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Research Report

Maternal separation alters serotonergic and HPA axis gene expression independent of separation duration in c57bl/6 mice

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ABSTRACT

Adverse early life experiences (aELEs), such as child abuse, neglect, or trauma, increase lifetime vulnerability for mental illness. In this study, aELEs were modeled in c57bl/6 mice using the maternal separation (MS) paradigm, in which pups were separated for 180 min/day (MS180), 15 min/day (MS15), or left undisturbed (AFR) from postnatal day 2–14. As adults, pups that experienced MS15 or MS180 demonstrated decreases in tryptophan hydroxylase 2 and serotonin transporter mRNA in the dorsal raphe dorsalis and ventralis, and increases in glucocorticoid receptor mRNA in the dentate gyrus of the hippocampus. To investigate factors underlying shared expression between MS conditions, dam on-nest time and DNA methylation at the TPH2 promoter and 5′ UTR were assessed. Post-reunion on-nest time increased as a function of separation duration, potentially serving as a mitigating factor underlying similar expression between MS conditions. TPH2 DNA methylation remained unchanged, suggesting changes in TPH2 mRNA are not mediated by changes in DNA methylation of this region. The shared pattern of expression between MS15 and MS180 conditions suggests a species- or strain- specific response to MS unique to c57bl/6 mice.

1. Introduction

Adverse early life experiences (aELEs), such as child abuse, neglect, and trauma, increase lifetime risk for mental illness. Limited clinical studies suggest immediate and long-term irregularities in stress responses (Heim et al., 2010). In parallel, abnormalities of the serotonin (5-HT) system, a neurotransmitter network implicated in modulating complex behaviors, is associated with the pathogenesis or vulnerability for mental illness (Lucki, 1998). However, much remains

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unknown regarding the long-term effects of aELEs on serotonergic function.

Polymorphisms in **tryptophan hydroxylase 2 (TPH2)** and **serotonin transporter (SERT)** are implicated in vulnerability for mental illness (Caspi et al., 2003; Waider et al., 2011). TPH2 is the rate-limiting enzyme in neuronal 5-HT synthesis (Walther et al., 2003), whereas SERT is the primary transporter responsible for reuptake of 5-HT (Blakely et al., 1991). TPH2 mRNA levels are increased in depressed suicides (Bach-Mizrachi et al., 2008) and polymorphisms are associated with risk for ADHD, anxiety disorders, suicide, and depression (Waider et al., 2011). Limited reports suggest that aELEs, such as immune system challenge (Sidor et al., 2010) or maternal separation (Gardner et al., 2009b) can alter TPH2 expression. Highlighting serotonergic and stress axis sensitivity to early life events, rhesus macaques carrying a hypomorphic TPH2 allele exhibit increased stress levels if exposed to low-quality peer care as infants (Chen et al., 2010). Similarly, SERT polymorphisms increase risk for depression and anxiety disorders, and interact with aELEs to increase risk for mental illness (Caspi et al., 2003).

A popular model of aELE is maternal separation (MS), in which rat pups are separated for 15 (MS15) or 180 (MS180) minutes per day from postnatal day (PND) 2–14. MS180 is associated with long-term increases in anxiety-related behaviors and abnormal stress axis function in rats. Conversely, MS15, the handling control, is associated with a "stress-resilient" profile characterized by lower anxiety-related behaviors and quicker feedback response (de Kloet et al., 2005). In part, long-term changes in behavior and stress response are thought to be mediated by changes in expression of the **glucocorticoid receptor (GR),** a receptor intimately involved in negative feedback and stress response (Anisman et al., 1998). Although the stress axis shares extensive anatomical and functional connections with the serotonergic system (Lopez et al., 1999), reports on serotonergic changes following MS have been inconsistent. For example, long-term SERT expression in MS180 rats is reported to increase (Gardner et al., 2009a), decrease (Lee et al., 2007), or remain unchanged (Oreland et al., 2009), highlighting difficulties in demonstrating a consistent effect on serotonergic development.

In parallel, emerging evidence suggests early experience-dependent changes in gene expression are mediated by epigenetic mechanisms (Weaver et al., 2004). Long-term decreases in anxiety behaviors and expression of corticotrophin releasing hormone (CRH), a stress peptide, following MS15, are linked to increases in the epigenetic regulator RE-1 Silencing Transcription Factor (REST) (Korosi et al., 2010), a known regulator of TPH2 (Patel et al., 2007). Conversely, prolonged separations, i.e. MS180, decreases REST mRNA in rats, and injections of a dominant-negative isoform, REST4, during postnatal but not adult life elevates anxiety (Uchida et al., 2010). This invites the possibility that early experience-dependent changes in expression of other REST-regulated genes, such as TPH2, are epigenetically mediated.

