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Social Behavioral Deficits Coincide with the Onset of Seizure Susceptibility in Mice Lacking Serotonin Receptor 2c

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Abstract

The development of social behavior is strongly influenced by the serotonin system. Serotonin 2c receptor (5-HT₂cR) is particularly interesting in this context considering that pharmacological modulation of 5-HT₂cR activity alters social interaction in adult rodents. However, the role of 5-HT₂cR in the development of social behavior is unexplored. Here we address this using Htr2c knockout mice, which lack 5-HT₂cR. We found that these animals exhibit social behavior deficits as adults but not as juveniles. Moreover, we found that the age of onset of these deficits displays similar timing as the onset of susceptibility to spontaneous death and audiogenic-seizures, consistent with the hypothesis that imbalanced excitation and inhibition (E/I) may contribute to social behavioral deficits. Given that autism spectrum disorder (ASD) features social behavioral deficits and is often co-morbid with epilepsy, and given that 5-HT₂cR physically interacts with Pten, we tested whether a second site mutation in the ASD risk gene Pten can modify these phenotypes. The age of spontaneous death is accelerated in mice double mutant for Pten and Htr2c relative to single mutants. We hypothesized that pharmacological antagonism of 5-HT₂cR activity in adult animals, which does not cause seizures, might modify social behavioral deficits in Pten haploinsufficient mice. SB 242084, a 5-HT₂cR selective antagonist, can reverse the social behavior deficits observed in Pten haploinsufficient mice. Together, these results elucidate a role of 5-HT₂cR in the modulation of social behavior and seizure susceptibility in the context of normal development and Pten haploinsufficiency.

Introduction

Serotonin (5-HT) is a key neurotransmitter that appeared early in evolution [1] and influences a variety of social processes [2] across species, from humans [3] to primates [4], rodents [5] and flies [6]. The serotonin receptor 5-HT₂cR, encoded by the 5Htr2c gene, is a G protein-coupled receptor (GPCR) that is coupled to Gq/G11 and modulates cellular excitability [7].
Pharmacological studies in animal models have demonstrated roles for 5-HT₂cR in communication [8, 9] and social interaction. For example, activation of 5-HT₂cR by mCPP [10, 11], a non-selective agonist, or by SSRI (selective serotonin reuptake inhibitor, indirect agonist via inhibition of the serotonin transporter SLC6A4) [12] reduces social interaction in rodents. Conversely, administration of the selective 5-HT₂cR antagonist SB 242084 in rats increases social interaction [11] and rescues social deficits caused by stress [13] or mCPP [10, 11].

Dysregulated activity of 5-HT₂cR has been implicated in autism spectrum disorder (ASD), which features deficits in social interaction and communication (DSM-V, American Psychiatric Publishing, 2013). For example, hyperactivity of 5-HT₂cR has been reported in two mouse models of ASD risk factors: in mice with chromosome 15q11-13 duplication due to overexpression of the 5-HT₂cR-editing snoRNA MBI52 [14], and in mice with a loss of function mutation for methyl-CpG binding protein 1 [15]. In addition, there is evidence of a physical interaction between 5-HT₂cR and Pten [16, 17], a negative regulator of the PI3-kinase pathway [18] and a risk factor for ASD [19–22]. However, it is not known how chronic alteration in 5-HT₂cR activity during development affects social behavior. Thus, we examined the social behavior of juvenile and adult Htr2c knockout mice, which lack 5-HT₂cR.

Interestingly, null mutant mice lacking 5-HT₂cR are extremely susceptible to audiogenic seizures [23, 24], suggesting an underlying elevation of the ratio of cellular excitation to inhibition (E/I balance) in these mice. It has been speculated that social and cognitive deficits might arise from a modification in this E/I balance, for example, through increased activity in excitatory neurons or reduced inhibitory neuron activity [25–29]. Supporting this idea, elevation of the E/I balance in the prefrontal cortex in mice elicits a profound impairment of social behavior [30]. To examine this relationship, we also investigated the onset of audiogenic seizure susceptibility in Htr2c knockout mice.

