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# Host Microbiota Regulates Central Nervous System Serotonin Receptor 2C Editing in Rodents

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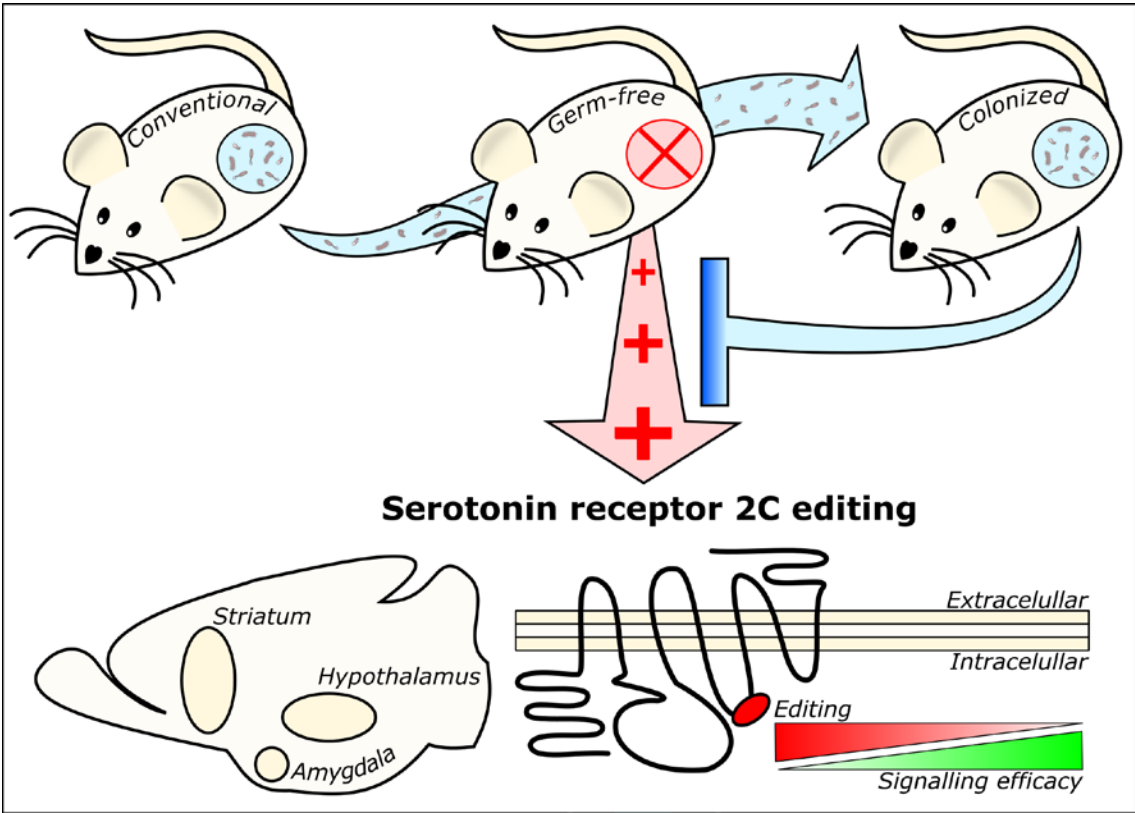
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## **Abstract**

Microbial colonization of the gastrointestinal tract plays a crucial role in the development of enteric and central nervous system functionality. The serotonergic system has been heavily implicated in microbiota-gut-brain axis signaling, particularly in proof-of-principle studies in germ-free (GF) animals. One aspect of the serotonergic system that has been left unexplored in relation to the microbiota is the unique ability of the serotonin receptor 2c (5-HT<sub>2C</sub>) to undergo post-transcriptional editing, resulting in decreased receptor functionality. We investigated whether GF mice, with absent microbiota from birth, have altered 5-HT<sub>2C</sub> receptor expression and editing in the brain and if colonization of the microbiota is able to restore editing patterns. Next, we investigated whether microbiota depletion later in life using a chronic antibiotic treatment in rats could affect 5-HT<sub>2C</sub> receptor editing patterns. We found that GF mice have an increased prevalence of the edited 5-HT<sub>2C</sub> receptor isoforms in the amygdala, hypothalamus, prefrontal cortex and striatum, which was partially normalized upon colonization post-weaning. However, no alterations were observed in the hypothalamus after microbiota depletion using an antibiotic treatment in adult rats. This suggests that alterations in the microbiome during development, but not later life, could influence 5-HT<sub>2C</sub> receptor editing patterns. Overall, these results demonstrate that the microbiota affects 5-HT<sub>2C</sub> receptor editing in the brain and may inform novel therapeutic strategies in conditions in which 5-HT<sub>2C</sub> receptor editing is altered, such as depression.

