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Serotonin 5-HT$_{1B}$ receptor-mediated behavior and binding in mice with the overactive and dysregulated serotonin transporter Ala56 variant

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Abstract

Rationale—Elevated whole-blood serotonin (5-HT) is a robust biomarker in ~ 30% of patients with autism spectrum disorders, in which repetitive behavior is a core symptom. Furthermore, elevated whole-blood 5-HT has also been described in patients with pediatric obsessive-compulsive disorder. The 5-HT$_{1B}$ receptor is associated with repetitive behaviors seen in both disorders. Chronic blockade of serotonin transporter (SERT) reduces 5-HT$_{1B}$ receptor levels in the orbitofrontal cortex (OFC) and attenuates the sensorimotor deficits and hyperactivity seen with the 5-HT$_{1B}$ agonist RU24969. We hypothesized that enhanced SERT function would increase 5-HT$_{1B}$ receptor levels in OFC and enhance sensorimotor deficits and hyperactivity induced by RU24969.

Objectives—We examined the impact of the SERT Ala56 mutation, which leads to enhanced SERT function, on 5-HT$_{1B}$ receptor binding and 5-HT$_{1B}$-mediated sensorimotor deficits.

Methods—Specific binding to 5-HT$_{1B}$ receptors was measured in OFC and striatum of naïve SERT Ala56 or wild-type mice. The impact of the 5-HT$_{1A/1B}$ receptor agonist RU24969 on prepulse inhibition (PPI) of startle, hyperactivity, and expression of cFos was examined.

Results—While enhanced SERT function increased 5-HT$_{1B}$ receptor levels in OFC of Ala56 mice, RU24969-induced PPI deficits and hyperlocomotion were not different between genotypes. Baseline levels of cFos expression were not different between groups. RU24969 increased cFos expression in OFC of wild-types and decreased cFos in the striatum.

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Conclusions—While reducing 5-HT$_{1B}$ receptors may attenuate sensorimotor gating deficits, increased 5-HT$_{1B}$ levels in SERT Ala56 mice do not necessarily exacerbate these deficits, potentially due to compensations during neural circuit development in this model system.

Keywords

Serotonin; 5-HT$_{1B}$; Autism spectrum disorder; ASD; Obsessive-compulsive disorder; OCD; Sensorimotor gating; Prepulse inhibition of startle; Orbitofrontal cortex; RU24969

Introduction

Elevated whole-blood serotonin (5-HT) levels are the most robust biomarker in autism spectrum disorder (ASD) (Gabriele et al. 2014; Muller et al. 2016) and have also been described in familial pediatric obsessive-compulsive disorder (OCD) (Hanna et al. 1991), suggesting that the serotonin system is perturbed in both disorders. ASD and OCD share repetitive behavior as a core symptom domain and frequently co-occur in the same individual (Bejerot 2007; Leyfer et al. 2006). Sensorimotor gating deficits have also been found in both ASD and OCD (Ahmari et al. 2012, 2016; Frankland et al. 2004; Hoenig et al. 2005; Kohl et al. 2013; McAlonan et al. 2002). Furthermore, serotonin 5-HT$_{1B}$ receptor agonists disrupt sensorimotor gating, and increased sensitivity to 5-HT$_{1B}$ agonists has been described in both ASD and OCD (Hollander et al. 2000; Novotny et al. 2004).

Prepulse inhibition (PPI) of startle has been used to study sensorimotor gating deficits in ASD and OCD (Ahmari et al. 2012, 2016; Frankland et al. 2004; Hoenig et al. 2005; Kohl et al. 2013). In rodents, 5-HT$_{1B}$ agonists attenuate PPI (Dulawa et al. 1997; Shanahan et al. 2009). The mixed 5-HT$_{1A/1B}$ agonist RU24969 diminishes the PPI response in wild-type (Dulawa et al. 1998) and 5-HT$_{1A}$ receptor knockout mice (Dulawa et al. 2000) but not in 5-HT$_{1B}$ receptor knockout mice (Dulawa et al. 1997, 1998, 2000), suggesting that RU24969 selectively modulates PPI through the 5-HT$_{1B}$ receptor. Furthermore, perseverative hyperlocomotion induced by RU24969 is also mediated by the 5-HT$_{1B}$ receptor (Shanahan et al. 2009).

While 5-HT$_{1B}$ receptors are most densely located on striatal neuron terminals in the globus pallidus and substantia nigra, and on Purkinjie neuron terminals in the deep cerebellar nuclei (Boschert et al. 1994; Pazos and Palacios 1985), 5-HT$_{1B}$ receptors in the orbitofrontal cortex (OFC) have been established as necessary and sufficient to induce PPI deficits and hyperlocomotive behavior in mice (Shanahan et al. 2011). Additionally, positive correlations between 5-HT$_{1B}$ receptor binding in the OFC and PPI in controls and OCD patients were observed (Pittenger et al. 2016). Functional neuroimaging studies found increased activity in the OFC and striatum in OCD patients (Saxena and Rauch 2000). The correlation between 5-HT$_{1B}$ receptor binding in the dorsal striatum was also implicated in PPI in rodent studies (Baldan Ramsey et al. 2011; Swerdlow et al. 2001), and PPI was significantly different from the correlation in OCD patients (Pittenger et al. 2016). Finally, 5-HT$_{1B}$ receptor agonism increased cFos expression, a marker of cellular activity, in the striatum of mice (Ho et al. 2016). As a result of these collective data across species, the 5-HT$_{1B}$ receptor has been

suggested as a potential mediating factor in repetitive behavior seen in OCD (Dulawa et al. 1997; Pittenger et al. 2016).

