<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Behavioural and neurochemical consequences of chronic gut microbiota depletion during adulthood in the rat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Hoban, Alan E.; Moloney, Rachel D.; Golubeva, Anna V.; McVey Neufeld, Karen A.; O'Sullivan, Orla; Patterson, Elaine; Stanton, Catherine; Dinan, Timothy G.; Clarke, Gerard; Cryan, John F.</td>
</tr>
<tr>
<td><strong>Publication date</strong></td>
<td>2016-10-11</td>
</tr>
<tr>
<td><strong>Type of publication</strong></td>
<td>Article (peer-reviewed)</td>
</tr>
</tbody>
</table>
[http://dx.doi.org/10.1016/j.neuroscience.2016.10.003](http://dx.doi.org/10.1016/j.neuroscience.2016.10.003) |
| **Access to the full text of the published version may require a subscription.** |
| **Rights** | © 2016, International Brain Research Organization. Published by Elsevier Ltd. All rights reserved. This manuscript version is made available under the CC-BY-NC-ND 4.0 license  
[http://creativecommons.org/licenses/by-nc-nd/4.0/](http://creativecommons.org/licenses/by-nc-nd/4.0/)  
[https://creativecommons.org/licenses/by-nc-nd/4.0/](https://creativecommons.org/licenses/by-nc-nd/4.0/) |
| **Embargo information** | Access to this article is restricted until 12 months after publication by request of the publisher. |
| **Embargo lift date** | 2017-10-11 |
| **Item downloaded from** | [http://hdl.handle.net/10468/3290](http://hdl.handle.net/10468/3290) |

Downloaded on 2020-09-16T16:12:35Z
Behavioural and Neurochemical Consequences of Chronic Gut Microbiota Depletion during Adulthood in the Rat

A.E. Hoban, R.D. Moloney, A.V. Golubeva, K.A. McVey Neufeld, O. O’Sullivan, E. Patterson, C. Stanton, T.G. Dinan, G. Clarke, J.F. Cryan

PII: S0306-4522(16)30512-7
DOI: http://dx.doi.org/10.1016/j.neuroscience.2016.10.003
Reference: NSC 17363

To appear in: Neuroscience

Accepted Date: 2 October 2016

Please cite this article as: A.E. Hoban, R.D. Moloney, A.V. Golubeva, K.A. McVey Neufeld, O. O’Sullivan, E. Patterson, C. Stanton, T.G. Dinan, G. Clarke, J.F. Cryan, Behavioural and Neurochemical Consequences of Chronic Gut Microbiota Depletion during Adulthood in the Rat, Neuroscience (2016), doi: http://dx.doi.org/10.1016/j.neuroscience.2016.10.003

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Behavioural and Neurochemical Consequences of Chronic Gut Microbiota Depletion during Adulthood in the Rat

A.E. Hoban\textsuperscript{a,b}, R. D. Moloney\textsuperscript{a}, A.V. Golubeva\textsuperscript{a}, K.A. McVey Neufeld\textsuperscript{a}, O. O’Sullivan\textsuperscript{d}, E. Patterson\textsuperscript{d}, C. Stanton\textsuperscript{d}, T.G. Dinan\textsuperscript{a,c}, G. Clarke\textsuperscript{a,c}, J.F. Cryan\textsuperscript{a,b#}

\textit{For Submission to: Neuroscience}

\textsuperscript{a} APC Microbiome Institute, University College Cork, Ireland
\textsuperscript{b} Department of Anatomy and Neuroscience, University College Cork, Ireland
\textsuperscript{c} Department of Psychiatry and Neurobehavioural Science, University College Cork, Ireland
\textsuperscript{d} Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland

# Corresponding author:
John F. Cryan, Ph.D.
Professor & Chair, Dept Anatomy & Neuroscience
Principal Investigator, Alimentary Pharmabiotic Centre
University College Cork,
Room 386, Western Gateway Building,
Western Rd., Cork
Ireland
Tel: +353 21 420 5426
Fax:+353 21 427 3518
E-Mail: j.cryan@ucc.ie
Abstract

