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Altered 5-HT$_{2C}$ receptor agonist-induced responses and 5-HT$_{2C}$ receptor RNA editing in the amygdala of serotonin transporter knockout mice

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Abstract

Background: The serotonin 5-HT$_{2C}$ receptor (5-HT$_{2C}$R) is expressed in amygdala, a region involved in anxiety and fear responses and implicated in the pathogenesis of several psychiatric disorders such as acute anxiety and post traumatic stress disorder. In humans and in rodent models, there is evidence of both anxiogenic and anxiolytic actions of 5-HT$_{2C}$ ligands. In this study, we determined the responsiveness of 5-HT$_{2C}$R in serotonin transporter (SERT) knockout (-/-) mice, a model characterized by increased anxiety-like and stress-responsive behaviors.

Results: In the three-chamber social interaction test, the 5-HT$_{2B/2C}$ agonist mCPP decreased sociability and sniffing in SERT wildtype (+/+) mice, both indicative of the well-documented anxiogenic effect of mCPP. This 5-HT$_{2C}$-mediated response was absent in SERT -/- mice. Likewise, in the open field test, the selective 5-HT$_{2C}$ agonist RO 60-0175 induced an anxiogenic response in SERT +/+ mice, but not in SERT -/- mice. Since 5-HT$_{2C}$R pre-mRNA is adenosine-to-inosine (A-to-I) edited, we also evaluated the 5-HT$_{2C}$R RNA editing profiles of SERT +/+ and SERT -/- mice in amygdala. Compared to SERT +/+ mice, SERT-/- mice showed a decrease in less edited, highly functional 5-HT$_{2C}$ isoforms, and an increase in more edited isoforms with reduced signaling efficiency.

Conclusions: These results indicate that the 5-HT$_{2C}$R in the amygdala of SERT -/- mice has increased RNA editing, which could explain, at least in part, the decreased behavioral responses to 5-HT$_{2C}$ agonists in SERT -/- mice. These alterations in 5-HT$_{2C}$R in amygdala may be relevant to humans with SERT polymorphisms that alter SERT expression, function, and emotional behaviors.

Keywords: 5-HT$_{2C}$ RNA editing, SERT, anxiety, amygdala

Background

The serotonergic system has been implicated in the pathophysiology and treatment of mood and anxiety disorders, as well as schizophrenia [1,2]. The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) influences neuronal activity via 14 5-HT receptors termed 5-HT$_1$ through 5-HT$_7$ (for a review, see [3]). The 5-HT$_{2C}$ receptor (5-HT$_{2C}$R) has been implicated in normal and altered function of neural circuitries involved in these neuropsychiatric disorders via genetic, immunohistochemical and pharmacological approaches [2,4,5]. The 5-HT$_{2C}$R is a G-protein coupled receptor (GPCR) coupled to PLC and PLA$_2$, although additional signaling cascades have also been described [6-8].

Our previous work indicates that 5-HT transporter (SERT) knockout (-/-) mice are a valid model to study anxiety-related behaviors. These mice exhibit a complex phenotype dominated by anxiety, exaggerated stress responsiveness, and other physiological effects such as obesity and type 2 diabetes-like symptoms, all of which have been previously associated with 5-HT$_{2C}$R genetic deficiencies ([9-12]; for a full review of SERT -/- mice, see [1]). Qu and colleagues [13] found a reduction in 5-HT$_2$R-induced arachidonic acid release in multiple brain regions including the basolateral amygdaloid complex of SERT -/- mice [13,14]. Further, we previously showed increased 5-HT$_{2C}$R binding sites with no mRNA

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changes in the amygdala of SERT -/- mice compared SERT +/+ mice [15]. The exact mechanisms responsible for the anxiety-like phenotype of SERT-/- mice are, however, not completely understood.

In humans and rodents, $5\text{-HT}_{2C}R$ pre-mRNA is subject to adenosine-to-inosine (A-to-I) RNA editing [16,17]. These base changes may result in an amino acid/protein different from those encoded by genomic DNA. It has been shown that RNA editing alters the G-protein efficiency of the $5\text{-HT}_{2C}R$ and its intracellular downstream effects and interactions with both endogenous and exogenous receptor agonists, as well as desensitization mechanisms and constitutive activity [17-20]. It is noteworthy that the $5\text{-HT}_{2C}R$ is the only example among the hundreds of GPCRs which exhibits this post-transcriptional processing [5].

A body of evidence suggests there are alterations in the $5\text{-HT}_{2C}R$ editing pattern in patients with certain neuropsychiatric diseases, and it has been suggested that $5\text{-HT}_{2C}R$ RNA editing may play a role in anxiety and depression [21-25]. The aim of the current study was to evaluate the status of $5\text{-HT}_{2C}R$-mediated anxiety-like behaviors in SERT -/- mice. The finding that SERT -/- mice were unresponsive to systemic administration of $5\text{-HT}_{2C}R$ agonists at doses that elicited anxiogenic responses in SERT +/+ mice prompted us to further investigate the RNA editing profile of the $5\text{-HT}_{2C}R$ in SERT -/- mice. We chose the amygdala as our primary target, since this region is critical in anxiety-related behaviors in rodents, non-human primates and humans [2,26-28].