Effective OCD treatments have been used to validate the use of 5-HT_{1B} agonism in modeling OCD-related behavioral abnormalities. Serotonin reuptake inhibitors (SRIs) provide effective pharmacological therapy for OCD (Hollander and Pallanti 2002). In mice, treatment for 28 days, but not 7 days, with selective SRIs (SSRIs) prevented PPI attenuation by RU24969 (Shanahan et al. 2009). Knockout of the 5-HT transporter (SERT) similarly prevents PPI attenuation by RU24969 (Shanahan et al. 2009). Treatment with ketamine, a promising treatment for OCD (Rodriguez et al. 2013), for 24 h reduced the preservative hyperlocomotion deficits induced by RU24969 (Thompson et al. 2020). Chronic SRI treatment also ameliorated RU24969-induced preservative hyperlocomotion in mice, while desipramine, a norepinephrine reuptake inhibitor that is not effective at treating OCD, did not affect RU24969-induced hyperlocomotion (Ho et al. 2016; Thompson et al. 2020). Thus, the PPI and hyperlocomotor deficits seen in mice after RU24969 administration are meaningful behavioral readouts for examining OCD-related circuit abnormalities associated with 5-HT_{1B} receptors.

Abnormal SERT function could explain elevated whole-blood 5-HT levels in autism and linkage to autism has been identified on the 17q region harboring the *SLC6A4* gene that encodes SERT, particularly in families with affected males, inherited from their unaffected mothers (International Molecular Genetic Study of Autism Consortium 1998; Sutcliffe et al. 2005). Multiple rare variants of *SLC6A4* have been identified in families with ASD and linkage to chromosome 17q, with the most common amino acid variant being a glycine to alanine change at position 56 in the SERT protein (Gly56Ala, referred to as Ala56) (Sutcliffe et al. 2005); however, de novo variants in SERT have not been identified at increased rates in autism (Cappi et al. 2016; Feliciano et al. 2019). A rare SERT variant, Ile425Val, has also been identified in an extended family with OCD (Wendland et al. 2008). In cellular models and lymphoblastoid cell lines, the Ala56 variant leads to increased SERT function, as do multiple other identified variants, including Val425 (Prasad et al. 2009; Prasad et al. 2005; Quinlan et al. 2019).

We previously created SERT Ala56 knock-in mice (Veenstra-Vanderweele et al. 2009; Veenstra-VanderWeele et al. 2012) that display enhanced and dysregulated SERT function as well as elevated whole-blood 5-HT levels, mimicking elevated whole-blood levels of 5-HT seen in approximately 30% of ASD patients (Gabriele et al. 2014; Piven et al. 1991). These animals also have altered firing properties of dorsal raphe neurons, including decreased basal firing and enhanced inhibition by 5-HT, as well as enhanced responses to 5-HT_{1A} and 5-HT_2 receptor agonists (Veenstra-VanderWeele et al. 2012). In addition, relative to wild-type littermates, Ala56 knock-in mice exhibit reduced ultrasonic vocalizations, decreased social dominance, and increased repetitive hanging/climbing behaviors. Here we sought to understand the impact of chronic enhanced SERT function on 5-HT_{1B} binding and 5-HT_{1B}-mediated PPI, hyperlocomotion, and immediate early gene activation. We hypothesized that the enhanced SERT function in Ala56 mice would lead to altered 5-HT_{1B} receptor binding and enhanced sensitivity to RU24969, paralleling what we observed at 5-HT_{1A} and 5-HT_2 receptors in these animals.

Methods

Animals

Male homozygous SERT Ala56/Ala56 (hereafter referred to as SERT Ala56 or Ala56) (Veenstra-Vanderweele et al. 2009) and wild-type littermate control mice (Gly56/Gly56) were generated from heterozygous breeding on a pure 129S6/SvEvTac background. Only male mice were used because the SERT Ala56 variant only affects males in the human population (Sutcliffe et al. 2005). Mice were housed with same-sex littermates, two to five per cage with a 12-h light/dark cycle and food and water available ad libitum. Mice were between 8 and 14 weeks of age for experiments. All procedures were approved by the Institutional Animal Care and Use Committee at Vanderbilt University, where the experiments took place, and designed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

5-HT$_{1B}$ binding assay

Adult, naïve wild-type and Ala56 mice were sacrificed via decapitation; the brains were rapidly removed and frozen by immersion in isopentane refrigerated with liquid nitrogen. Eight series of coronal sections (10 μm) were obtained with a cryostat at − 20 °C and thaw-mounted onto poly-L-lysine-coated slides. Right and left hemispheres were tracked. Each slide contained one brain from each genotype.

Half of the brains were processed on 1 day and the other half were processed on a different day, balanced for genotype. The sections were thawed at room temperature for 60 min and pre-incubated for 60 min in binding buffer (65 mM Tris-HCL, 0.93 mM MgCl$_2$ in water) at room temperature. The edges of the slides were then dried and marked with a PAP-pen (Abcam, Cambridge, MA). Total binding (TB) and nonspecific binding (NSB) solutions were prepared in binding buffer. TB: [^{125}I]-iodocyanopindolol (1 μL/mL), pargyline (10^{-2} M), isoproterenol (10^{-2} M), 8-OH-DPAT (10^{-4} M), ascorbic acid (1%), and bovine serum albumen (3%). NSB: [^{125}I]-iodocyanopindolol (1 μL/mL), pargyline (10^{-2} M), isoproterenol (10^{-2} M), 8-OH-DPAT (10^{-4} M), GR127935 (200 μM), ascorbic acid (1%), and bovine serum albumen (3%). Slides were allowed to incubate for 120 min in 1 mL of TB or NSB solution in a covered tray at room temperature, after which point the reactions were terminated by submerging the slides in ice-cold binding buffer followed by two washes (7.5 min each) in ice-cold binding buffer. The slides were then dipped in ice-cold water and allowed to air-dry for 4 h at room temperature. Once dry, the slides were exposed to film in the presence of radioactive standards (0.063–2.15 μCi/g) for 24 h. The films were developed using a Mini-Medical Series developer. Once dry, the films were scanned on an Epson Perfection V550 Photo Scanner at 1200 dpi.

