiz and Avant, 2005; Cottrell and Seckl, 2009). Severe maternal distress during pregnancy activates the maternal hypothalamic–pituitary–adrenal (HPA) axis and initiates the release of glucocorticoids (GCs) such as cortisol in humans or corticosterone (CORT) in rats, which may produce excessive GC levels that enter the fetal circulation to affect the developing brain (Takahashi et al., 1997, 1998; Takahashi, 1998; Williams et al., 1999). Consequences of excessive fetal GC exposure may particularly alter the developmental trajectories of the hippocampus and prefrontal cortex (Golub et al., 2011; Palacios et al., 2011). For example, prenatal stress may reduce hippocampal neurogenesis, alter dendritic morphology, and diminish hippocampal volume (Lemaire et al., 2000; Lui et al., 2011; Mychasiuk et al., 2012) along with global increases in cerebral blood flow and changes in energy metabolism (Bryan, 1990).

Modifications in brain development by prenatal stress are generally associated with lasting behavioral

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Abbreviations: CORT, corticosterone; *Epha5*, ephrin receptor A5; *Fgf12*, fibroblast growth factor 12; GC, glucocorticoid; HPA, hypothalamic–pituitary–adrenal; MEMRI, manganese-enhanced magnetic resonance imaging; MPS, multigenerational prenatal stress; *Negr1*, neuronal growth regulator; P, postnatal day; ROI, region of interest; S, stress; *Snap25*, synaptosomal-associated protein 25; qRT-PCR, quantitative real-time PCR.

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impairments, such as exacerbated stress responses later in life (Glover et al., 2009) and elevated risk of mental illness such as depression (Nestler et al., 2002a), anxiety (Glover et al., 2009) and schizophrenia (Malaspina et al., 2008). For example, patients with depression display reduced hippocampal volume and cell density associated with elevated HPA axis activity and functionality (Nestler et al., 2002b; Bremner et al., 2008). Similarly, changes in temporal lobe asymmetry along with changes in stress sensitivity due to prenatal stress have been suggested as causal factors in schizophrenia (Weinstock, 2001).

The morphological and behavioral consequences of prenatal stress appear to be sex-specific, with more prominence in male offspring (Mychasiuk et al., 2012). The sexually dimorphic behavioral consequences of prenatal stress have been proposed to lead to heightened vulnerability to neurodevelopmental disorders, such as schizophrenia, in males (Mueller and Bale, 2008). Greater resilience to prenatal stress in females has been linked to sex-specific genetic and epigenetic regulation (Van den Hove et al., 2013) and differential endocrine and neurotransmitter responses (Wei et al., 2013; Yao et al., 2014). However, recurrent prenatal stress across generations produces changes that differ from the consequences of prenatal stress in a single generation (Migicovsky and Kovalchuk, 2011; Veenendaal et al., 2013). Notably, intergenerational transfer of altered stress response (Skinner, 2008; Franklin et al., 2010; Babenko et al., 2012; Crews et al., 2012; Zucchi et al., 2012; Zucchi et al., 2013) may have maladaptive changes, thus enhancing vulnerability to disease (Hao and Metz, 2013; Metz et al., 2015).

While previous studies focused on stress in the F1 generation and its transgenerational effects (Franklin et al., 2010; Yao et al., 2014), there has been little investigation of the effects of recurrent, multigenerational prenatal stress (MPS) resembling a population living in a continuously hostile environment. Our work indicates that MPS disrupts maternal behavior in adult offspring (Ward et al., 2013), promotes hyperactivity (Erickson et al., 2014), causes a functional simplification of brain signaling (Skelin et al., 2015) and hemispheric dominance shift (Ambeskovic et al., 2016) in males along with altered epigenetic regulation (Yao et al., 2014).

Here we proposed that recurrent prenatal stress exposure across three generations of the maternal lineage will gradually aggravate stress sensitivity and increase the risk of emotional disturbances in the offspring. We examined if behavioral changes and altered gene expression induced by MPS in the F3 generation are associated with altered brain activation and morphology using *in vivo* manganese-enhanced magnetic resonance imaging (MEMRI), T_2-relaxometry, and cytoarchitectonics.

EXPERIMENTAL PROCEDURES

Animals

This study involved 141 female Long-Evans rats bred and raised at the University of Lethbridge vivarium. The animals were housed in pairs or triplets under a 12-h light/dark cycle with lights on at 7:30 AM. The room temperature was maintained at 20 °C with relative humidity at 30%. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Lethbridge Animal Welfare Committee.

Experimental design

The design of the MPS animal model is illustrated in Fig. 1. Three generations (F0, F1, F2) of timed-pregnant dams were bred under standardized conditions and mated with non-stressed males. Animals in the MPS lineage were stressed daily from gestational day 12–18 by restraint and forced swimming applied in a semi-random sequence (Yao et al., 2014). Groups of female F1, F2, and F3 adult offspring underwent blood sampling for CORT assays on postnatal day (P) 90 and open field testing on P110 to determine HPA axis programing across generations. Each generation used yoked non-stress controls which were used as comparison for open field behavior. CORT values of stressed F1, F2 and F3 animals were compared to the naïve parental F0 generation to minimize potential influences of secondary bystander effects (see Mychasiuk et al., 2011).

In the F3 generation, 120-day old MPS and non-stress control rats were implanted with osmotic mini-pumps for $MnCl_2$ delivery for MEMRI measurements. At P130 animals were sacrificed for histological analysis. Age-matched naïve MPS and control animals were sacrificed to collect prefrontal cortex tissue for gene expression analyses.

Corticosterone analysis

Blood was sampled from females between 8:00 and 9:00 in the morning hours in the non-stress F0 (n = 5), and stressed F1 (F1-S; n = 7), F2 (F2-SS; n = 6), F3 (F3-SSS; n = 7) generations. Rats were anesthetized with 2% isoflurane in oxygen and maintained for approximately 5 min in which 0.6 ml of blood was

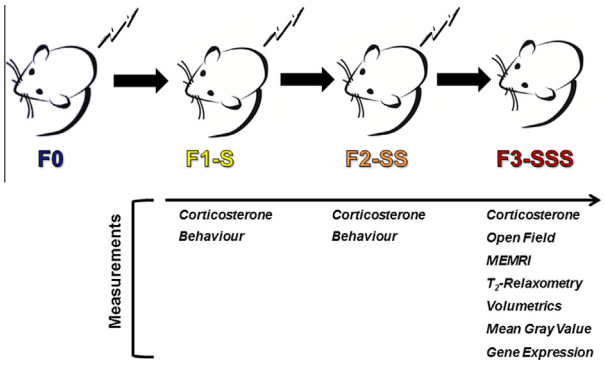

Fig. 1. Schematic diagram of the experimental design using multigenerational exposure to prenatal stress across three generations. The parental generation (F0), their daughters (prenatal stress, F1-S) and grand-daughters (F2-SS) underwent restraint and swim stress during pregnancy resulting in multigenerationally stressed great-grand-daughters (F3-SSS). Female rats derived from a non-stressed lineage were used as controls. Specific measurements performed are listed for each generation.