**Material and Methods**

**Animals**

Strains used were B6.129-Htr2ctm1Jke [31] (from The Jackson Laboratory) and B6.129-Htr2ctm1Jul [24] (from The Jackson Laboratory). Both lines were crossed to a C57BL/6J background for at least 10 generations to reach congenicity. Mice of the B6.129-Ptenmt1Rps line [32] were obtained from the repository at the National Cancer Institute at Frederick, where they were already backcrossed onto a congenic C57BL/6J background by the Donating Investigator. The line has been maintained by backcrossing to C57BL/6J mice for more than 10 generations. For behavioral experiments female Htr2ctm1Jke/+ mice were crossed with C57BL/6J males, thus producing Htr2ctm1Jke/Y (referred to as Htr2c/Y) and Htr2c+/Y (referred to as wild-type) male offspring. For the analysis of spontaneous death, female Htr2ctm1Jul/+ or Htr2ctm1Jke/+ mice were crossed with Ptenmt1Rps/+ males, resulting in Htr2+/Y; Ptenmt1Rps/+ (referred to as Pten+/+) and Htr2Jke/Y; Ptenmt1Rps/+ (referred to as Htr2c+/Y; Pten+/+) male offspring. No distinction is made between the Htr2ctm1Jul and Htr2ctm1Jke lines in the analysis of spontaneous death as they are phenotypically equivalent. Due to the localization of the Htr2c gene on the X chromosome and to random X chromosome inactivation, no female Htr2c+/+ mice were used in this study to avoid complications arising from mosaicism.

Behavioral testing occurred between postnatal days 21 to 26 (P21–26) for juveniles and P85–90 for adults. All animals were housed in mixed-genotype groups of 2–5 mice per cage, with no differences in housing between genotypes. Food and water were provided ad libitum and animals were kept on a reversed 12-h light/dark cycle. All behavioral testing was performed during the dark (active) phase of the light cycle. Experiments were performed in accordance with National Institute of Health and Association for Assessment and Accreditation of
Laboratory Animal Care guidelines and approved by The Scripps Research Institute’s Institutional Animal Care and Use Committee.

**Three-chamber social approach and social novelty**

Juvenile and adult mice were tested as previously described [33, 34] under white-light conditions. Briefly, test mice were each placed into the center of a black acrylic center arena (60 x 30 x 30 cm) that was divided into three equal compartments (each 20 x 30 x 30 cm). Mice were habituated to the empty arena for 5 min on each of the two days prior to testing. The test day consisted of three phases: 5 min acclimation to the empty arena, 10 min sociability testing [choice between two acrylic tubes (20 cm tall, 10 cm in diameter, with 16 ¾” diameter holes in the bottom half of the cylinder), one containing a novel, same-sex conspecific (location counterbalanced across mice), the other being empty], and 10 min social novelty testing (novel, same-sex conspecific placed in the previously empty tube). Tubes and chambers were cleaned with quatricide and paper towel-dried between mice. Ethovision (Noldus Information Technology, Wageningen, The Netherlands) was used to score the time spent in each chamber, as well as the velocity and distance traveled, for each mouse. Different cohorts of mice were used for juvenile and adult experiments.

**Audiogenic seizure (AGS) testing**

Juvenile (P25) and adult animals (P90) were moved to the testing area and left undisturbed for 1–3h prior to testing. AGS testing was performed using a Phenotyper box (29.2 x 29 x 30.5 cm, Pten-T10/N, Noldus Information Technology, Wageningen, The Netherlands) equipped with a speaker and clear walls, each inside a noise-attenuating box with fans on. Behavior was monitored via a CCD camera mounted on the ceiling of the box and recorded by Ethovision. After a 30 s period of acclimation to the chamber, a 108 dB white noise stimulus was maintained for 60 s or until overt seizure had occurred. Mice that exhibited no sign of seizure during the stimulus were then monitored for 30 min. The motor response to audiogenic stimulus was classified as described previously [35]: no response (NR), wild running (WR), clonic seizure (CS), tonic seizure (TS), respiratory arrest/death (RA).

**Lifespan Study**

A total of 68 mice were used in the lifespan study with 20 wild-type mice, 21 Htr2c+/Y; Pten+/−, 15 Htr2c−/Y; Pten+/− and 12 Htr2c−/Y; Pten−/−. Mice were maintained in standard conditions with 5 mice per cage and were permitted to live out their lives until death due to natural causes. The mice used in the lifespan study were not disturbed except to check on the mice twice each day and were euthanized if any sign of distress was observed following AAALAC recommendations.