Optical densities were measured from acquired images using ImageJ, as described in O'Reilly et al. (2016), and converted to radioactivity (μCi/g) from the standards. Readings were taken from the OFC (medial, ventral, and lateral orbitofrontal cortices) and the dorsal striatum (dSTR) (Supplemental File 1). Readings were taken from the left and right hemispheres from ~ 5 sections per hemisphere for the OFC and from ~ 3 sections per hemisphere for the dSTR. The location of each section along the anterior-posterior axis was

tracked in order to determine if there was an effect of the anterior-posterior location on the binding levels. The nonspecific binding radioactivity was subtracted from the total binding radioactivity to estimate the specific binding.

Behavior

Wild-type ($n = 12$) and Ala56 ($n = 14$) mice were 8–12 weeks at the time of testing and habituated to the open-field chamber for 90 min 2 days prior to behavioral testing. Mice received an intraperitoneal injection of 0 or 10 mg/kg body weight of the 5-HT$_{1B}$ agonist, RU24969 dissolved in 0.9% saline, and were immediately placed in the open-field chamber for 20 min. A 10-mg/kg dose of RU24969 was chosen because it has been shown to reliably affect PPI (Dulawa et al. 1997). Locomotor activities were assessed using activity monitors measuring 27.9×27.9 cm (MED Associates). Each apparatus contained 16 photocells in each horizontal direction, as well as 16 photocells elevated 4 cm to measure rearing, as described previously (Veenstra-VanderWeele et al. 2012).

Following the open-field test, the mice were immediately tested for prepulse inhibition of acoustic startle using Acoustic Startle Reflex Test Compartments (MED Associates). By placing the animals in the open field immediately following injections, the start of the prepulse inhibition paradigm is similar to the timing used previously, which was 30 min post-injection (Dulawa et al. 1997). Mice were acclimated to background white noise of 65 dB for 5 min before being presented with seven trial types in six discrete, randomized blocks of trials (for a total of 42 trials) with an intertrial interval of 10 to 20 s. One trial measured baseline movement and one trial measured response to the 120 dB, 50-ms startle stimulus alone. The other five trials used an acoustic prepulse of 70, 76, 82, or 88 dB preceding the acoustic startle stimulus by 100 ms. Startle amplitude was measured every millisecond over a 65-ms period beginning at the onset of the startle stimulus. Prepulse inhibition was calculated by dividing the difference between baseline startle and startle following prepulse by baseline startle as in Veenstra-VanderWeele et al. (2012). Two days later, the same mice received the opposite drug treatment and underwent the same behavioral analysis.

cFos immunostaining

Two hours after the second injection (RU24969 or saline), Ala56 mice were anesthetized with sodium pentobarbital and transcardially perfused with 1× phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. The brains were extracted, stored overnight in 4% PFA at 4 °C, and transferred to PBS containing 30% sucrose until they were cut on a microtome (40-μm sections) in the coronal plane and stored in a cryoprotectant (PBS, ethylene glycol, and glycerol) at − 20 °C.

cFos immunostaining was performed on sections containing OFC and striatal regions. The sections were washed (4×5 min) in PBS containing 0.3% Triton X-100 (PBST), followed by incubation in 0.3% H_2O_2 for 30 min at room temperature and were then washed (3×5 min) again with PBST. The sections were blocked in PBST containing 5% Normal Donkey Serum and then incubated with rabbit polyclonal anti-cFos primary antibody (1:1000 dilution in PBST, 5% NDS, Abcam, Cambridge, MA) for 3 days at 4 °C with constant agitation. The sections were washed (PBST, 4×5 min), followed by

incubation in donkey anti-rabbit biotinylated secondary antibody (1:250 dilution in PBST, 5% NDS, Jackson Immuno Secondary, catalog number) for 1.5 h at 4 °C. The sections were then washed (PBST, 4 ×5 min); incubated in PBST containing VECTASTAIN® Elite® avidin/biotin complex (ABC) reagent; washed (PBST, 4 × 5 min); and reacted with 3,3′-diaminobenzidine (DAB) substrate (DAB substrate kit, Vector Labs, Burlingame, CA) according to manufacturer's instructions. The sections were washed three times in PBS and mounted on Superfrost slides.

Sections were visualized under a brightfield light microscope and images were obtained using AxioVision software. Activated cells in the ventral OFC and dSTR were counted using the ImageJ software (US National Institutes of Health, Bethesda, MD). The area of activated cells as a percentage of the total area was used for quantification.