**Keywords (6):** Germ-free, Microbiota, Serotonin, Serotonin 2C receptor, Editing, Brain

Graphical abstract



## 1. Introduction

There is an increasing understanding of the gut microbiota's ability to interact with neurophysiology and behavior, in what is currently recognized as the microbiota-gut-brain axis <sup>1-3</sup>. A well-established model for proof-of-principal studies to investigate the involvement of the microbiota in various physiological and behavioral parameters are animals devoid of any microbiota (germ-free, GF) <sup>4</sup>. Using this model, previous studies have shown that the serotonergic system is particularly affected by the gut microbiota <sup>5</sup>. For instance, GF mice have decreased levels of colonic and serum serotonin (5-HT), which is normalized upon colonization of the microbiota (colonized germ-free; CGF) <sup>6, 7</sup>. In the brain, male GF mice have increased hippocampal 5-HT levels, whereas no differences were seen in the expression of the 5-HT receptor 1a, 2c and 6 <sup>8</sup>.

Interestingly, the 5-HT receptor 2c (5-HT<sub>2C</sub>) is unique, compared to the other 5-HT receptors, in the way that it can undergo post-transcriptional adenosine-to-inosine RNA editing <sup>9</sup>. 5-HT<sub>2C</sub> receptor editing occurs in a site-specific manner on five specific nucleotide positions (A-E), resulting in 32 possible RNA isoforms, which can translate into 24 differential protein sequences <sup>10</sup>. Editing of the 5-HT<sub>2C</sub> receptor results in a decreased agonist-induced signaling, constitutive activity, desensitization and heterodimerization-signaling interactions with other receptors like the ghrelin receptor - GHS-R1a <sup>11-15</sup>. Initial studies investigating the phenotype of mice solely expressing the fully edited VGV isoform, compared to the non-edited INI isoform, revealed that these mice had a reduction in body weight despite compensatory hyperphagia, a constitutively active sympathetic nervous system, increased energy expenditure and decreased grip strength as weanling, similar to what has been found in individuals suffering from Prader-Willi syndrome <sup>16, 17</sup>. Alternatively, mice expressing the unedited INI isoform show increased plasma corticosterone levels and decreased fear-dependent memory and depressive-like behavior <sup>18</sup>. Particularly the latter is of interest, as 5-HT<sub>2C</sub> receptor editing in the cortex has been associated with suicide in major depression <sup>19-22</sup>.

Various environmental influences have already been shown to affect 5-HT<sub>2C</sub> receptor editing, including aging, spinal cord injury-induced inflammation, chronic stress and fluoxetine treatment

<sup>23-26</sup>. Interestingly, many of these conditions, such as obesity, stress and aging, have been associated with changes in the composition of the gut microbiota <sup>27-30</sup>. This may indicate a role for the gastrointestinal microbiota in modulating central 5-HT<sub>2C</sub> receptor editing. Understanding which factors influence 5-HT<sub>2C</sub> editing allows for a more in-depth comprehension of these conditions and may result in improved therapeutic targets. Considering recent work from our lab has shown that the microbiota is able to influence RNA splicing and editing in GF mice <sup>31, 32</sup>, we hypothesized that the host microbiota could regulate 5-HT<sub>2C</sub> receptor editing in the central nervous system using the GF mouse model. Here we investigated HT<sub>2C</sub> receptor and isoform gene expression levels in the amygdala, prefrontal cortex, hypothalamus and striatum. In tandem, we investigated whether depletion of the gastrointestinal microbiota using an antibiotic cocktail (ABX) could affect 5-HT<sub>2C</sub> receptor editing in the hypothalamus of rats. Finally, we sought to further understand the underlying mechanisms by measuring the gene expression of the enzymes involved in 5-HT<sub>2C</sub> receptor editing; adenosine deaminase acting on RNA 1 and 2 (ADAR1 and ADAR2 respectively) <sup>10</sup>.

## 2. Results

### Frequency of 5-HT<sub>2C</sub> receptor isoforms in the amygdala and prefrontal cortex

We re-analyzed RNA sequencing data from a previous study<sup>31</sup>, and found that the amygdala of GF mice had a decreased prevalence of the unedited INI isoform of the 5-HT<sub>2C</sub> receptor ( $p = 0.008$ ), which was not found in CGF mice (**Fig 1**). In line with this, we found a trend towards an increased prevalence of the VNV isoform (transcript variant ABD) ( $p = 0.061$ ), as well as an increased prevalence of the VNI isoform (transcript variant AB) ( $p = 0.005$ ). These differences were also absent in CGF mice. Similar effects were seen in the prefrontal cortex, even though no significant differences were observed (s**Fig 1**).

### Expression of 5-HT<sub>2C</sub> receptor isoforms in the hypothalamus and striatum

We further aimed to explore whether the microbiome affects 5-HT<sub>2C</sub> receptor editing in other brain regions using qRT-PCR. Analysis of the hypothalamus revealed that full-length 5-HT<sub>2C</sub> expression remained unaffected by microbiota status (**Fig 2A**). A two-way ANOVA comparing microbiome status and the different 5-HT<sub>2C</sub> receptor isoforms revealed a significant microbiome effect only ( $F(2, 149) = 19.237, p < 0.001$ ), indicating that microbiome status had a similar effect on all different 5-HT<sub>2C</sub> receptor isoforms. Subsequent analysis of the 5-HT<sub>2C</sub> INI unedited isoform showed an increased expression in GF mice (**Fig 2B**), which wasn't present after colonization ( $F(2, 29) = 4.926, p = 0.015$ ). Surprisingly, the same was seen in the analysis of the edited VNV isoform (**Fig 2C**) ( $F(2, 29) = 9.538, p = 0.001$ ). No significant differences were found in the VNI, VSV and VGV isoforms in GF mice, even though subsequent colonization induced a trend towards decreased expression of these isoforms (**Fig 2D-F**) ( $F(2, 29) = 3.136, p = 0.060$ ;  $F(2, 28) = 2.717, p = 0.085$ ;  $F(2, 29) = 3.244, p = 0.054$ , respectively).