Gut microbiota colonization is a key event for host physiology that occurs early in life. Disruption of this process leads to altered brain development which ultimately manifests as changes in brain function and behaviour in adulthood. Studies using germ-free mice highlight the extreme impact on brain health that results from life without commensal microbes, however the impact of microbiota disturbances occurring in adulthood is less studied. To this end, we depleted the gut microbiota of 10-week-old male Sprague Dawley rats via chronic antibiotic treatment. Following this marked, sustained depletion of the gut bacteria, we investigated behavioural and molecular hallmarks of gut-brain communication. Our results reveal that depletion of the gut microbiota during adulthood results in deficits in spatial memory as tested by Morris water maze, increased visceral sensitivity and a greater display of depressive-like behaviours in the forced swim test. In tandem with these clear behavioural alterations we found changes in altered CNS serotonin concentration along with changes in the mRNA levels of corticotrophin releasing hormone receptor 1 and glucocorticoid receptor. Additionally, we found changes in the expression of BDNF, a hallmark of altered microbiota-gut-brain axis signaling. In summary, this model of antibiotic-induced depletion of the gut microbiota can be used for future studies interested in the impact of the gut microbiota on host health without the confounding developmental influence of early-life microbial alterations.
Introduction

There is growing appreciation for the importance of the gut microbiota in shaping brain function and behavior (Mayer 2011, Cryan and Dinan 2012). Alterations in the bidirectional microbiota-gut-brain axis are reported to be involved in the pathogenesis of many well described disorders of the gut including irritable bowel syndrome (IBS) and inflammatory bowel disorder (IBD) (De Palma et al., 2014; Distruitti et al., 2016; Staudacher and Whelan, 2016). More recently brain disorders such as autism spectrum disorder (Mayer et al., 2014), and mood disorders have been associated with dysfunctional microbiota-gut-brain axis communication (Cryan and Dinan 2012, Foster and McVey Neufeld 2013).

Multiple approaches have been used to study the modulatory effects of gut microbiota on gut-brain interactions, and in particular the use of germ-free (GF) animals have highlighted an important role for the microbiota in stress-related behaviors and neurochemistry (Sudo et al., 2004). However, GF studies have several limitations (Luczynski et al., 2016; Lundberg et al., 2016) and ultimately there is no translational equivalent, especially during critical neurodevelopmental windows. For example, GF animals are born under aseptic conditions where they are maintained in the absence of any colonizing bacteria throughout their lifetime (Williams, 2014; Luczynski et al., 2016). Therefore, there is a rapidly growing need for more informative and gut-specific approaches such a humanized microbiota-associated mice (Arrieta et al., 2016) to investigate microbiota-mediated signaling along the gut-brain axis.

There is a wide array of reported differences in brain biochemistry of GF animals compared to their conventionally raised counterparts, highlighting the essential role of the gut microbiota in normal brain development (Stilling et al. 2014a; Stilling et al. 2014b). This includes altered hypothalamic-pituitary-adrenal (HPA) axis responses/programming (Sudo et al., 2004), BNDF expression (Gareau et al., 2010; Heijtz et al., 2011; Neufeld et al., 2011; Stilling et al., 2015) and serotonergic system alterations (Clarke et al., 2013). Along with these alterations, many changes in behavioral outputs have been noted including social, anxiety-related and cognitive outputs (Gareau et al., 2010; Heijtz et al., 2011; Neufeld et al., 2011; Clarke et al., 2012; Desbonnet et al., 2014; Arentsen et al., 2015; Buffington et al., 2016). Alternative models must be evaluated to fully appreciate the role the gut microbiota plays in these aspects of brain function in the absence of the confounding impact of alterations to early-life trajectories of microbiota assembly.
The use of antibiotics has attracted attention in this regard as it allows us to effectively and specifically knock-down some of the gut microbiota for prolonged periods of time and at different developmental time points and then examine the impact on brain development and behaviour (Desbonnet et al. 2015; Bercik et al. 2011; Verdu et al. 2006). One recent study highlighted the impact of short 7 day exposure to antibiotics in adulthood (Fröhlich et al., 2016) however, few have looked at the impact of chronic long term treatment in adulthood (Möhle et al., 2016). Subsequently the use of antibiotics in order to deplete intestinal microbiota is a more controlled animal model as these animals are not hindered by any additional physiological differences as in the GF animals which have underdeveloped immune systems as well as major differences in metabolic processing. Therefore, in order to more precisely appraise the role of the gut microbiota during adulthood we set out to establish an effective rat model of microbiota depletion via chronic antibiotic administration. In particular, we assess the effects of gut microbiota depletion on visceral sensitivity, cognitive, emotional behaviors and biological markers of microbiota-gut-brain axis dysfunction.

