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Exposure to Acute and Chronic Fluoxetine has Differential Effects on Sociability and Activity of Serotonergic Neurons in the Dorsal Raphe Nucleus of Juvenile Male BALB/c Mice

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Abstract—Although the neurobiological mechanisms underlying autism spectrum disorder (ASD) are still unknown, dysregulation of serotonergic systems has been implicated in the etiology of ASD, and serotonergic antidepressant drugs are often prescribed to treat some symptoms of ASD. The BALB/c strain of mice express a dysregulated serotonergic system and a phenotype that is relevant to ASD. In this study, juvenile male BALB/c mice were exposed to the selective serotonin reuptake inhibitor fluoxetine either chronically (18 mg/kg/-day in drinking water, post-natal day (PND) 28–39) or acutely (18 mg/kg, i.p.; PND40), or to vehicle control conditions (0.9% sterile saline, i.p.; PND40), prior to being exposed to the three-chambered sociability test (SAT; PND40). One cohort of mice then received an injection of the aromatic amino acid decarboxylase inhibitor, NSD-1015, and one hour later brain tissue was collected for quantification of 5-hydroxytryptophan accumulation in the dorsal raphe nucleus (DR) as a measure of TPH2 activity. For the second cohort, brain tissue was collected ninety minutes after the onset of the social phase of the SAT and prepared for immunohistochemical staining for c-Fos and TPH2 to measure the activation of serotonergic neurons within subregions of the DR. Acute fluoxetine decreased social behavior, while chronic fluoxetine increased social behavior compared with vehicle-treated controls. Furthermore, acute and chronic fluoxetine treatments were without effect on TPH2 activity but differentially affected populations of serotonergic neurons in the DR. These data are consistent with the hypothesis that serotonergic systems are implicated in social behavior that is relevant for ASD.

Key words: serotonin, antidepressant, fluoxetine, anxiety, autism, dorsal raphe.

INTRODUCTION

Autism spectrum disorders (ASD) are highly heterogeneous neurodevelopmental disorders affecting over 1 in 68 individuals in the United States (Christensen, 2016; Bent et al., 2017). Although restricted and repetitive behaviors and interests are typical characteristics of ASD (American Psychiatric Association, 2013), low sociability, or a reduced drive to seek social interactions, is one of the most prominent and disabling symptoms (Brodkin, 2007). Despite significant research, the neurobiological basis of some common behavioral symptoms of ASD, such as reduced sociability, remain unclear.

Growing evidence supports a dysregulation of the serotonergic systems in the etiology of ASD. For example, allelic variations in the *TPH2* gene, which encodes tryptophan hydroxylase 2, the rate-limiting enzyme for the biosynthesis of brain serotonin, are associated with increased risk for ASD (Yang et al., 2012; Singh et al., 2013). Functional evidence further indicates that the peak in brain serotonin synthesis capacity during the first five years seen in typically developing children is absent in ASD (Chugani et al., 1999), while pharmacologically decreasing brain serotonin levels increases anxiety and exacerbates behavioral symptoms in adults with ASD (McDougle et al., 1996). As the serotonergic system is involved in early brain development

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Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; 5-HTP, 5-hydroxytryptophan; AADC, aromatic amino acid decarboxylase; ASD, autism spectrum disorder; DR, dorsal raphe nucleus; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; HPLC, high performance liquid chromatography; i.p., intraperitoneal; NSD-1015, m-hydroxybenzylhydrazine; PND, post-natal day; SAT, three-chambered social approach test; SNP, single nucleotide polymorphism; SSRI, selective serotonin reuptake inhibitor; *TPH2*, tryptophan hydroxylase 2, human, gene/mRNA; *Tph2*, tryptophan hydroxylase 2, mouse/rat, gene/mRNA; TPH2, tryptophan hydroxylase 2, protein.

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(Bonnin et al., 2006), dysregulation of this system may have important implications for ASD-related behaviors.

Selective serotonin reuptake inhibitors (SSRIs) are often prescribed for individuals with ASD (Hollander et al., 2003), including children (Oswald and Sonenklar, 2007). Preliminary evidence suggests some degree of efficacy of SSRIs in improving social behaviors and communication in ASD (for reviews, see Moore et al., 2004; Posey et al., 2006). However, controlled studies are lacking due to the high heterogeneity of the disorder. In preclinical studies, the BALB/c mouse has been used to study the role of the serotonergic systems in behaviors that are relevant for ASD as it carries a loss-of-function polymorphism (C1473G) in the *Tph2* gene and exhibits low baseline sociability. In this model, chronic SSRI treatment produces antidepressant-like responses (Dulawa et al., 2004; Jiao et al., 2011) and has anxiolytic-like effects (Dulawa et al., 2004; Holick et al., 2008). However, little is known about the effect of SSRI treatment on social behavior in this strain.