**Drug treatment**

20 minutes prior to testing, mice tested for social approach with 5-HT2cR antagonist were given an intraperitoneal injection of SB 242084 (Sigma-Aldrich) diluted to 0.3mg/ml in a 10% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich) in sterile 25mM citric acid vehicle, or equivalent volume vehicle. For all injections, care was taken to handle animals gently to minimize stress.

**Data analysis**

Independent-sample t-tests were used to assess the effects of genotype (wild-type, Htr2c+/Y) on behavior, and paired-sample t-tests were used to analyze chamber preferences for the
three-chamber social approach (% time in mouse chamber vs. % time in empty tube chamber) and novelty test (% time in novel mouse chamber vs. % time in familiar mouse chamber) for each genotype. Additionally, approach-avoidance scores [time in chamber with a social stimulus minus time in chamber with the empty tube; [34, 36]] were calculated, and genotypes were compared using independent-sample t-tests (wild-type, Htr2c<sup>+/Y</sup>) or two-way analyses of variance (drug: vehicle, 0.3mg/kg SB 242084; genotype: wild-type, Htr2c<sup>+/Y</sup>). Kaplan-Meier survival analysis was used with Log-Rank test followed by pairwise comparison (Holm-Sidak) to analyze the data. For the pharmacogenetic study, planned comparisons between genotypes for each drug (vehicle: wild-type vs. Htr2c<sup>+/Y</sup>, SB 242084: wild-type vs. Htr2c<sup>+/Y</sup>) and between drug treatment for each genotype (wild-type: vehicle vs. SB 242084, Htr2c<sup>+/Y</sup>; vehicle vs. SB 242084), were performed using independent-sample t-tests. In all cases, normality was assessed using Levene’s test. All statistics were performed using PASW 18 (IBM Corporation, Armonk, NY, USA), with significance set at \( p < 0.05 \). All graphs represent mean +/- SEM.

Results

We assessed the development of social behavior in Htr2c knockout mice using the three-chamber social approach and social novelty assay [33, 34] as juveniles (P21–26) or adults (P85–90). Both wild-type and Htr2c<sup>+/Y</sup> juvenile males spent significantly more time in the chamber with the novel social stimulus versus the chamber with the object control during the social approach assay (\( t(8) = 2.39, p < 0.05 \) and \( t(8) = 2.35, p < 0.05 \) respectively, Fig 1A left) and in the chamber with the novel social stimulus versus the chamber with the familiar social stimulus during the social novelty assay (\( t(8) = 2.54, p < 0.05 \) and \( t(8) = 2.64, p < 0.05 \) respectively, Fig 1A right). Correspondingly, we did not find a significant difference between wild-type and Htr2c<sup>+/Y</sup> juvenile mice for the approach-avoidance score (\( t(16) = 0.30, p = 0.77 \), Fig 1B). In order to determine the proportion of mice that show a strong preference for the mouse chamber, we designed a preference index, calculated as: (number of mice where the time in chamber 1 (containing stimulus mouse in cage) was ≥ 10% of the time spent in chamber 3 (containing empty cage)) – (number of mice where the time in chamber 1 was < 10% of the time spent in chamber 3) / (total number of mice). Both wild-type mice (55.5%) and Htr2c<sup>+/Y</sup> mice (33.3%) presented a high positive preference index, showing that more than half of the mice had a strong preference for the mouse chamber (Fig 1C). Additionally, there was no significant difference in velocity or distance traveled in the social approach phase (\( t(16) = 0.73, p = 0.47 \) and \( t(16) = 0.62, p = 0.55 \) respectively, Fig 1D). These results suggest that Htr2c deletion does not impact social behavior of juvenile mice. Next, we wanted to test if constitutive deletion of Htr2c affects social behavior in adult mice.