Statistics

5-HT$_{1B}$ binding—Univariate analysis of variance (ANOVA) was used to determine if there was an effect of section location along the anterior-posterior axis on 5-HT$_{1B}$ binding levels within each hemisphere for the wild-type and Ala56 genotypes combined. Once it was determined that there was no effect of section, the activity was averaged for each brain region within each hemisphere and a univariate ANOVA was used to determine if there was an effect of hemisphere. Once it was determined that there was no effect of hemisphere, the radioactivity for each region was averaged over both hemispheres. The primary hypothesis was that 5-HT$_{1B}$ binding would be altered in the OFC, particularly in the ventral OFC (VO), where activation of 5-HT$_{1B}$ receptors was sufficient to induce PPI deficits (Shanahan et al. 2011). We hypothesized that binding could also be altered in the medial and lateral OFC (MO and LO, respectively) where drugs infused into the VO could potentially diffuse to and where a few cannula were placed in the Shanahan et al.'s (2011) study. Our secondary analysis focused on binding in the dSTR where cFos expression has been shown to be increased after RU24696 exposure (Ho et al. 2016). To analyze receptor binding activity in the OFC, a multivariate analysis of variance (MANOVA) was performed with MO, VO, and LO as dependent variables and genotype as the independent variable. A univariate ANOVA was used to determine if there was an effect of genotype in the dSTR. Differences were considered significant at $p < 0.05$.

Behavior—For the open field, the total distance traveled was broken into 5-min time bins over the 20-min trial. A three-way ANOVA was used to examine the effect of genotype, time bin, and RU24969 exposure. For the prepulse inhibition paradigm, a three-way ANOVA was used to examine effects of prepulse intensity, genotype, and RU24969 exposure. Post hoc analysis was performed to examine the effect of drug exposure on startle response by performing a *t* test at each prepulse intensity for the two genotypes combined. Bonferroni's correction was done to account for multiple comparisons. Differences were considered significant at $p < 0.05$.

cFos staining—Two-way ANOVA evaluating genotype (wild-type or Ala56) and drug treatment (Saline or RU24969) was used to establish group differences or significant

interactions. Differences were further investigated using Tukey's HSD post hoc analysis. Differences were considered significant at $p < 0.05$.

Results

5-HT$_{1B}$ receptor binding is increased in the orbitofrontal cortex

There was no effect of section location along the anterior-posterior axis from which 5-HT$_{1B}$ receptor binding levels were measured within either hemisphere for any brain region on specific 5-HT$_{1B}$ binding, nor was there an effect of hemisphere (Supplemental File 2). There was a trend for genotype to affect 5-HT1B binding in the OFC ($F_{3,7} = 4.19$, $p = 0.05$). Compared to wild-type, Ala56 mice had increased specific 5-HT$_{1B}$ binding in the VO and a trend for increased binding in the LO (Fig. 1a; VO—$F_{1,9} = 6.14$, p value: 0.04; LO—$F_{1,9} = 5.03$, $p = 0.052$). Differences in 5-HT$_{1B}$-specific binding were not detected between the wild-type and Ala56 mice in the MO (Fig. 1a; $F_{1,9} = 1.49$, p value: 0.25) or in the dSTR (Fig. 1b; $F_{1,9} = 1.87$, $p = 0.21$).

Ala56 mice show no difference in RU24969-induced hyperlocomotion or PPI deficits

Locomotor activity, measured as distance traveled, was assessed in the SERT Ala56 and wild-type mice in response to saline or RU24969 administration in the open field (Fig. 2a). There was an effect of time ($F_{1.12,28.24} = 19.90$, $p < 0.0001$) and RU24969 administration ($F_{1,24} = 180.70$, $p < 0.0001$) on locomotor activity, and there was an interaction between these two factors (time × drug $F_{2.12,50.79} = 83.50$, $p < 0.0001$). Differences in locomotor activity were not detected due to genotype (genotype $F_{1,24} = 0.01$, $p = 0.93$), nor was there an interaction between time and genotype ($F_{3,72} = 0.70$, $p = 0.56$), drug and genotype ($F_{1,24} = 0.36$, $p = 0.56$) or among the three factors (time × drug × genotype $F_{3,72} = 1.30$, $p = 0.28$). Although there was an effect of prepulse intensity ($F_{3,72} = 80.60$, $p < 0.0001$), RU24969 exposure ($F_{1,24} = 15.48$, $p < 0.001$), and an interaction between intensity and drug exposure ($F_{3,72} = 9.94$, $p < 0.0001$), there was no effect of genotype ($F_{1,24} = 1.31$, $p = 0.26$) on PPI (Fig. 2b), nor were there interaction effects between genotype and prepulse intensity ($F_{3,72} = 1.84$, $p = 0.15$); genotype and RU24969 exposure ($F_{1,24} = 2.72$, $p = 0.11$); or genotype, prepulse intensity, and RU24969 exposure ($F_{3,64} = 1.25$, $p = 0.30$). Because we found no genotype differences in response to RU24969, we combined both genotypes to evaluate the interaction between prepulse intensity and drug exposure. We found that RU24969 enhanced the startle effect at prepulse intensities of 70, 76, and 80 dB (70 dB: $t_{192} = 4.594$, $p < 0.0001$; 76 dB: $t_{192} = 3.846$, $p < 0.001$; PPI 82: $t_{192} = 2.965$, $p < 0.01$) but the effect was no longer present at 88 dB ($t_{192} = 0.4328$; $p > 0.9999$).

RU24969 administration alters immediate early gene expression in the orbitofrontal cortex and striatum

To examine the effect of RU24969 administration on cellular activity, cFos expression was examined in the VO of the OFC and the dSTR (Supplemental File 3) using the region delineations used to assess 5-HT$_{1B}$ binding (Supplemental File 1). A two-way ANOVA revealed there was no effect of drug ($F_{1,19} = 2.58$, $p = 0.12$) or genotype ($F_{1,19} = 0.05$, $p = 83$) on cFos expression in the VO (Fig. 3a), but there was an interaction ($F_{1,19} = 8.27$, $p = 0.01$). Post hoc analysis revealed that exposure to RU24969 significantly increased cFos

expression in the OFC of the wild-type mice (Tukey's HSD $p = 0.03$, Supplemental File 2), with no significant difference in the SERT Ala56 mice ($p = 0.78$).