We subsequently sought out to investigate striatal 5-HT<sub>2C</sub> receptor expression and the prevalence of its isoforms. Here we found that both GF and CGF mice had an increased full-length 5-HT<sub>2C</sub> expression (**Fig 3A**) ( $F(2, 22) = 4.214, p = 0.030$ ). A two-way ANOVA comparing microbiome status and the different 5-HT<sub>2C</sub> receptor isoforms revealed a significant microbiome effect and interaction effect ( $F(2, 66) = 4.564, p = 0.042$ ;  $F(4, 66) = 4.224, p = 0.015$ ), indicating that microbiome status had a different impact on the various 5-HT<sub>2C</sub> receptor isoforms. Similar to the hypothalamus, GF mice had an increased expression of the VNV isoform (**Fig 3B**), which was



downregulated after colonization ( $F(2, 23) = 6.498, p = 0.006$ ). GF mice also had an increased expression of the VGV isoform (**Fig 3C**), even though this remained unaffected by colonization ( $F(2, 21) = 5.485, p = 0.013$ ). Finally, CGF mice showed a trend towards increased expression of the VNI isoform compared to GF mice (**Fig 3D**) ( $F(2, 20) = 3.152, p = 0.103$ ). The INI and VSV isoforms were not reliably detected in the striatum.

### **Expression of editing enzymes ADAR1 and ADAR2 in the hypothalamus and striatum**

To gain a better understanding of what may be driving changes in 5-HT<sub>2C</sub> receptor isoform expression, we investigated the expression of the enzymes responsible for 5-HT<sub>2C</sub> receptor editing; ADAR1 and ADAR2<sup>10</sup>. Interestingly, hypothalamic ADAR1 and ADAR2 expression remained unaffected in GF mice (**Fig 4A-B**), even though colonization induced an increase compared to CON mice ( $F(2, 27) = 3.448, p = 0.048$ ;  $F(2, 28) = 3.047, p = 0.065$ ). We observed a similar absence of difference in the striatum where ADAR1 and ADAR2 expression remained unaltered in GF mice (**Fig 4C-D**), whereas colonization induced a trend towards increased ADAR1 expression only ( $F(2, 21) = 3.201, p = 0.063$ ).

### **Expression of 5-HT<sub>2C</sub> receptor isoforms in the hypothalamus of antibiotic-treated rats**

Considering that we observed the most substantial effect of the microbiome on 5-HT<sub>2C</sub> receptor editing patterns in the hypothalamus, we wanted to investigate whether depletion the gastrointestinal microbiota using antibiotics could affect 5-HT<sub>2C</sub> receptor editing<sup>33</sup>. To this end, we used the hypothalamus of antibiotic-treated rats<sup>34, 35</sup>. We observed no statistically significant differences in gene expression levels of the full-length 5-HT<sub>2C</sub> receptor, as well as the INI, VNV, VNI and VGV isoforms (**Fig 5**). The VSV isoform was not reliably detected in the hypothalamus.

### 3. Discussion

The host microbiota has been increasingly implicated in both the peripheral and central serotonergic system<sup>6-8</sup>. In the present study, we report that GF mice have an altered gene expression profile of 5-HT<sub>2C</sub> receptor editing isoforms in the amygdala, hypothalamus, and striatum. Many of these changes were normalized after colonization of the microbiota, further indicating that the host microbiota controls 5-HT<sub>2C</sub> receptor editing. However, no significant changes in 5-HT<sub>2C</sub> receptor editing in the prefrontal cortex of GF mice and hypothalamus of ABX-treated rats were observed.

Interestingly, our analysis revealed that microbiome status had a similar effect on all different 5-HT<sub>2C</sub> receptor isoforms in the hypothalamus, whereas 5-HT<sub>2C</sub> receptor isoforms were differentially impacted by microbiome status in the amygdala. For instance, the 5-HT<sub>2C</sub> receptor isoform VNI was increased in the hypothalamus of GF mice, which was not observed in the amygdala. These findings indicate that the microbiota can affect 5-HT<sub>2C</sub> receptor isoforms in a brain region-dependent manner. It is also interesting to note that no significant changes in 5-HT<sub>2C</sub> receptor editing were observed in the prefrontal cortex, which were observed in the amygdala. This is likely due to the 5-HT<sub>2C</sub> receptor not being as highly expressed in the prefrontal cortex. This results in a decreased sequencing depth of the 5-HT<sub>2C</sub> receptor and its isoforms, and these findings therefore need to be interpreted with care. Nonetheless, it has previously been reported that GF mice have an increased expression of genes involved in myelination in the prefrontal cortex, but not frontal cortex, hippocampus, cerebellum, amygdala and striatum<sup>36</sup>. This supports the notion that the gut microbiota can influence the brain in a region-dependent manner.