Wild-type adult males spent significantly more time with the social stimulus during social approach (\( t(15) = 7.90, p < 0.001 \), Fig 2A left), and with the novel social stimulus during social novelty (\( t(15) = 3.28, p < 0.01 \), Fig 2A right), while Htr2c<sup>+/Y</sup> adult males showed no significant differences in chamber time during these assays (\( t(16) = 1.19, p = 0.25 \) and \( t(16) = 1.31, p = 0.21 \) respectively, Fig 2A). Analyzing these data using a social approach-avoidance score, we found that the time spent interacting with a stimulus mouse was significantly less in Htr2c<sup>+/Y</sup> than in wild-type mice (\( t(22.3) = 2.47, p < 0.05 \), Fig 2B). The individual approach-avoidance score of Htr2c<sup>+/Y</sup> mice showed a bimodal distribution that was reflected by a higher variation in this score than in wild-type mice (Levene’s test for equality of variance: \( F = 14.91, p < 0.01 \), Fig 2C). Using the preference index, we found a high preference index for wild-type mice (87.5%) while Htr2c<sup>+/Y</sup> mice showed a negative score (-12.5%; Fig 2D) confirming that less than half of these mice showed a preference for the mouse chamber. Moreover, we found that velocity and distance traveled in the social approach assay were significantly increased in Htr2c<sup>+/Y</sup> compared to wild-type mice.
Fig 1. Three-chamber social approach and social novelty test in Htr2c−/Y juvenile male mice. (A) Time spent in each chamber. (B) Approach-avoidance scores. (C) Preference index: [(number of mice where the time in the mouse chamber was ≥ 10% than the time spent in the tube chamber)–(number of mice where the time in the mouse chamber was < 10% than the time spent in the tube chamber)]/total number of mice. (D) Velocity and distance traveled. n = 9 per genotype. *, p<0.05, NS: non-significant difference with paired samples t-test (A) and independent-samples t-test (B and D).

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mice (t(30) = 2.13, p<0.05 and t(30) = 2.46, p<0.05 respectively, Fig 2E) confirming previous studies [31, 37]. Thus, constitutive deletion of Htr2c decreased social interaction and increased locomotor activity in adult mice.

Adult Htr2c knockout mice feature spontaneous death and audiogenic seizures (AGS), which has been interpreted as reflecting a role for 5-HT2cR in tonic inhibition of excitability in neuronal networks [24, 38]. E/I imbalance within neural networks has been hypothesized as underlying social behavioral deficits in ASD and a variety of other neuropsychiatric disorders [25–28]. In light of this hypothesis, we find it interesting that adult, but not juvenile, Htr2c knockout mice display deficits in sociability. To further explore the relationship between social behavioral deficits and seizure susceptibility, we tested for AGS at P25 and P90, after social behavioral testing was complete in juvenile and adult cohorts of mice, respectively. No Htr2c−/Y mice displayed seizures at P25, confirming previous findings that juvenile Htr2c−/Y mice are not susceptible to AGSs [23]. At P90, we found that all Htr2c−/Y mice, but no wild-type mice, exhibited tonic-clonic seizures manifested by a brief forelimb and hindlimb flexion followed by a rigid and prolonged hindlimb extension (Fig 3). The latency to the tonic-clonic phase was extremely short, ranging from 2 to 6 seconds after the start of the white noise (average: 4.12 s). This confirms that the timing of social behavior deficits coincides with the onset of seizure susceptibility in Htr2c mice. It remains to be determined whether a causal relationship exists between these two phenotypes.

Pten is a significant risk factor for ASD in humans [19–22] and various mouse models of Pten deletion show a deficit in social behavior [33, 34, 39, 40]. It has been shown previously that haploinsufficiency for the Slc6a4 gene, encoding the serotonin transporter, can exacerbate the social behavior deficits observed in Pten+/− mice [34]. Moreover, Pten and 5-HT2cR receptor interact biochemically in dopaminergic neurons of the ventral tegmental area [17], making mutations in Pten a strong candidate for modifying the phenotypes observed in Htr2c−/Y mice. As Htr2c deletion did not affect the preference of the juvenile mice in the 3-chamber assay (Fig 1), we aimed to investigate the social behavior of Htr2c−/Y; Pten+/− adults. However, we found that only 20% of Htr2c−/Y; Pten+/− mice survived to P90 (Fig 4). This made adult social behavioral testing impractical in these animals. Wild-type (n = 20) and Pten+/− mice (n = 21) did not exhibit any spontaneous death within the timeframe examined (up to P180). An autopsy of mice that died spontaneously did not reveal any noticeable health problems such as hemorrhage, infarction, ischemia, hamartomas or tumors. Thus, as previously observed for Htr2c−/Y mice [24], we conjecture that the early lethality of Htr2c−/Y; Pten+/− may be attributed to an increase in spontaneous epileptic seizures. Using a Kaplan-Meier analysis, we found that there was a significant effect of genotype on survival (Log-Rank test: F(2) = 36, p<0.001). Pairwise comparisons revealed a significant difference on the survival of Pten+/− mice vs. Htr2c−/Y (p<0.001) and Htr2c−/Y; Pten+/− mice (p<0.001) and also between Htr2c−/Y and Htr2c−/Y; Pten+/− mice (p<0.05). We interpret these results as consistent with mutations in Pten and Htr2c interacting to influence spontaneous death, possibly via an exacerbation of the epileptic mechanism present in Htr2c knockout mice. However, because of the accelerated age of death, we were prevented from testing social behavior in these compound mutant animals.