A two-way ANOVA on cFos expression in the dSTR (Fig. 3b, Supplemental File 3) revealed an effect of drug ($F_{1,21} = 6.75$, $p = 0.02$) on cFos expression, but no effect of genotype ($F_{1,21} = 0.07$, $p = 0.79$), and no interaction effect ($F_{1,21} = 3.59$, $p = 0.22$).

Discussion

Across species, serotonin 5-HT$_{1B}$ receptor agonism has been suggested to mediate prepulse inhibition deficits and repetitive behavior observed in OCD and ASD (Dulawa et al. 1997; Pittenger et al. 2016). Chronic administration of SRIs and genetic ablation of SERT result in decreased levels of 5-HT$_{1B}$ receptors in the OFC of mice, which have been shown to be necessary and sufficient for RU24969-induced hyperlocomotion and PPI deficits (Shanahan et al. 2011). Consistent with decreased SERT function yielding decreased 5-HT$_{1B}$ receptor levels in the OFC, Ala56 mice, whose SERT function is enhanced and dysregulated, have increased baseline levels of 5-HT$_{1B}$ binding in the OFC. However, the relationship between 5-HT$_{1B}$ receptor levels in the OFC and behavioral response to RU24969 of Ala56 mice do not neatly parallel-blunted RU24969 response to decreased 5-HT$_{1B}$ receptor levels. We saw no increase in either baseline or RU24969-induced hyperlocomotion or PPI deficits in Ala56 mice compared to wild-type mice. Our PPI results are, however, consistent with prior work showing that RU24969 no longer has an effect on startle response at the 88-dB prepulse intensity in this background strain (Dulawa and Geyer 2000).

The 10-mg/kg dose of RU24969 is considered a high dose and was used because it reliably alters PPI (Dulawa et al. 1997), but PPI deficits induced by 2.5-, 5-, and 10-mg/kg doses of RU24969 are similarly different from saline administration (Dulawa and Geyer 2000). While administration of 1-, 3-, 5-, or 10-mg/kg RU24969 significantly increased the distance traveled in the open field compared to saline administration in the wild-type mice (Ho et al. 2016), the doses did not linearly increase locomotor activity: locomotor activity induced by 1- and 10-mg/kg doses differed from that induced by 3-mg/kg activity, whereas the 5-mg/kg dose did not elicit a locomotor response that differed from 1, 3, or 10 mg/kg (Ho et al. 2016). These data imply that, although 10 mg/kg is a high dose, lower doses would not necessarily yield different results.

Although we did not measure the path complexity of the observed hyperlocomotion in the open field, previous data suggest that locomotor path complexity is less robustly associated with RU24969 response than is hyperlocomotion itself (Shanahan et al. 2011). Based on these data, it is possible that having fewer 5-HT$_{1B}$ receptors is protective against sensorimotor gating deficits, while having more receptors does not necessarily exacerbate these deficits.

It is also possible that developmental alterations in non-serotonergic circuits within the SERT Ala56 animals could offset changes in 5-HT$_{1B}$ receptor expression. Chronic SERT blockade with fluoxetine has been shown to reduce striatal dopamine and dopamine metabolite levels; this effect was also seen in SERT knockout mice (Morelli et al. 2011).

Natural variations in SERT function have also been suggested to mediate dopamine-related behavioral phenotypes (Carneiro et al. 2009), and Ala56 mice have altered dopamine-related molecular pathways in the medial and lateral amygdala compared to the wild-type mice (O'Reilly et al. 2020). Dopamine receptor agonists, such as apomorphine, induce PPI deficits in mice (Dulawa and Geyer 1996). Thus, the discordant results between 5-HT1B binding and RU24969-induced behavior could be explained by complex effects of the SERT Ala56 variant on multiple neurotransmitter systems.

5-HT$_{1B}$ receptors are expressed on terminals of serotonergic neurons, where they inhibit release of 5-HT (Boschert et al. 1994; Hen 1992). Here, we find that exposure to RU24969 increased cFos activity in the OFC, perhaps due to serotonergic neurons sending an excitatory projection to inhibitory neurons in the OFC, as is seen in the PFC and hippocampus (Sun et al. 2019; Szonyi et al. 2016). RU24969 may therefore reduce the excitatory effect of serotonergic projections on inhibitory neurons, allowing primary neurons to have enhanced firing. However, 5-HT$_{1B}$ receptors are also present on terminals of glutamate, GABA, dopamine, and acetylcholine projecting neurons and also inhibit neurotransmitter release from these neurons (reviewed in (Sari 2004)). Thus, the role of the 5-HT$_{1B}$ receptor in modulating release of these other neurotransmitters may play a part in increased cFos expression in the OFC of the wild-type mice. The maintained cFos activity in Ala56 mice after RU24969 seems likely to be due to a compensatory mechanism, such as a shift in the amount of inhibition or neurotransmission in the OFC. For example, an increase in the number of 5-HT$_{1B}$ receptors in the OFC may lead to a baseline reduction in inhibitory signaling in Ala56 mice, resulting in slightly increased levels of excitatory activity. Further reducing the 5-HT signaling by RU24969 agonism may thus not have much of an impact on excitation in the OFC of Ala56 mice. Alternatively, having an increased number of 5-HT$_{1B}$ receptors in the OFC of Ala56 mice may lead to altered neurotransmission of multiple neurotransmitters, resulting in no net change in cFos activity. Neither of these possibilities was directly assessed.