Results
Behavioral analysis
Social interaction test
In the social interaction test we first assessed "sociability" (the preference for spending time in the stranger side vs. the empty side) [main effects of side ($F_{1,22} = 61.53$, $p < 0.0001$); genotype ($F_{1,22} = 0.92$, N.S.) and drug ($F_{1,22} = 1.72$, N.S.); side × genotype interaction ($F_{1,22} = 1.10$, N.S.), side × drug interaction ($F_{1,22} = 1.18$, N.S.), genotype × drug interaction ($F_{1,22} = 0.56$, N.S.) and side × drug × genotype interaction ($F_{1,22} = 6.87$, $p = 0.016$)]. In vehicle-treated mice, there were no significant differences in sociability between SERT +/+ and -/- mice (Figure 1A). In SERT +/+ mice, administration of mCPP reduced sociability, indicative of its anxiogenic effect, whereas mCPP had no effect in SERT -/- mice, reflecting a diminished responsiveness of the $5\text{-HT}_{2C}R$ (Figure 1A).

We next assessed "sniffing" (time spent sniffing the stranger cage vs. the empty cage) [main effects of side ($F_{1,22} = 183.26$, $p < 0.0001$), genotype ($F_{1,22} = 5.59$, $p = 0.027$) and drug ($F_{1,22} = 8.97$, $p = 0.007$); side × genotype interaction ($F_{1,22} = 0.002$, N.S.), side × drug interaction

($F_{1,22} = 6.98$, $p = 0.015$), genotype × drug interaction ($F_{1,22} = 10.13$, $p = 0.004$), and side × drug × genotype interaction ($F_{1,22} = 11.61$, $p = 0.003$)]. Vehicle-treated SERT +/+ and -/- mice both spent more time sniffing the stranger vs. the empty cage (Figure 1B). However, mCPP-treated SERT +/+ mice spent significantly less time sniffing the stranger cage compared to vehicle-treated SERT +/+ mice, whereas mCPP was without such an effect in SERT -/- mice (Figure 1B).

To rule out a possible role for changes in locomotor activity, we also assessed the number of entries to each side chamber [main effects of side ($F_{1,22} = 28.23$, $p < 0.0001$), genotype ($F_{1,22} = 5.69$, $p = 0.026$) and drug ($F_{1,22} = 0.56$, N.S.); side × genotype interaction ($F_{1,22} = 0.20$, N.S.), side × drug interaction ($F_{1,22} = 0.03$, N.S.), genotype × drug interaction ($F_{1,22} = 0.56$, N.S.), and side × drug × genotype interaction ($F_{1,22} = 0.33$, N.S.)]. There were no significant differences in the number of entries to the side chambers based on genotype or drug administration, suggesting that differences in locomotor activity did not play a role in the differences in anxiogenic responses elicited by mCPP in SERT +/+ mice (Figure 1C).

Administration of the selective $5\text{-HT}_{2C}R$ antagonist RS 102221 15 min prior to mCPP blocked the anxiogenic effect of mCPP on sociability in wildtype C57BL/6J mice (Figure 2), confirming that the mCPP-induced anxiogenic response in the social interaction test was mediated by $5\text{-HT}_{2C}R$ [main effects of side ($F_{1,36} = 64.29$, $p < 0.0001$) and drug ($F_{3,36} = 0.64$, N.S.); side × drug interaction ($F_{3,36} = 6.43$, $p = 0.001$). Neither RS 102221 nor mCPP, administered alone or in combination, affected locomotor activity (data not shown).