Our data reveal increased gene expression levels of the unedited INI and edited VNV 5-HT<sub>2C</sub> receptor isoforms, as well as a trend towards increased levels of the VNI and VSV isoforms in GF mice. This indicates that the microbiota is able to regulate 5-HT<sub>2C</sub> receptor editing profiles in the hypothalamus, even though it remains unclear whether GF mice have increased editing, as increases in both the unedited INI and edited VNV were observed. Subsequent analysis of the hypothalamus of ABX-treated rats did not reveal any differences. Even though we did observe a non-significant increase in the 5-HT<sub>2C</sub> receptor isoforms, but this is likely explained by an increased 5-HT<sub>2C</sub> receptor expression overall. The absence of differences in the hypothalamus of adult ABX-treated rats suggests that alterations in the microbiome during development as observed

in GF mice, but not later life, might influence 5-HT<sub>2C</sub> receptor editing patterns. It is also interesting to note that increased hypothalamic 5-HT<sub>2C</sub> receptor editing may be implicated in disorders characterized by hyperphagia, as the hypothalamus is central for regulating food intake and energy homeostasis<sup>37</sup>. Indeed, mice solely expressing the fully edited VGV isoform show hyperphagia<sup>16, 17</sup>. It is also important to note that the 5-HT<sub>2C</sub> receptor is colocalized in the hypothalamus with the orexigenic ghrelin receptor - GHS-R1a, where it regulates ghrelin's orexigenic effect<sup>38</sup>. Editing of the 5-HT<sub>2C</sub> receptor impairs the ability of the 5-HT<sub>2C</sub> receptor to reduce GHS-R1a agonist-mediated signaling through receptor dimerization<sup>14</sup>. As such, hypothalamic 5-HT<sub>2C</sub> receptor editing could represent a promising therapeutic target to treat conditions associated with hyperphagia such as Prader-Willi syndrome and obesity. However, we did not investigate any measures of hyperphagia, so more research is warranted to see whether microbiota-targeted strategies can affect hyperphagia through modulation of hypothalamic 5-HT<sub>2C</sub> receptor editing.

We also investigated the gene expression of ADAR1 and ADAR2 in the hypothalamus and striatum, the enzymes involved in post-transcriptional adenosine-to-inosine RNA editing<sup>10</sup>. Here, we observed no changes in ADAR1 and ADAR2 gene expression corresponding to the differences found in 5-HT<sub>2C</sub> receptor editing. This is aligned with results from a genetic leptin-deficient mouse model of obesity (*ob/ob*), where an increase in 5-HT<sub>2C</sub> receptor editing was found in the hypothalamus, but no differences in ADAR1 and ADAR2 expression<sup>39</sup>. The dissimilarity between 5-HT<sub>2C</sub> receptor editing and ADAR1 and ADAR2 expression may potentially be due to dissimilarities between gene expression and protein levels/enzyme activity. Alternatively, these discrepancies could also be explained by changes in ADAR1 and ADAR2 expression in cell types that do not express the 5-HT<sub>2C</sub> receptor.

The data presented here demonstrate that the microbiota can influence 5-HT<sub>2C</sub> receptor editing, and therefore potentially impact 5-HT<sub>2C</sub> receptor signaling. These results, combined with the changes observed in the ADAR1 and ADAR2 gene expression, might also postulate that the microbiota could be able to modulate A-to-I editing of other substrates. Nonetheless, it is important to emphasize that this study used the proof-of-principle GF mouse model and that more research needs to be done investigating the mechanisms underpinning these findings using various microbiota-altering interventions (e.g., antibiotics, fecal matter transplant) or microbial-derived metabolites (e.g., short-chain fatty acids, bile acids). Overall, the findings presented in this

manuscript provide an exciting starting point for future research investigating microbiota-targeted strategies for modulating 5-HT<sub>2C</sub> receptor signalling by adenosine-to-inosine RNA editing in the CNS.

## **4. Materials and methods**

### **Animals**

Male Swiss Webster mice (Taconic, Germantown, New York, USA) from F1-generation offspring from conventionally-raised (CON) and germ-free (GF) breeding pairs were used as previously described<sup>31, 40</sup>. Colonized germ-free (CGF) mice were removed from the GF unit shortly after weaning (postnatal day 21), placed on CON-used bedding and housed next to CON mice in the standard animal unit to allow colonization of microbes present in the environment. CON mice were housed 2–5/cage under controlled conditions (temperature 20–21 °C, 55–60% humidity) on a 12 h light/dark cycle. GF mice were housed in groups of 2–4/cage in a flexible-film gnotobiotic isolator under the same 12-h light/dark cycle. All mice received the same autoclaved, pelleted diet (Special Diet Services, product code 801010). At ten weeks of age, animals were sacrificed, brain regions were rapidly dissected from fresh brains as adapted from<sup>41</sup>, stored in RNAlater (Qiagen, 76106) at 4 °C for 24 h and finally transferred to –80 °C.