Prior studies in rodents found no change in cFos mRNA in the OFC following RU24969 exposure (Ho et al. 2016), which contrasts our results showing that cFos protein expression is enhanced in the OFC of the wild-type mice. 5-HT$_{1B}$ binding in the OFC was significantly higher in the VO and tended to be higher in the LO of Ala56 mice compared to wild-type, which may indicate subregional differences in receptor expression. It is therefore possible that cFos mRNA expression was not different in previous studies due to the inclusion of MO (Ho et al. 2016).

In the dSTR, RU24969 exposure reduced cFos expression. This directionality is opposite to what is expected based on previous reports in which cFos mRNA or protein levels are increased by exposure to RU24969 (Ho et al. 2016; Wirtshafter and Cook 1998). Although both male and female mice respond similarly to RU24969 (Thompson and Dulawa 2019), sex differences or strain differences (C57BL/6J vs. 129S6/SvEvTac) could account for the discrepancies between the current cFos results from male mice and those reported previously in female mice (Ho et al. 2016). Additionally, although previous reports indicate that cFos levels normalize by 24 h post-injection with RU24969 (Wirtshafter and Cook 1998), we cannot discount the impact of experience on cellular function: the saline group received an

injection of RU24969 48 h prior to the saline injection given for the cFos expression studies along with exposure to the open field and PPI paradigm. We note that because we did not see an interaction effect of genotype × drug exposure, we did not perform post hoc analyses to determine if the cFos expression was significantly different specifically in the wild-type mice.

The SERT Ala56 variant, as do other identified SERT variants found in the ASD population, leads to increased and dysregulated SERT function (Prasad et al. 2009; Prasad et al. 2005; Quinlan et al. 2019) and, presumably, decreased 5-HT signaling, as supported by increased 5-HT clearance from the extracellular space and increased sensitivity to 5-HT_{1A} and 5-HT_{2A} receptor agonists (Veenstra-VanderWeele et al. 2012). Notably, the SERT Ala56 variant leads to increased repetitive climbing/hanging behavior in mice (Veenstra-VanderWeele et al. 2012). Other gain-of-function SERT variants have been described in human genetic studies but not in mouse models; although a transgenic SERT overexpressing mouse has been created but not evaluated for response to RU24969 or baseline PPI (Dawson et al. 2011; Jennings et al. 2006, 2008). Here, we did not detect genotype differences in PPI or locomotor activity between SERT Ala56 and control mice even with relatively large sample sizes. Similarly, SERT knockout mice do not differ from control mice in PPI, although SERT knockout mice display lower levels of locomotor activity in the open field (Shanahan et al. 2009). Thus, alterations in SERT function minimally alter behavior on their own, but the downstream effects of altering SERT function may be substantial, as is seen in the SERT knockout response to RU24969 (Shanahan et al. 2009).

Although Ala56 mice display increased sensitivity to 5-HT_{1A} and $5\text{-HT}_{2A/2C}$ receptor agonists (Veenstra-VanderWeele et al. 2012), the current results suggest that not all 5-HT receptors are similarly affected. Of note, it is also possible that the increased 5-HT_{1A} sensitivity that we previously observed counteracted any differential effect of RU24969 at 5-HT_{1B} receptors. Because SERT inhibitors attenuate RU24969-induced PPI deficits and hyperactivity, we expected the SERT Ala56 mice to have enhanced PPI deficits and hyperactivity in response to RU24969. While we did not find altered behavioral effects in response to RU24969 administration to SERT Ala56 mice compared to the wild-type mice, our 5-HT_{1B} binding and cFos staining results suggest that there is a difference in neural response to 5-HT_{1B} agonists. The lack of behavioral difference in the context of altered neural response may indicate complex developmental effects of the SERT Ala56 variant or compensatory mechanisms that offset the impact of increased 5-HT uptake on 5-HT_{1B} receptor signaling or on downstream circuit function. Thus, while SRI treatments may be effective at treating OCD, in part because they reduce 5-HT_{1B} levels, increased 5-HT_{1B} receptor levels due to enhanced SERT function do not fully account for the sensorimotor gating deficits seen in ASD and OCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Funding