Open field test
To further explore this apparent reduction in responsiveness of $5\text{-HT}_{2C}R$ observed in the social interaction test, we tested the effects of the 5-HT_{2C} agonist RO 60-0175 in the open field test. For the frequency in the center of the open field, there was a significant main effect of genotype ($F_{1,34} = 20.70$, $p < 0.0001$), a significant main effect of drug condition ($F_{1,34} = 9.75$, $p = 0.004$) and a significant genotype × drug condition interaction ($F_{1,34} = 4.78$, $p = 0.036$). Following vehicle, SERT -/- mice made fewer visits to the center of the open field compared to SERT +/+ mice (Figure 3A). In SERT +/+ mice, treatment with RO 60-0175 decreased the frequency of visits to the center of the open field to levels observed in SERT -/- mice, suggestive of an anxiogenic effect, whereas RO 60-0175 had no effect in SERT -/- mice (Figure 3A). Regarding the total distance traveled, there was a significant main effect of genotype ($F_{1,34} = 8.23$, $p = 0.007$), a significant main effect of drug condition ($F_{1,34} = 8.05$, $p = 0.008$) and a significant genotype × drug condition interaction ($F_{1,34} = 5.50$, $p = 0.025$). At baseline, SERT -/- mice displayed less locomotor activity than SERT +/+ mice
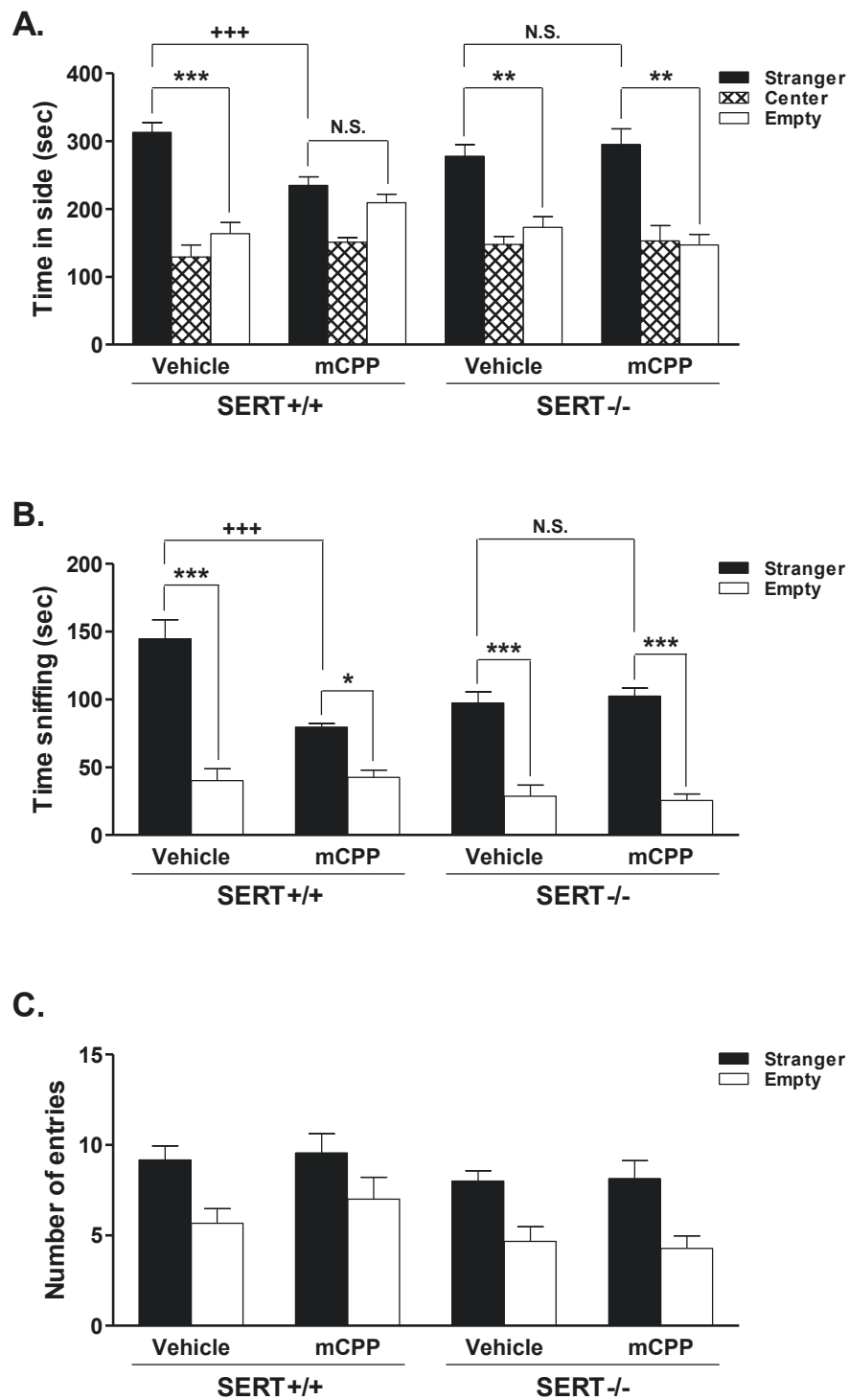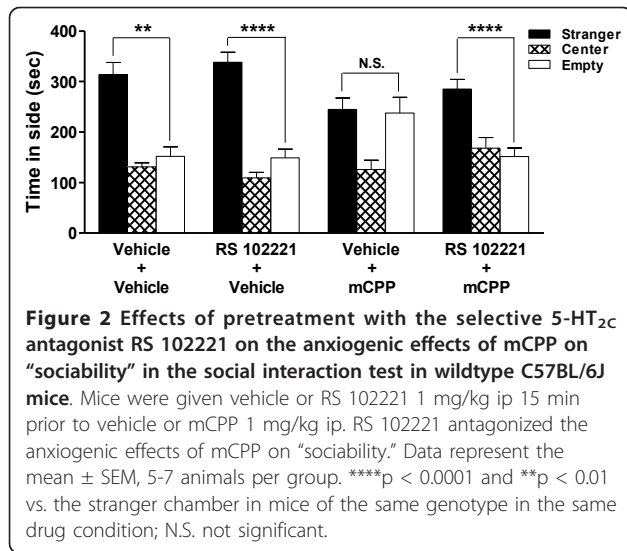
Figure 1 Effects of mCPP in the social interaction test in SERT +/+ and -/- mice. 1A. Effects of mCPP on "sociability". SERT +/+ and -/- mice were given vehicle or mCPP 1 mg/kg ip 30 min prior to testing. mCPP decreased sociability (indicating increased anxiety) in SERT +/+ mice, with no effects in SERT -/- mice. **1B.** Effects of mCPP on "sniffing." SERT +/+ and SERT -/- mice were given vehicle or mCPP 1 mg/kg ip 30 min prior to testing. mCPP decreased sniffing in SERT +/+ mice (indicating increased anxiety), with no effects in SERT -/- mice. **1C.** Effects of mCPP on locomotor activity. There was no effect of mCPP 1 mg/kg ip administered 30 min prior to testing on the number of entries into the different chambers. Data represent the mean ± SEM, 7 animals per group. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ vs. the stranger chamber in mice of the same genotype in the same drug condition; +++ $p < 0.01$ vs. mice of the same genotype given vehicle; N.S. not significant.