Although predominantly a rat model of aELE, there is interest in adapting the MS paradigm in mice. This is important for the validation and applicability of findings for clinical research, as well as combining a well-validated aELE model with the extensive genetic toolkit readily available in mice. However, although some studies report expected changes in behavior and stress axis response to MS (Romeo et al., 2003; Veenema et al., 2007; Veenema and Neumann, 2009), others have suggested a resiliency to MS in c57bl/6 mice (Millstein and Holmes, 2007; Parfitt et al., 2007). Yet, to our knowledge, the only reports of MS-related epigenetic changes have been in c57bl/6 mice, implying the strain is ideal for investigating aELE-mediated epigenetic changes (Bordner et al., 2011; Murgatroyd et al., 2009). Therefore to clarify and explore the MS paradigm in c57bl/6 mice, we performed MS to investigate (i) long-term changes in the expression of TPH2, SERT, and GR mRNA, (ii) maternal response to MS, and (iii) DNA methylation of the TPH2 promoter and 5′ untranslated region.

2. Results

To assess whether maternal separation (MS) affected gross development, mice were weighed on postnatal day (PND) 2, 14, 21, 35, and 60. Weight was not significantly different between groups on all days (data not shown). Because analysis incorporates expression data from the original and replicate study, optical density from MS15 and MS180 sections were normalized against the AFRs of each study. Raphe expression was assessed across 10 sections from –4.35 to –4.80 bregma (Paxinos, 2001), covering the dorsal raphe dorsalis (DRd), ventralis (DRv), lateral wings (DRlw), median raphe (MR), and paramedian raphe (pMR) (Fig. 1). Analyses of TPH2 mRNA and SERT mRNA levels across raphe subdivisions revealed significant differences contingent upon having experienced MS, but largely independent of the duration of separation. TPH2 mRNA in the DRd $[F(2,20)=13.12;\ p<0.001]$ and DRv $[F(2,20)=16.39;\ p<0.001]$ was decreased in MS15 $[\text{DRd: } p<0.001;\ \text{DRv: } p<0.001]$ and MS180 mice $[\text{DRd: } p=0.001;\ \text{DRv: } p<0.001]$. However, only MS15 mice $[\text{MR: } p=0.02]$ had decreased TPH2 mRNA in the MR $[F(2,20)=3.78;\ p=0.04]$ and no significant changes in TPH2 mRNA was found in the DRlw $[F(2,20)=2.39;\ p=0.12]$ and pMR $[F(2,20)=2.21;\ p=0.14]$ (Fig. 3**A**). A similar pattern was observed for SERT mRNA in raphe, with decreased levels in the DRd $[F(2,20)=4.27;\ p=0.03]$ and the DRv $[F(2,20)=7.53;\ p<0.01]$ for MS15 $[\text{DRd: } p<0.05;\ \text{DRv: } p<0.01]$ mice, decreased in the DRv but not DRd for MS180 $[\text{DRd: } p=0.06;\ \text{DRv: } p<0.01]$ mice, and not changed in the DRlw $[F(2,21)=0.27;\ p=0.77]$, MR $[F(2,20)=3.02;\ p=0.07]$, and pMR $[F(2,20)=0.95;\ p=0.41]$ for either MS15 or MS180 mice (Fig. 3**B**).

Reports on MS-related changes in TPH2 and SERT mRNA levels are limited. As GR has been widely reported to be sensitive to early life experience, we used it as a marker to assess the effectiveness of MS180 as an aELE in c57bl/6 mice. Anterior hippocampal GR mRNA was analyzed in the dentate gyrus (DG), CA3 region, and CA1|CA2 region (Fig. 2). GR mRNA was increased in MS15 $[\text{DG: } p=0.04]$ and MS180 $[\text{DG: } p=0.03]$ conditions in the DG $[F(2,20)=4.55;\ p=0.02]$, but unchanged in the CA3 $[F(2,20)=2.25;\ p=0.13]$ and CA1|CA2 regions $[F(2,20)=1.60;\ p=0.23]$ (Fig. 3**C**).

To investigate whether changes in TPH2 mRNA were epigenetically regulated, DNA methylation was assessed at the TPH2 promoter and 5′ UTR using sodium bisulfite sequencing. This region was chosen for its high number of CpG dinucleotides, high CG content (50%+), and inclusion of a REST binding site. However, despite differences in TPH2