Male adult Sprague Dawley rats were housed in five per cage in standard cages and were under a strict 12-h light/dark cycle. Rats received the same autoclaved diet as mice in the previous experiment (Teklad Global 18% Protein Rodent Diet, product code 2018S). At 9 weeks of age, rats were exposed to a cocktail of antibiotics for a total of 13 weeks to deplete the gut microbiota. The antibiotic cocktail consisted of ampicillin (1 g/L), vancomycin (500 mg/L), ciprofloxacin HCL (20 mg/L), imipenem (250 mg/L) and metronidazole (1 g/L) in autoclaved water, as previously discussed<sup>34, 35</sup>. Drinking water was renewed every 3 days. At the end of the treatment, animals were sacrificed, brain regions were rapidly dissected from fresh brains as adapted from<sup>41</sup>, stored in RNAlater (Qiagen, 76106) at 4 °C for 24 h and finally transferred to –80 °C.

All experiments were approved by the Animal Experimentation Ethics Committee of University College Cork and Health Products Regulatory Authority (HPRA) and were conducted in accordance with European Directive 86/609/EEC.

### **RNA isolation and cDNA synthesis**

RNA from the hypothalamus and striatum was isolated using the mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific, AM1560), concentrations and quality were assessed using the NanoDrop™ spectrophotometer (ThermoFisher Scientific), and finally, mRNA was synthesized into cDNA with the cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814). cDNA

was stored at  $-20^{\circ}\text{C}$  until gene expression analysis. Samples used for sequencing were assessed for RNA integrity using Bioanalyzer (Agilent), and equal amounts of RNA from two to three animals were subsequently pooled to yield four samples per group.

### **Sequencing and bioinformatics pipeline**

Data from amygdala and prefrontal cortex sequencing was obtained from a previous study<sup>31</sup>. Briefly, Library preparation, sequencing, and Fastq-file generation were done by Beckman Coulter Genomics service (Danvers, MA, USA). Paired-end reads of  $2 \times 100$  bp were produced on an Illumina HiSeq2500 sequencer.

Fastq-format reads were quality filtered and trimmed using Trimmomatic (v0.32)<sup>42</sup> with the following non-default parameters: AVGQUAL: 20; SLIDINGWINDOW: 4:20; LEADING: 10; TRAILING: 10; MINLEN: 60. Alignment to the mouse reference genome (GRCm38.p3) was achieved using the STAR aligner (v2.4.0f1)<sup>43</sup> with default options and an index compiled with gene models retrieved from the Ensembl database (release 78).

Known RNA-editing positions in the mouse reference genome (mm10/GRCm38 coordinates) were retrieved from a previous publication<sup>39</sup> and compiled to a non-redundant list of 17,831 positions. This list was subsequently used as an input for the REDIttools algorithm REDIttoolKnown.py<sup>44, 45</sup>. Further input arguments were a list of splice sites (taken from UCSC table browser), the mouse genome sequence and Ensemble database release 78 gene models. Non-default parameters were -C 1000, -c 0, -q 10, -m 10, -v 1, -n 0.001, -t 4. The relative abundance of 5-HT<sub>2C</sub> receptor RNA isoforms was calculated as a percentage of total 5-HT<sub>2C</sub> receptor RNA abundance. However, the abundance of all 5-HT<sub>2C</sub> receptor isoforms was similar to previous work (**sTable 1**)<sup>39</sup>, indicating that these findings are unlikely to be explained by false-positives.

### **qRT-PCR analysis**

Gene expression analysis of hypothalamic and striatal tissue was carried out by qRT-PCR using various probes from Applied Biosystems, of which the ones used for detecting edited 5-HT<sub>2C</sub> receptor isoforms were custom-made as designed according to a previously described method (**Table 1**)<sup>39, 46</sup>. The same probes for the 5-HT<sub>2C</sub> receptor isoforms were used for both mouse and rat samples, as they contain the same mRNA sequences<sup>10</sup>. The TaqMan™ Universal Master Mix II, no UNG (ThermoFisher Scientific, 4440040) was used. Multiplex qRT-PCR was performed

using the ABI7300 Real-time PCR machine (Applied Biosystems, Warrington, UK) by performing a pre-denaturation step for 10 min at 95 °C, followed by 50 cycles of amplification by melting at 95 °C and annealing at 60 °C for 1 min. Data were normalized using Actb as endogenous control and transformed using the  $2^{-\Delta\Delta CT}$  method. All procedures were carried out according to the manufacturers' instructions.