Experimental Procedures

Animals and treatments

Adult male Sprague Dawley rats (n=10/group) were housed 5 per cage in standard rat cages. All animals were housed in our animal facility and maintained under a 12-h light/dark cycle. All experimental groups received the same autoclaved diet (Teklad Global 18% Protein Rodent diet, Product code 2018S). All experiments were conducted in accordance with European Directive 86/609/EEC. Approval by the Animal Experimentation Ethics Committee of University College Cork was obtained before commencement of all animal related experiments. In order to sufficiently deplete the gut microbiota rats were treated with a combination of antibiotics for 6 weeks at adulthood (10 weeks old at start of treatment) and throughout behavioral testing (total of 13 weeks treatment). The antibiotic cocktail was administered in drinking water to avoid any adverse effects from chronic stress induced by alternative administration methods such as oral gavage. The antibiotic cocktail consisted of ampicillin (1g/L), vancomycin (500mg/L), and ciprofloxacin HCL (20mg/L), imipenem (250mg/L) and metronidazole (1g/L) and was chosen based on published protocols from our group (Kelly et al., 2016) and others (Heimesaat et al., 2013) and was made up in autoclaved
water and changed every 3 days. Control animals received autoclaved water without any antibiotics which was also changed every 3 days. Bottle weights were taken before and after replenishment. Animal weights were taken every 3 days to ensure animals were not losing excessive body weight. Along with regular weight monitoring, weekly fresh fecal pellets were collected to ensure microbiota depletion. Cages were cleaned every second day to reduce the risk of re-establishment of a standard gut microbiota. Behavioral assessment commenced after 6 weeks of antibiotic treatment. Administration of antibiotics continued throughout all behavioral assessments until the animals were culled 13 weeks after beginning treatment (Figure 1 A, B). All 20 animals (n=10/group) completed each behavioural test and their accompanying tissue was used for all molecular assessments except for microbiota sequencing which is highlighted in figure 7.

**Open Field (OF)**

The open field is a novel stressful environment, where the animals are placed in an open arena that is brightly lit from above and was carried out as previously described (O’Mahony et al. 2014b). 30 minutes before behavioural testing, animals were habituate to the room. The apparatus consisted of a white round arena measuring 90cm, brightly lit to 1000 lux. At the beginning of the test animals were placed into the center of the arena and allowed to explore for 10 minutes. After testing animals were returned to their home cage in the housing facility. The arena was cleaned with 70% ethanol to ensure that no cue smell remained form the previous trail. Faecal output was manually scored. Total distance travelled, distance moved in the inner zone, velocity and transitions were analyzed using a tracking software system (Ethovision, Noldus, The Netherlands).

**Elevated plus maze (EPM)**

The EPM is a commonly used behavioral paradigm used to investigate anxiety-like behaviors in rodents and was carried out as previously described (O’Mahony et al. 2014b). Animals were habituated to the testing room for 30 minutes prior to test. The maze is elevated 750mm from the floor, comprising of two open and closed arms (100mm x 500mm x 400mm walls; W x L x H). All arms of the maze were cleaned with 70% ethanol before introduction of the animal. Facing the open arm, each animal was placed into the center platform for 5 minutes.
The time spent in each arm and number of entries to the open and closed arm were manually scored.