As an alternative approach to explore whether Pten and 5-HT2cR interact to influence social behavior, we used SB 242084, a selective antagonist of 5-HT2cR that has been shown to increase social interaction behavior [11] and rescue social investigation deficits associated with stress.
[13] without increasing seizure susceptibility [11] in rodents. We used adult female Pten haploinsufficient and wild-type female mice as social approach deficits in female Pten+/− mice are well described [33, 34]. We administered 5-HT2cR antagonist SB 242084 systemically at 0.3 mg/kg, a dose that has been shown to significantly increase social interactions in rats [11, 13], and tested social approach behavior using a three-chamber social approach apparatus. Vehicle-treated wild-type animals spent significantly more time in the chamber containing the social stimulus mouse than the chamber containing an empty tube, while vehicle-treated Pten+/- mice did not display this preference (t(15) = 5.23, p<0.001 and t(16) = 0.40, p = 0.70, Fig 5A), indicating a deficit in sociability consistent with previous findings in untreated Pten+/- animals [33, 34]. Wild-type and Pten+/- mice treated with SB 242084 both displayed a significant preference for the chamber containing the social stimulus mouse (t(14) = 4.32, p<0.001 and t(15) = 4.38, p<0.001, Fig 5A). Analyzing these data using a social approach-avoidance score, we found a significant effect of genotype or SB 242084 treatment on the time spent to interacting with the stimulus mouse (Two-way ANOVA: Effect of genotype: F (1, 63) = 5.32, p<0.05. Effect of SB 242084 treatment: F (1, 63) = 6.32, p<0.05. Interaction between genotype and SB 242084 treatment: F (1, 63) = 1.97, p = 0.17 (n.s.), Fig 5B). Planned comparisons revealed a significant difference between Pten+/- mice treated with vehicle only and Pten+/- mice treated with SB 242084 (t(31) = 2.73, p<0.01) and between wild-type mice treated with vehicle and Pten+/- mice treated with vehicle (t(31) = 2.82, p<0.01), but no significant difference between wild-type treated with vehicle and wild-type treated with SB 242084 (t(29) = 0.76, p = 0.45) and between wild-type mice treated with SB 242084 and Pten+/- mice treated with SB 242084 (t(29) = 0.56, p = 0.58). Additionally, we found that, while Pten+/- mice treated with vehicle presented a very low preference index (5%), wild-type mice treated with vehicle or SB 242084 and Pten+/- mice treated with SB 242084 all presented a high positive preference index (50, 60 and 37.5% respectively). There was no spontaneous death or other evidence of seizures in Pten+/- mice treated with SB 242084. These results show that SB 242084 treatment suppresses the social behavior deficits exhibited by Pten+/- mice.