Although the precise mechanisms underlying the effects of chronic administration of SSRIs on behavior are not fully understood, several hypotheses have been proposed. For example, several lines of evidence suggest that regulation of serotonin synthesis through the modulation of TPH2 enzyme activity, which can be assessed using the decarboxylase inhibition method, is important for the therapeutic effects of SSRIs. Using this method, chronic treatment with the SSRI citalopram decreased 5-HTP accumulation, thereby indicating decreased TPH2 activity, in forebrain regions (Honig et al., 2009; Bosker et al., 2010). Alternatively, a role for modulation of serotonergic neuronal firing rates in the dorsal raphe nucleus (DR), which contains the majority of the forebrain-projecting serotonergic neurons, has been suggested. Acute injection of SSRIs inhibit DR neuronal firing rates (Evrard et al., 1999; Rasmussen et al., 2004; El Mansari et al., 2005), which is associated with changes in behavioral measures (Hajós et al., 1995; Teissier et al., 2015). Importantly, the DR contains subsets of anatomically and functionally organized serotonergic neurons (for review, see Hale and Lowry, 2011) and subpopulations of serotonergic neurons in the DR are differentially activated following social and anxiety-related stimuli (Donner et al., 2012; Hale et al., 2012). However, to date, no study has looked at the effect of SSRIs on subpopulations of DR serotonergic neurons.

To examine the role of serotonergic systems in social behaviors that are relevant for ASD, juvenile male BALB/c mice were treated either acutely or chronically with the SSRI fluoxetine and exposed to the three-chambered social approach test. TPH2 enzyme activity and activation of serotonergic neurons were subsequently measured across subregions of the DR.

EXPERIMENTAL PROCEDURES

Animals

Forty-eight male BALB/c mice were obtained from the Animal Research Centre (ARC, Western Australia) and housed at the La Trobe Animal Research and Teaching Facility (Cohort 1; TPH2 activity, $N = 24$; Cohort 2; c-Fos/TPH2 immunostaining, $N = 24$). An additional sixteen age-matched conspecific mice were used as stimulus mice in the social approach test (Nadler et al., 2004). Mice arrived at post-natal day (PND) 21 and were given seven days to habituate to the facility prior to the start of the experiment. Adolescence in mice consists of early adolescence (prepubescent or juvenile; PND 21–34), middle adolescence (periadolescent; PND 34–46), and late adolescence (PND 46–59) time periods (Spear, 2000). Thus, all mice were juveniles (or prepubescent; PND 28) at the start of the experiment and middle adolescence (periadolescent, PND 40) following 12 days of SSRI or vehicle administration in the drinking water. Mice were group housed, four per cage, in autoclaved IVC cages (39.1 cm W × 19.9 cm D × 16 cm H; Greenline Sealsafe Plus GM500; Tecniplast, Rydalmere, NSW, Australia) with standard bedding, on a 12 h:12 h reverse light–dark cycle (lights on at 1900 h), with temperature and humidity levels maintained constant (21 °C ± 2 °C, 45% humidity), and with *ad libitum* food and water. Following the seven-day habituation period, mice were randomly assigned to one of three treatment groups: control ($n = 16$), acute fluoxetine ($n = 16$) or chronic fluoxetine ($n = 16$). All procedures were conducted in accordance with the NHMRC Australian code for the care and use of animals for scientific purposes (8th edition, 2013) and approved by the La Trobe University Animal Ethics Committee. For an illustration of the experimental timelines, see Fig. 1. All efforts were made to minimize the number of animals used and their suffering.

Pharmacological treatment

The selective serotonin reuptake inhibitor fluoxetine hydrochloride (fluoxetine; Cat. No. F132-10MG; Lot# LRAA9180; Sigma–Aldrich, Castle Hill, NSW, Australia) was administered either chronically or acutely.

Mice in the chronic treatment group received fluoxetine dissolved in plain drinking water at a concentration of 0.14 mg/ml, for 12 consecutive days (days 1–12; PND 28 to PND 39); the control and acute groups received plain drinking water. Previous research indicates that this concentration of fluoxetine in the drinking water results in a dose of 18 mg/kg/day in BALB/c mice (Dulawa et al., 2004). This dose was selected as it is the lowest dose that produces chronic antidepressant-like responses in this strain (Dulawa et al., 2004). Water bottles were changed once, midway through the experiment (day 6). To ensure that mice receiving fluoxetine treatment received the correct dose of drug every day and to control for any effect of treatment on body weight, drinking bottles were weighed daily and mice were weighed twice per week.

On the test day (day 13; PND 40), mice in the acute treatment group received an intraperitoneal (i.p.) injection of 100 μl of 18 mg/kg of fluoxetine dissolved in 0.9% sterile saline; mice in the chronic treatment group and mice in the vehicle control group both received an i.p. injection of 100 μl of vehicle (0.9% sterile saline).