Conflict of interest

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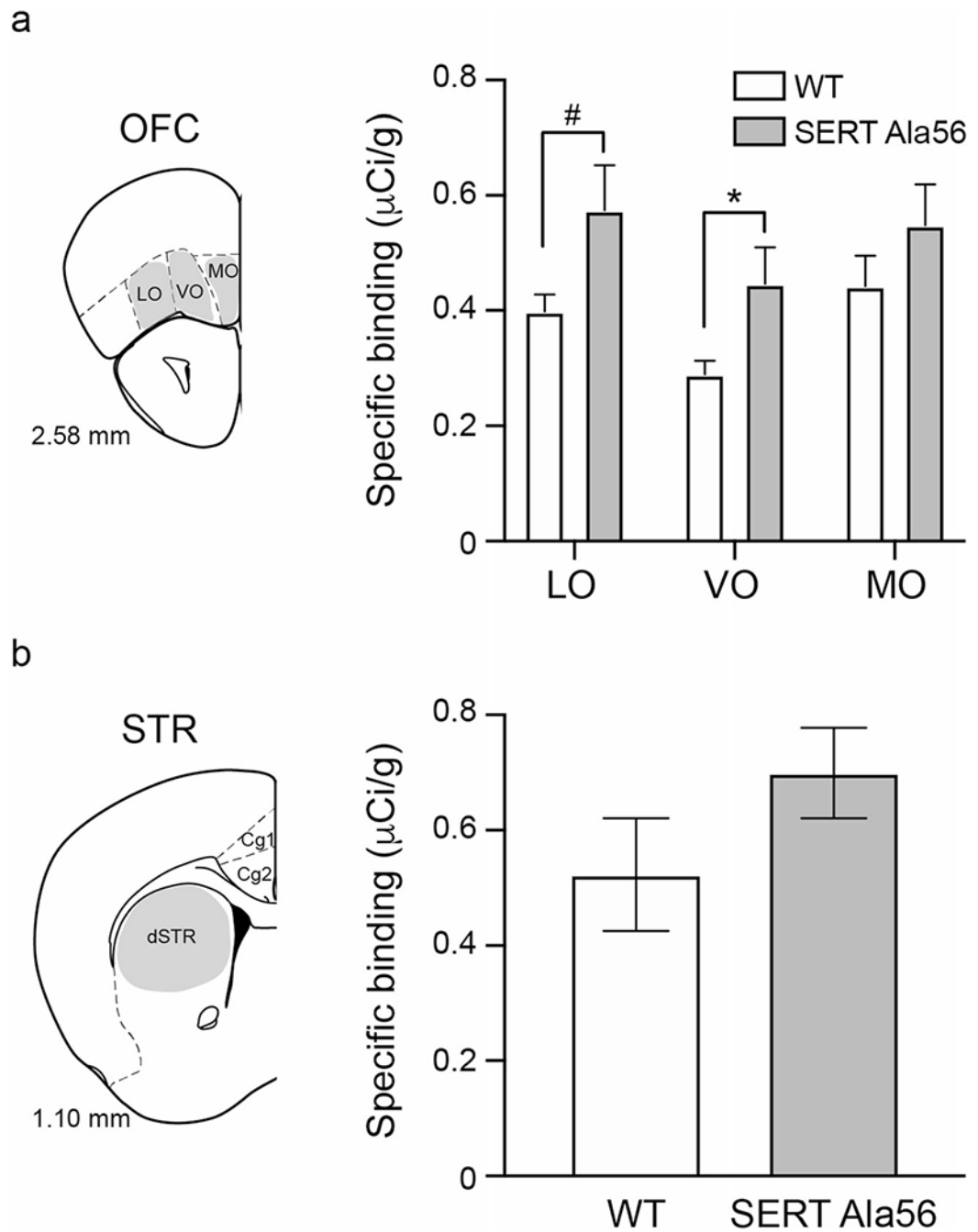
Fig. 1.
5-HT$_{1B}$ receptor binding is increased in the ventral OFC. **a** Specific binding to 5-HT$_{1B}$ receptors was measured in the OFC. There was more receptor binding in the ventral OFC of Ala56 than wild-type mice and a tendency for receptor binding to be increased on the lateral OFC of Ala56 mice. **b** There were no differences in the dorsal striatum in 5-HT$_{1B}$ binding between the two genotypes. LO = lateral OFC; VO = ventral OFC; MO = medial OFC. dSTR = dorsal striatum. Wild type: $n = 6$; Ala56: $n = 5$. Data are mean ± SEM. $*p < 0.05$; $^{\#}p < 0.1$
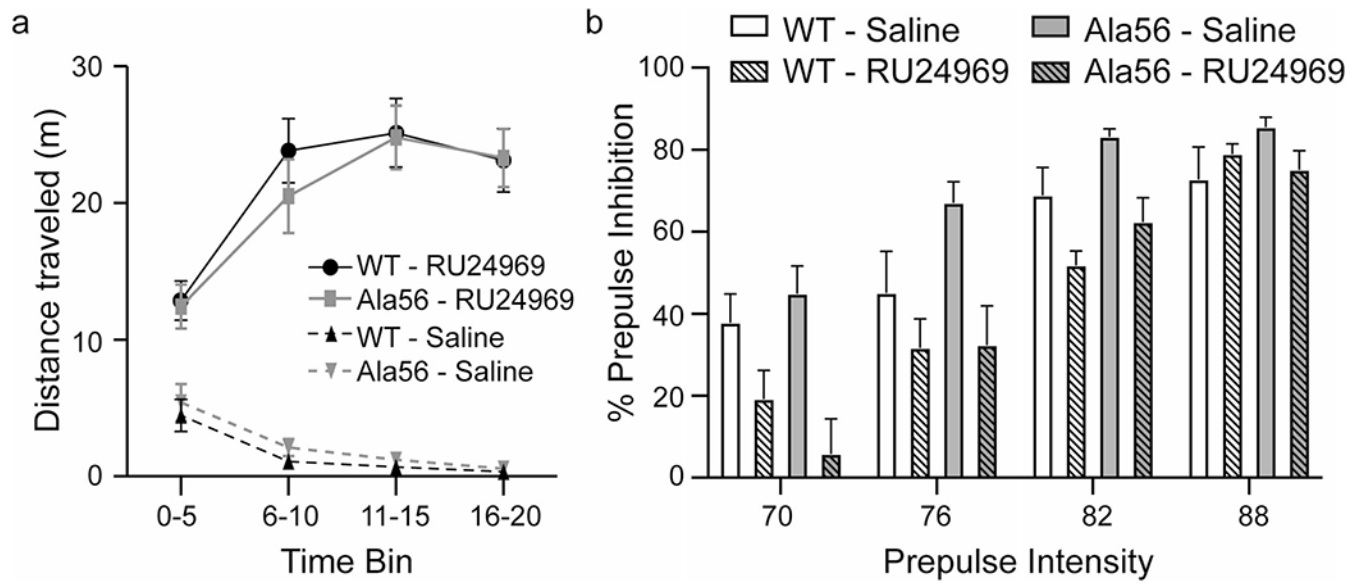
Fig. 2.
RU24969 enhances hyperlocomotive behavior and PPI similarly in Ala56 and wild-type mice. Wild-type ($n = 12$) and Ala56 ($n = 14$) mice were administered 10-mg/kg body weight RU24969 or an equivalent volume of saline and immediately tested in the open field. **a** Under saline conditions, both wild-type and Ala56 mice habituate to the open field, decreasing the distance they walk with time. Administration of RU24696 increases locomotor activity similarly in wild-type and