Figure 2 Effects of pretreatment with the selective 5-HT$_{2C}$ antagonist RS 102221 on the anxiogenic effects of mCPP on "sociability" in the social interaction test in wildtype C57BL/6J mice. Mice were given vehicle or RS 102221 1 mg/kg ip 15 min prior to vehicle or mCPP 1 mg/kg ip. RS 102221 antagonized the anxiogenic effects of mCPP on "sociability." Data represent the mean ± SEM, 5-7 animals per group. ****$p < 0.0001$ and **$p < 0.01$ vs. the stranger chamber in mice of the same genotype in the same drug condition; N.S. not significant.
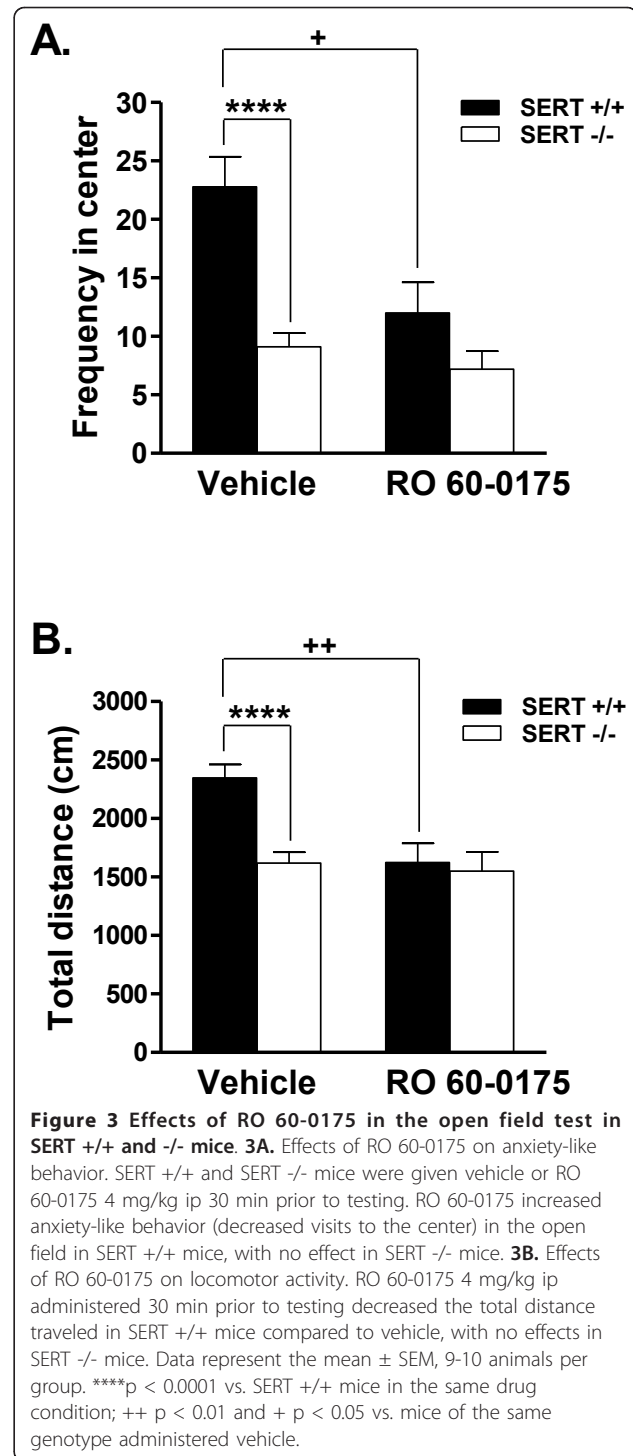
(Figure 3B). There was also a reduction in locomotion in SERT +/+ mice after RO 60-0175 administration, to levels comparable to SERT -/- mice; RO 60-0175 did not alter locomotion in SERT -/- mice. Activity in periphery, however, remained unchanged (data not shown).
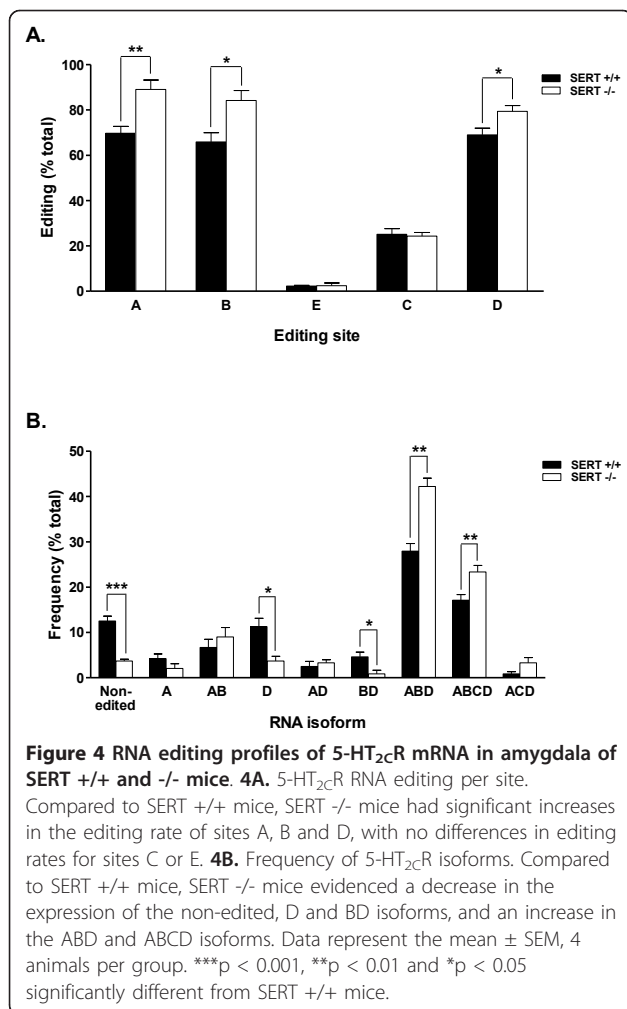
RNA editing