Discussion

We report here that adult Htr2c null mutant mice present social behavior deficits and that these deficits are restricted to adult mice and are not present in juvenile mice. It has been hypothesized that dysregulated E/I balance may contribute to social behavioral deficits in neuropsychiatric disorders such as ASD [25–29] and consistent with this idea, it has been shown that elevation of cellular E/I balance within the mouse medial prefrontal cortex elicits an impairment of social behavior [30]. Evidence supports that E/I imbalance is also responsible for seizure susceptibility and spontaneous death in Htr2c mutant mice [24, 41, 42]. Interestingly, similar to social behavior deficits, Htr2c null mutant mice present a susceptibility to AGS only in adulthood [23, 24, 31] (and Fig 3). Given this, together with the observation that pharmacological antagonism of 5-HT2cR does not increase susceptibility to seizures and can increase social investigation in rodents [10, 11, 13], it is reasonable to speculate that E/I imbalance in adult Htr2c knockout mice is responsible for both social behavior deficits and susceptibility to spontaneous and audiogenic seizures. Similarly, E/I imbalance might be responsible for both social behavioral deficits and epilepsy in some ASD patients, as it has been shown that
30% of ASD patients can present a co-morbidity for epilepsy [43]. Alternatively, it is possible that mutations in Htr2c lead to an early dysregulation of growth and connectivity that results in abnormal neural circuitry going into the critical period for social learning, with social deficits
not manifesting until after the critical period closes. It has been shown, for example, that social isolation that occurs in a critical period of 2 weeks after weaning alters prefrontal cortex function and myelination and these phenotypes are not reversible after reintroduction into a social environment [44]. For this hypothesis, dopaminergic neurons are a strong candidate cell type since they are important for social behavior [33, 45–49] and express 5-Ht2cR [50]. Future experiments using timed conditional deletions of Htr2c will help answer this question.

We also find that a second site mutation in Pten accelerates the spontaneous death rate observed in Htr2c/-Y mice [23, 24]. Consistent with previous reports in Htr2c/-Y mice [24], autopsy of these mice did not reveal any noticeable health problems suggesting that the early lethality of Htr2c/-Y; Pten+/- is caused by an increase in spontaneous epileptic seizures. An increased susceptibility to seizures has been reported in different models of Pten conditional deletion [39, 40, 51, 52], and ASD patients with a PTEN mutation can also present with epilepsy [53]. Although we did not observe any spontaneous seizures in germline Pten+/- mice, it is possible that Htr2c mutation uncovers a susceptibility masked by the C57BL/6 background, which is normally seizure resistant [54]. This enhancement of a seizure-prone phenotype in Htr2c/-Y; Pten+/- mice might seem surprising given that Pten physically interacts with 5-HT2cR to repress its activity [17], thus one might predict that Pten and Htr2c mutations would have opposing phenotypic effects. Indeed, we have demonstrated that pharmacological antagonism of 5-HT2cR with SB 242084 in Pten haploinsufficient mice is capable of restoring a preference for social investigation in a three-chamber social approach assay. In interpreting these results, it is worth considering that chronic treatment using a 5-HT2cR antagonist does not increase

Fig 4. Spontaneous death in Htr2c/-Y mice is accelerated by a second-site mutation in Pten. Time course of spontaneous death in Htr2c/-Y (n = 15), Pten+/- (n = 21) and Htr2c/-Y; Pten+/- (n = 12) mice.

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seizure susceptibility [11] and we did not observe any spontaneous seizures in Pten haploinsufficient mice treated with SB 242084, indicating that gross E/I balance is normal. Thus, it is possible that mutations in Htr2c and Pten may act in different cell types to influence E/I balance in a manner that is not recapitulated by pharmacological antagonism of 5-HT2cR. For example, mutations in Htr2c might result in decreased activity of GABAergic neurons [55–58] while Pten mutations result in increased activity of excitatory neurons [59], resulting in a synergistic elevation of E/I balance. Another possibility is that Pten and 5-HT2cR might have different periods and mechanisms of interaction: one complementary interaction in early development that sets up a later vulnerability to seizures, and another antagonistic interaction in mature circuitry that can reverse social behavioral deficits. Additionally, SB 242084 might rescue the social behavior deficits observed in Pten haploinsufficient mice through an anxiolytic effect, for example by modulating release of dopamine in the limbic system [60]. While the precise mechanism remains to be discovered, the above data are consistent with the hypothesis that 5-HT2cR interacts with Pten to modulate both E/I balance and the circuitry influencing social behavior. Important issues to address in future work will be to determine the degree to which modulating 5-HT2cR is significant for the ASD-therapeutic effects of drugs such as risperidone, fluoxetine and olanzapine, which target a broad range of molecules, including 5-HT2cR.

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Author Contributions

Conceived and designed the experiments: JS DL OK DP. Performed the experiments: JS DL OK DP. Analyzed the data: JS DL OK DP. Contributed reagents/materials/analysis tools: DP. Wrote the paper: JS DL OK DP.

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