**Table 1. Used qRT-PCR probes**

<b>Gene symbol</b>	<b>Common gene name</b>	<b>Probe ID</b>
Actb (Mice)	$\beta$ -actin	4352341E
Actb (Rats)	$\beta$ -actin	4351314E
ADAR1	Adenosine deaminases acting on RNA 1	Mm00508001_m1
ADAR2	Adenosine deaminases acting on RNA 2	Mm00504621_m1
Full-length 5-HT <sub>2C</sub> (Mice)	Serotonin receptor 2C	Mm00664865_m1
Full-length 5-HT <sub>2C</sub> (Rats)	Serotonin receptor 2C	Rn.Pt.58.46103231
<b>Gene symbol</b>	<b>Probe sequence</b>	
5-HT <sub>2C</sub> INI Unedited	[Fam]tagcaatacgtaatcctattg [MGB/NFQ]	
5-HT <sub>2C</sub> VNV Isoform	[Fam]tagcagtgcgtaatcctgttga [MGB/NFQ]	
5-HT <sub>2C</sub> VNI Isoform	[Fam]tagcagtgcgtaatcctattg [MGB/NFQ]	
5-HT <sub>2C</sub> VSV Isoform	[Fam]tagcagtgcgtagtcctgttg [MGB/NFQ]	
5-HT <sub>2C</sub> VGV Isoform	[Fam]tagcagtgcgtggtcctgttg [MGB/NFQ]	

### Statistical analysis

Isoform abundances retrieved from sequencing data were compared using a two-way ANOVA followed by Dunnet post hoc correction. Gene expression data were first analyzed using a two-way ANOVA (microbiome status X 5-HT<sub>2C</sub> isoforms) to investigate whether microbiome status affected 5-HT<sub>2C</sub> isoform expression overall, which was followed up with a Bonferroni post hoc correction. Data from individual isoforms were subsequently analyzed using a one-way ANOVA, followed Tukey's post hoc test. Statistical analysis was performed using SPSS software version 24 (IBM Corp). Data are expressed as mean  $\pm$  SEM. A p-value < 0.05 was deemed significant.



## **Abbreviations**

Antibiotic-treated: ABX, Adenosine deaminases acting on RNA 1: ADAR1, Adenosine deaminases acting on RNA 2: ADAR2, Controls rats: CTR, Conventionally raised: CON, Germ-free: GF, Colonized germ-free: CGF, Serotonin: 5-HT, Serotonin receptor 2C: 5-HT<sub>2C</sub>.

## **Author contributions**

MVDW, RMS, GC, TGD, JFC, and HS have contributed to the conception and design of the work, as well as critically revising it for intellectual content. Data acquisition, analysis, and interpretation were performed by MVDW, RMS, VLP, and AEA.

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## **Disclosure of interest**

The authors have no competing interests to declare.

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## Tables and figure legends

### Table 1. Used qRT-PCR probes

### sTable 1. Relative occurrence of 5-HT<sub>2C</sub> isoforms compared to previous work

**Figure 1. Germ-free mice have a decreased prevalence of the unedited INI 5-HT<sub>2C</sub> receptor isoform and increased prevalence of the VNV and VNI isoforms.** The amygdala of mice with a conventional microbiota (CON), mice without a microbiota (GF) and mice of which the microbiota was colonized (CGF), were investigated using RNA sequencing, after which the relative frequency of 5-HT<sub>2C</sub> receptor transcript variants was assessed. Only transcript variants are depicted of which relative frequency was higher than 1%. Data were analyzed using two-way ANOVA following Dunnet post hoc correction. Significant differences are depicted as: \*\* $p < 0.01$ ; compared to CON. All data are expressed as mean  $\pm$  SEM (n = 4).

**Figure 2. Germ-free mice have increased expression of specific hypothalamic 5-HT<sub>2C</sub> receptor isoforms, which are downregulated after colonization.** Hypothalamic gene expression was investigated of the full-length 5-HT<sub>2C</sub> receptor (A), as well as the INI, VNV, VNI, VSV and VGV (B-F respectively) isoforms of mice with a conventional microbiota (CON), germ-free mice (GF) and germ-free mice of which the microbiota was colonized (CGF). Data were analyzed using a one-way ANOVA, followed by Tukey's post hoc test. Significant differences are depicted as: \* $p < 0.05$ ; compared to CON, # $p < 0.05$ ; compared to GF. All data are expressed as mean  $\pm$  SEM (n = 9-11).

**Figure 3. Germ-free mice have increased striatal expression the 5-HT<sub>2C</sub> receptor, as well as the VNV and VGV isoforms.** Striatal gene expression was investigated of the full-length 5-HT<sub>2C</sub> receptor (A), as well as the VNV, VGV, and VNI (B-D respectively) isoforms of mice with a conventional microbiota (CON), germ-free mice (GF) and germ-free mice of which the microbiota was colonized (CGF). Data were analyzed using a one-way ANOVA, followed by Tukey's post hoc test. Significant differences are depicted as: \* $p < 0.05$ , \*\* $p < 0.01$ ; compared to CON, # $p < 0.05$ ; compared to GF. Non-significant differences are depicted as: ns. All data are expressed as mean  $\pm$  SEM (n = 7-8).