Positively sequenced clones were used to compare the 5-HT$_{2C}$R RNA editing profiles of SERT +/+ and -/- mice. The change in the editing rate at each specific editing site is shown in Figure 4A. Compared to SERT +/+ mice, SERT -/- mice had significant increases in the editing rate of site A (89.1% vs. 69.7%, $p = 0.009$), site B (84.2% vs. 65.9%, $p = 0.0227$) and site D (79.3% vs. 68.9%, $p = 0.04$). No differences in editing rates between the two SERT genotypes were found for sites C or E. The frequency of the RNA isoforms expressed at least 3% in one of the genotypes is shown in Figure 4B. Compared to SERT +/+ mice, SERT -/- mice evidenced a significant decrease in the expression of the non-edited (3.7% vs. 12.5%, $p = 0.0003$), D (3.7% vs.11.3%, $p = 0.0117$) and BD (4.6% vs.1.1%, $p = 0.0356$) isoforms. Further, two of the 5-HT$_{2C}$R RNA isoforms were significantly increased in SERT -/- mice compared to SERT +/+ mice; ABD (42.2% vs.27.9%, $p = 0.0012$) and ABCD (23.4% vs. 17.1%, $p = 0.016$). Overall, this comparison of 5-HT$_{2C}$R RNA editing profiles shows an increase in editing in SERT -/- mice vs. SERT +/- mice which results in a shift from non/low editing isoforms toward highly/full edited isoforms.

Discussion

To our knowledge, the present data document the first assessments of anxiety-related behavioral alterations elicited by 5-HT$_{2C}$R agonists in SERT -/- mice. Specifically, in the social interaction test, there were no significant

Figure 3 Effects of RO 60-0175 in the open field test in SERT +/+ and -/- mice. **3A.** Effects of RO 60-0175 on anxiety-like behavior. SERT +/+ and SERT -/- mice were given vehicle or RO 60-0175 4 mg/kg ip 30 min prior to testing. RO 60-0175 increased anxiety-like behavior (decreased visits to the center) in the open field in SERT +/+ mice, with no effect in SERT -/- mice. **3B.** Effects of RO 60-0175 on locomotor activity. RO 60-0175 4 mg/kg ip administered 30 min prior to testing decreased the total distance traveled in SERT +/+ mice compared to vehicle, with no effects in SERT -/- mice. Data represent the mean ± SEM, 9-10 animals per group. ****$p < 0.0001$ vs. SERT +/+ mice in the same drug condition; ++ $p < 0.01$ and + $p < 0.05$ vs. mice of the same genotype administered vehicle.

differences in baseline assessments (vehicle administration) between SERT +/+ and SERT -/- mice. However, the anxiogenic response induced by the 5-HT$_2$R agonist mCPP in SERT +/+ mice was absent in SERT -/- mice. The role of 5-HT$_{2C}$R in this anxiogenic response was confirmed by pretreatment with the selective 5-HT$_{2C}$R

Figure 4 RNA editing profiles of 5-HT$_{2C}$R mRNA in amygdala of SERT +/+ and -/- mice. **4A.** 5-HT$_{2C}$R RNA editing per site. Compared to SERT +/+ mice, SERT -/- mice had significant increases in the editing rate of sites A, B and D, with no differences in editing rates for sites C or E. **4B.** Frequency of 5-HT$_{2C}$R isoforms. Compared to SERT +/+ mice, SERT -/- mice evidenced a decrease in the expression of the non-edited, D and BD isoforms, and an increase in the ABD and ABCD isoforms. Data represent the mean ± SEM, 4 animals per group. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ significantly different from SERT +/+ mice.

antagonist RS 102221, which blocked the anxiogenic effect of mCPP in wildtype C57BL/6J mice. Others have previously shown that both systemic and local (intra-amygdala) administration of mCPP increases anxiety levels in rodents, indicating that these receptors - possibly in the basolateral amygdala - are responsible for the anxiogenic effect of mCPP [29,30].We also replicated previous findings from our lab showing that SERT -/- mice exhibit increased baseline anxiety-like behaviors in the open field test (for a review, see [1]). In addition, we showed that the anxiogenic response induced by the selective 5-HT$_{2C}$ agonist RO 60-0175 in the open field test in SERT +/+ mice is abolished in SERT -/- mice.