Ala56 mice. **b** Immediately following testing in the open field, mice were tested for PPI. There were no differences in Ala56 and wild-type in percent of prepulse inhibition at any prepulse intensity. RU24969 decreased the percent of prepulse inhibition similar for both genotypes. Data are mean ± SEM
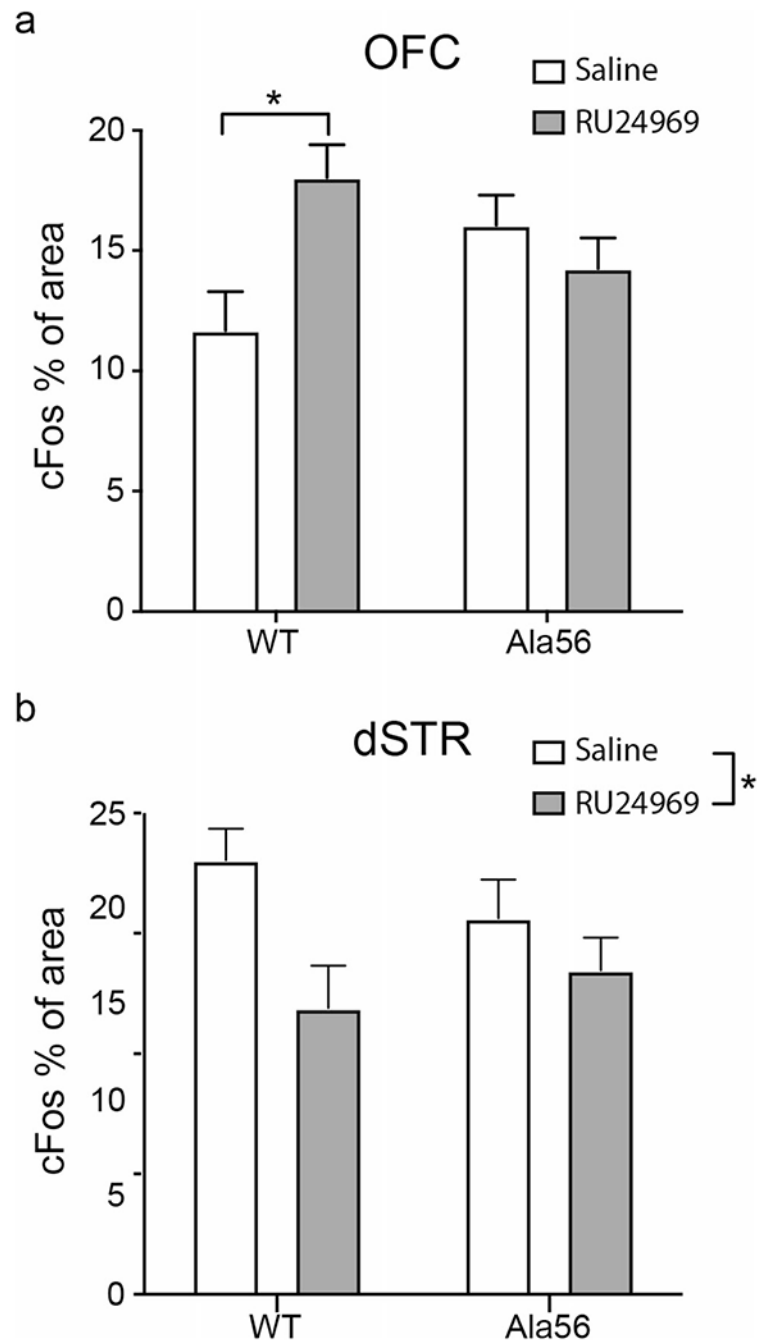
Fig. 3.

RU24969 has no effect on cFos activity in SERT Ala56 mice. Two hours following saline or RU24969 injection, brains were collected for cFos immunostaining. There were no differences between wild-type and Ala56 cFos staining in any brain region after saline injection. **a** cFos staining was increased in the ventral OFC (VO) of wild-type mice after RU24969 administration. **b** RU24969 decreased cFos staining in the dorsal striatum (dSTR). There was no effect of genotype nor was there an interaction. VO: wild-type, saline: $n = 5$; wild-type, RU24969: $n = 5$. Ala56, saline: $n = 5$; Ala56, RU24969: $n = 5$; dS: wild-type,

saline: $n = 5$: wild-type, RU24969: $n = 6$; Ala56, saline: $n = 5$; Ala56, RU24969: $n = 9$; vS: wild-type, saline: $n = 4$; wild-type, RU24969: $n = 6$; Ala56, saline: $n = 5$; Ala56, RU24969: $n = 9$. Data are mean ± SEM. *$p < 0.05$