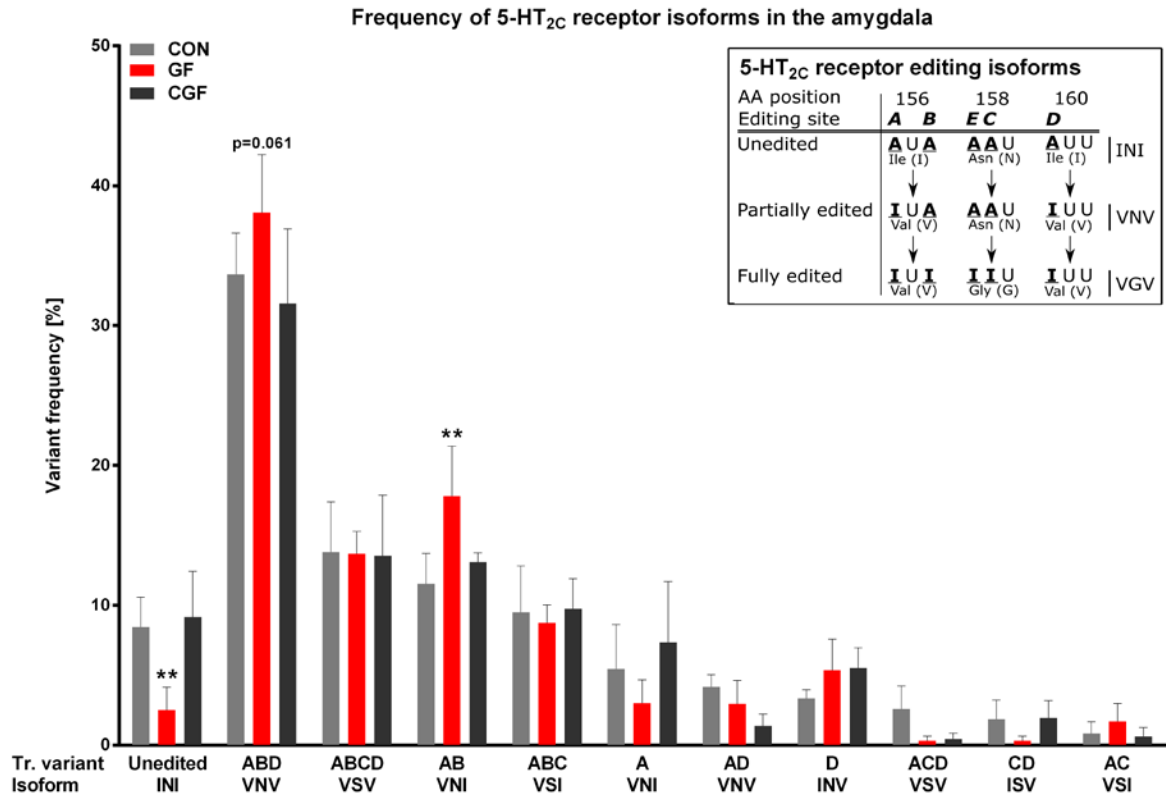
**Figure 4. Hypothalamic and striatal ADAR1 and ADAR2 expression remain unaffected in germ-free mice.** The hypothalamus and striatum were assessed for the expression of both

adenosine deaminases acting on RNA enzymes 1 and 2 (ADAR1 and ADAR2 respectively) of mice with a conventional microbiota (CON), germ-free mice (GF) and germ-free mice of which the microbiota was colonized (CGF). Data were analyzed using a one-way ANOVA, followed by Tukey's post hoc test. Significant differences are depicted as: \* $p < 0.05$ ; compared to CON. All data are expressed as mean  $\pm$  SEM (n = 7-11).

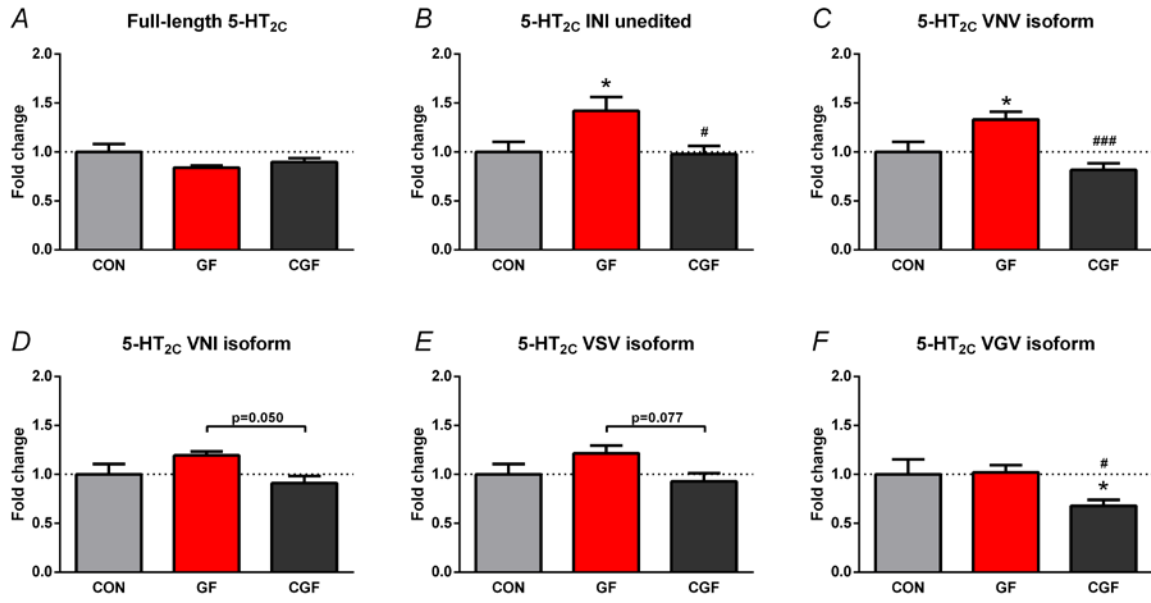
**Figure 5. Hypothalamic 5-HT<sub>2C</sub> gene expression patterns in rats receiving an antibiotic cocktail remain unaffected.** Hypothalamic gene expression was investigated of the full-length 5-HT<sub>2C</sub> receptor (A), as well as the INI, VNV, VNI and VGV (B-E respectively) isoforms of control (CTR) and antibiotic-treated (ABX) rats. Data were analyzed using a one-way ANOVA, followed by Tukey's post hoc test. All data are expressed as mean  $\pm$  SEM (n = 9-10).