Using autoradiography to determine binding sites and *in situ* hybridization for mRNA content, previous reports from our lab indicate that 5-HT$_{2C}$R mRNA levels are unaltered in amygdala of SERT -/- mice, whereas 5-HT$_{2C}$R binding sites are significantly increased in this region [15]. We therefore hypothesized that differences in RNA editing levels might account for this apparent discrepancy between levels of binding sites

and the 5-HT$_{2C}$R responsiveness to agonist stimulation. Given previous reports indicating that intra-amygdala injections of mCPP were able to elicit anxiogenic responses in rodents [29,30], and the known role of this brain region in rodent and human anxiety, we focused our efforts on characterizing the RNA editing profile of 5-HT$_{2C}$R in amygdala of SERT -/- mice compared to that of their SERT +/+ littermates.

SERT -/- mice had significantly decreased frequencies of non-edited, D and BD isoforms, as well as a significantly increased frequencies of the ABCD and ABD isoforms, the latter being the major isoform present in both SERT +/+ and -/- mice. ABCD codes for the VSV variant of the 5-HT$_{2C}$R, and has been shown to exhibit reductions in receptor signaling both in agonist-elicited and intrinsic activity [16,31]. The major isoform ABD codes for the variant VNV together with the AD isoform, which was slightly increased in SERT -/- mice compared to SERT +/+ mice. Previous studies also show that the VNV is the major 5-HT$_{2C}$R isoform present in C57BL/6J mice [32,33]. The VNV variant has reduced basal activity with no alteration in the potency of 5-HT stimulation [16,31]. Thus, the present results suggest that SERT gene deletion shifts the RNA editing profile of the 5-HT$_{2C}$R pre-mRNA population toward more edited, less active isoforms. This might explain the lack of effect of 5-HT$_{2C}$ agonists in both the social interaction and open field tests in SERT -/- mice at doses which were anxiogenic in SERT +/+ mice. It is important, however, to emphasize that the aforementioned reports indicating pharmacological differences among 5-HT$_{2C}$R RNA editing isoforms were conducted with human and rat clones of each RNA isoform heterologously expressed, therefore caution is required when comparing the values from the present *in vivo* study conducted in mice [16,31].

Previous reports have shown that pharmacological manipulations of serotonergic tone have an impact on 5-HT$_{2C}$R RNA editing, either by direct 5-HT$_{2C}$R activation by the non-selective 5-HT$_2$ agonist DOI or by chronic fluoxetine (a selective serotonin reuptake inhibitor (SSRI)) treatment. Gurevich and colleagues [21] found that 129Sv mice treated with chronic fluoxetine exhibit significantly increased editing in site D and significantly decreased editing in site E. Chronic fluoxetine treatment in C57BL/6J mice, however, led to modest, non-significant changes in 5-HT$_{2C}$R RNA editing, whereas the same treatment in BALB/c mice led to significant increases in editing of sites A, B, C and D [22]. In SERT -/- mice, extracellular 5-HT levels are increased 3-6 fold in brain [1,34]. Our current results suggest that, as a result of a targeted gene deletion of SERT, the increased extracellular levels of 5-HT alters 5-HT$_{2C}$R RNA editing. In addition, these results suggest

that targeted SERT gene deletion has a more profound impact than 28 days of treatment with fluoxetine in C57BL/6J wildtype mice [21]. However, it is important to note that the prior studies analyzed 5-HT$_{2C}$R RNA editing in forebrain neocortex [21,22,35], whereas the current study analyzed 5-HT$_{2C}$R RNA editing in amygdala; thus, a direct comparison of these studies with the current studies is limited by the anatomical differences.

The observed increase in the frequency of 5-HT$_{2C}$R RNA editing in SERT -/- mice might also explain the apparent paradoxical upregulation of the number of 5-HT$_{2C}$R binding sites previously observed in SERT -/- mice [15]. It has been shown that RNA editing also alters the ratio of alternative splicing variants, promoting the generation of the full mRNA variant coding for the functional protein *in vitro* [36], and recently *in vivo* [23]. Thus, the increased RNA editing observed here in SERT -/- mice might be related to the previously observed increase in surface expression of 5-HT$_{2C}$R [15]. Another plausible link between RNA editing and 5-HT$_{2C}$R upregulation is receptor desensitization [5]. It is known that the non-edited 5-HT$_{2C}$R isoform exhibits the highest constitutive activity and is present mainly intracellularly, whereas more edited isoforms are present largely as membrane-bound receptors and are more resistant to desensitization, at least *in vitro* [37,38]. The observed increase in 5-HT$_{2C}$R RNA editing in SERT -/- mice, which generates receptor isoforms with less efficacious signaling and reduced basal activity, is in line with previous findings of a reduction in DOI-induced arachidonic acid release in several brain regions, including the basolateral amygdaloid complex, in SERT -/- mice [13,14]. However, the concomitant activation of 5-HT$_{2A}$ receptors does not allow a claim to be made for reduced activity of 5-HT$_{2C}$R in those studies, especially as other signaling pathways for 5HT$_{2C}$R receptors exist (e.g., PLC/IP$_3$).

There is considerable evidence suggesting the involvement of 5-HT$_{2C}$R in anxiety-related behaviors, although there is still debate about the precise role of this receptor in anxiety (for a review, see [2]). For example, it has been shown that activation of 5-HT$_{2C}$R mediates the anxiogenic-like effects elicited by the non-selective 5-HT$_{2C}$ agonist mCPP in rodents, replicated in the current studies, and in humans [2,39,40]. Similarly, selective 5-HT$_{2C}$R antagonists have been shown to exert anxiolytic effects in several animal models of anxiety in some reports [41,42], but not in others [43,44]. The current results are in line with reports of initial anxiogenic-like effects of SSRIs treatment in both humans and in several animal models of anxiety [45]. The current data also show that, as indicated above, varying SERT expression can have profound consequences on the functional status of postsynaptic 5-HT$_{2C}$R receptors, as expected

from the marked increases in extracellular levels of 5-HT found in SERT -/- mice [34]. These results also suggest that polymorphisms affecting SERT expression might exert a modulatory effect on the functional status of 5-HT$_{2C}$R in humans. This might have implications for personalized medicine, as several selective 5-HT$_{2C}$R agonists are being proposed as anti-obesity agents that have now advanced to clinical trials [46], in addition to the reported potential use of 5-HT$_{2C}$R antagonists as anxiolytics [41].

The current studies focused on the analysis of RNA editing in amygdala, a key region involved in fear and anxiety. However, the circuit controlling anxiety-related traits and responses spans multiple regions. It will be of interest for future research to examine different brain areas to evaluate potential region-specific alterations in 5-HT$_{2C}$R RNA editing frequencies, based on previous studies showing brain region-specific alterations in tissue 5-HT content, and in 5-HT synthesis and turnover rates [9,47,48]. A detailed characterization of the role of 5-HT$_{2C}$R in amygdala control and in alterations in its RNA editing profile might also require a finer dissection (such as laser-caption microdissection) of the different subregions of the heterogeneous amygdala structure.

Conclusions

In summary, for the first time, we report functional alterations of 5-HT$_{2C}$R-mediated responses to agonist stimulation in SERT -/- mice, as observed in the social interaction and open field paradigms. Further, we suggest that this alteration could be, at least in part, be explained by the significant increases in RNA editing of 5-HT$_{2C}$R in the amygdala of SERT -/- mice that generates less active receptor isoforms. These findings will help to unravel the role of 5-HT neurotransmission in amygdala activity, especially in terms of alterations in SERT expression reported in humans with different alleles for the SERT promoter (5-HTTLPR s and l alleles) and other polymorphisms affecting SERT expression that have been found to be relevant in neuropsychiatric disorders [49-51]. Additional efforts are needed to further dissect the role of the 5-HT$_{2C}$R among different amygdala subnuclei and in different neuronal types, to further understand the physiological relevance of 5-HT$_{2C}$R editing in this and other brain regions, in addition to the role of 5-HT$_{2C}$R in neuropsychiatric disorders.

Methods
Animals
Male SERT +/+ and -/- mice were originally produced by homologous recombination in ES cells as previously described [52], and are currently the product of ~20-24 heterozygous backcrosses with wildtype mice on a

C57BL/6J genetic background. Commercial wildtype C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used for the antagonism experiment. The animals weighed ~20-35 g at the time of the experiments, and were housed in groups of 3-5 per cage with food and water available *ad libitum.* The animals were maintained on a 12-h light/dark cycle (lights on 0600 hours) in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All experiments adhered to the guidelines of the National Institutes of Health, and were approved by the National Institute of Mental Health Animal Care and Use Committee.

Drugs and drug administration

The following compounds were used: (i) the 5-HT$_{2B/2C}$ agonist 1-(3-chlorophenly)piperazine (mCPP) (Tocris Bioscience, Ellisville, MO), (ii) the selective 5-HT$_{2C}$ agonist (αS)-6-Chloro-5-fluoro-α-methyl-1H-indole-1-ethanamine fumarate (RO 60-0175), and (iii) the selective 5-HT$_{2C}$ antagonist 8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride (RS 102221) (Tocris Bioscience, Ellisville, MO). mCPP was administered at a dose of 1 mg/kg and RO 60-0175 was administered at a dose of 4 mg/kg, based on previous investigations that showed behavioral effects at these doses [29,43] and preliminary pilot studies performed in our lab. RS 102221 was administered at 1 mg/kg based on previous investigations [53]. mCPP and RO 60-0175 were dissolved in saline (sterile 0.9% NaCl solution), and RS 10221 was dissolved in 1% DMSO and saline. Drugs were injected via intraperitoneal (ip) injection (injection volume 10 ml/kg) 30 min prior to behavioral testing. In the antagonism study, RS 102221 was injected 15 min prior to mCPP.