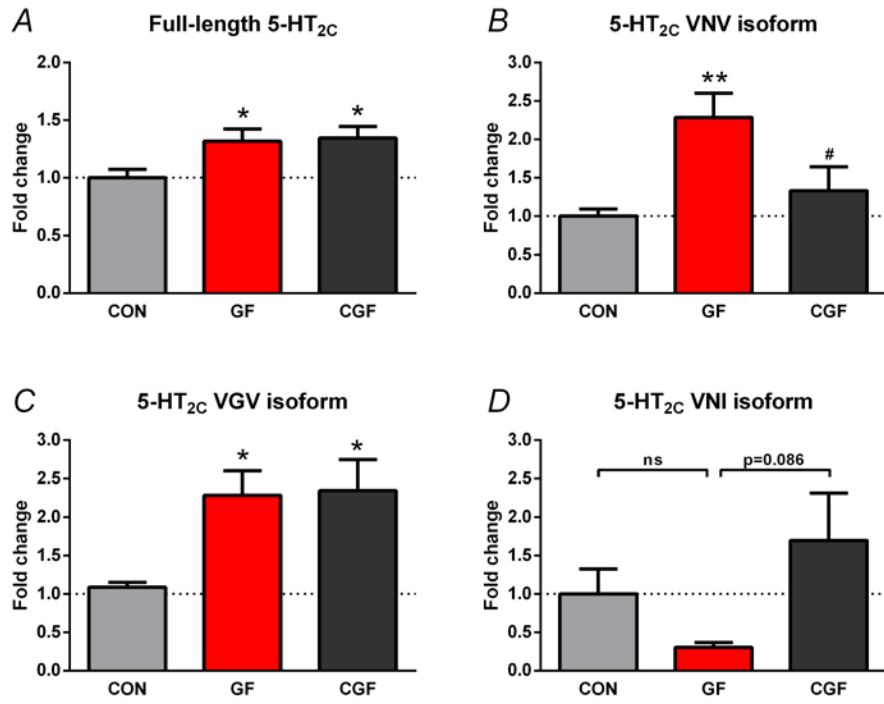
Figure 1



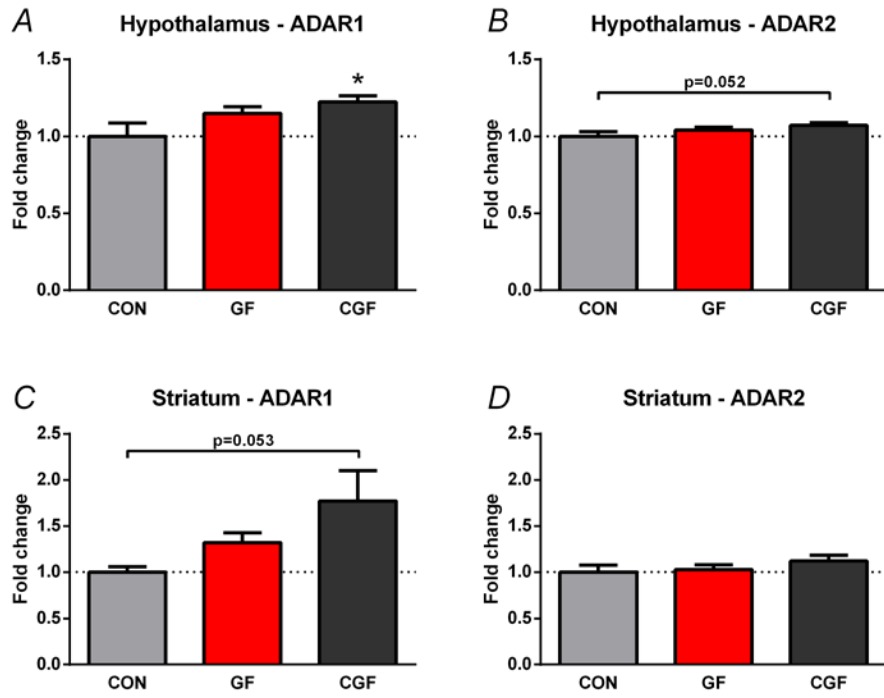
**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**