Behavioral paradigms

A separate cohort of animals was used for each behavioral study. On test days, animals were moved in their home cage to a dimly lit testing room 1 h prior to experiments. All behavioral experiments were carried out between 1000 and 1400 hours.

Social interaction test

The social interaction test was used because it can detect the anxiolytic and anxiogenic effects of serotonergic agents [54,55]. SERT +/+ and -/- mice were injected with either vehicle (saline) or mCPP. Thirty min later, mice were tested in an automated three-chamber box as described previously [56]. Dividing walls had retractable doorways allowing access into each chamber. The automated box had photocells embedded in each doorway to allow quantification of the number of entries and the duration in each chamber of the social test box. The chambers of the apparatus were cleaned with water and dried with paper towels between each trial. At the end of each test day, the apparatus was sprayed with 70% ethanol and wiped clean with paper towels. The test has three 10-min phases: (1) Center habituation - the test mouse was first placed in the middle chamber and allowed to explore, with the doorways into the two side chambers closed; (2) Side chamber habituation - the mouse was allowed to explore the entire social test box, with the doorways into the two side chambers open, and (3) Sociability - after the second habituation period, the test mouse was enclosed in the center compartment of the social test box, and an unfamiliar mouse ("stranger," an adult C57BL/6J male) was enclosed in a wire cage (11 cm height, 10.5 bottom diameter, bars spaced 1 cm apart; Galaxy Cup; Spectrum Diversified Designs, Inc., Streetsboro, OH) and placed in a side chamber, and a similar empty wire cage was placed in the other side chamber. The location of the stranger alternated between the left and the right sides of the social test box between subjects. Following placement of the stranger mouse, the doors were reopened, and the subject was allowed to explore the entire social test box. The automated testing system recorded the amount of time spent and the number of entries in each chamber. In addition, the time spent sniffing each wire cage was recorded by an experimenter blind to the administered drug.

Open field test

As pilot studies indicated that a range of doses of mCPP (0 - 5 mg/kg) did not elicit anxiogenic effects in the open field test, we evaluated the effects of RO 60-0175, a selective 5-HT$_{2C}$R agonist. SERT +/+ and -/- mice were injected with either vehicle or RO 60-0175. Thirty min later, mice were placed in the corner of a novel open field arena (40 × 40 × 35) and were allowed to explore for 5 min. Behaviors, including distance traveled (cm) and frequency of visits to center (20 × 20 cm), were recorded using the Noldus Ethovision Video Tracking system (Noldus Information Technology, Leesburg, VA).

RNA editing

Determinations of RNA editing profiles were performed in a separate cohort of SERT +/+ and -/- mice. Amygdala samples were obtained as previously described [32]. Mice were sacrificed and brains were rapidly removed and placed in a brain block matrix. 1 mm coronal sections encompassing the amygdala region were dissected (posterior to the optic chiasm and anterior to the pons as ventral surface landmarks). From coronal sections, tissue containing visible amygdala nuclei was dissected using the *rhinal sulcus* as a guide. The tissues from both hemispheres were collected together.

Total RNA was extracted using miRvana PARIS Kit (Ambion, Austin, TX). 480 ng were used in first-strand cDNA synthesis using SuperScript III First-Strand SuperMix (Invitrogen, Carlsbad, CA) using the gene-specific primer CGGCGTAGGACGTAGATCGTTAAG [33]. Amplification of the edited region was performed using primers sense (5'-TGTGCTATTTTCAACTGC GTCCATCATG), antisense (5'-CGGCGTAGGACGTA-GATCGTTAAG) and Master Mix (Promega, Madison, WI). PCR products were cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and used for transformation in *E. coli*. From each animal, isolated colonies were randomly chosen for plasmid DNA isolation (Qiagen, Valencia, CA) and bidirectionally sequenced with M13 primers at the National Institute of Neurological Disorders and Stroke (NINDS) intramural DNA sequencing core facility. Raw chromatograms from 60 positively sequenced colonies per animal (240 per genotype) were analyzed for changes in the editing region previously described.

Statistical analysis

For each experiment, data were analyzed using two-way (genotype × drug condition) or three-way (genotype × drug condition × side) analyses of variance (ANOVAs), or by t-tests when only two groups were compared. Post-hoc comparisons between genotypes or between drug conditions were conducted using t-tests. Significance was based on $p < 0.05$.

Authors' contributions

PRM conceived, designed and supervised the experiments, analyzed and interpreted the data, and wrote the manuscript. MAF participated in the design of studies, performed the pilot studies, performed the statistical analysis for the behavioral section, and contributed significantly to the writing of the manuscript. CLJ carried out the cloning experiments. JLL carried out the social interaction test experiments. HTF carried out the open field test experiments. JRW participated in the design and execution of the molecular experiments and helped with the RNA editing sequence analysis. DLM supervised PRM's work, participated in the design and coordination of the experiments, and helped to write the manuscript. All authors read the manuscript, provided critical input, and approved the final manuscript.

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