**Forced swim test (FST)**

The FST is a behavioural test used in rodents to assess antidepressant-like behaviour and was carried out as previously described (Slattery and Cryan, 2012). All experimental animals were first habituate to the testing room 30 minutes prior testing. A pre-swim (15 min) was conducted first, 24 hours prior to the test swim. On test day, all animals were introduced again to the Plexiglas cylinder (46cm tall x 21cm in diameter) filled with water (24°C) to a depth of 30cm. Test sessions (5 minutes) were recorded by video camera positioned directly above the cylinder. Animals were removed from their homecage and placed into the tank. After 5 min, the animal was removed from the tank, dried and replaced back in its home cage. The tank was then emptied and fresh water replaced into the tank between animals. Analysis of behaviour was conducted by an experienced experimenter for the test 5 minutes. The parameters of interest were the length of time immobile, swimming and climbing. The predominant behaviours were scored every 5 seconds within a 5 minute time frame. Climbing was defined by the rat presenting its forepaws along the edge of the cylinder in an upwards movement. Any horizontal movement was classified as swimming. Finally, immobility was defined as no additional movement required for the animal to maintain its head above water. Latency to first immobile display was also measured.

**Morris water maze (MWM)**

The MWM is a behavioural test used to assess hippocampal-dependent spatial learning. The protocol was adapted from (Vorhees & Williams 2006; O'Mahony et al. 2014b). Animals were trained (acquisition days 1-5) with a hidden clear Plexiglas platform in a constant position located in one quadrant of the pool. Animals were subjected to four trials on each training day. For each trial the starting point varied randomly. Trials started with the rat facing the wall of the pool. The animal was released into the water at water-level. Time was started the moment the animal was released and measured until the rat located the submerged platform. On conditions where the animal was unable to locate the platform within the allocated time frame (120 seconds), it was then guided by the experimenter. Once on the
platform animals were left there for 30s. For the probe trial (day 6) the platform was removed and the animal was placed in a novel start position in the maze, facing the tank wall. The animal was then removed after 60s. Videos for the probe trial were scored for the first 30s.

**Colorectal distension (CRD)**
CRD was performed as previously described (O’Mahony et al., 2009). Isoflurane was used to anaesthetize animals before a latex polyethylene balloon (6cm in length) was inserted into the colon of each animal. The balloon was inserted 1 cm from the anus and inflated following 10 minutes recovery from anesthesia, the balloon was distended from 0 to 80 mmHg using a Distender Series IRRTM Barostat (G&J ElectronicsInc) over a period of 8 minutes. During this gradual inflation the pressure was increased 1 mmHg every 6s. DataTrax2 software was used to quantify the magnitude of the balloon pressure signals. Visceromotor responses (VMR), were assessed by an observer.

**Novel object recognition (NOR)**
The protocol used was adapted from (Bevins and Besheer, 2006). Animals were first habituated to the testing arena for 10 minutes and then removed. Following this two identical objects were placed in the arena, followed by the animal facing away from the objects. After the sample-object exposure time (10 minutes), the animal was removed and returned to home cage for 1 h. The testing arena was cleaned with 70% ethanol and one of the objects was exchanged with a new, novel object. The animal was placed back in the arena with the novel and familiar objects for 5 minutes. Videos were scored by trained observers and measures of direct contact (including any contact with mouth, nose or paw) were manually scored.

**Hot Plate**
The hot plate test was used to assess somatic pain sensitivity and was carried out as previously described (Gosselin et al., 2010). Animals were placed on a hot plate with all four paws facing the plate. The plate was heated to 50°C (Plantar Test Analgesia Meter, Stoelting, IL, US) as previously described (Karlsten, Gordh Jr et al. 1990). The response to the hot plate was measured as latency in seconds to first hind paw lick or jump in response to heat. In order to avoid tissue damage a cut off time of 30s was put in place.
High-performance liquid chromatography (HPLC): brain monoamine analysis

The monoamines noradrenaline (NA), serotonin (5-HT), dopamine (DA), monoamine metabolites 5-Hydroxyindoleacetic acid (5-HIAA), 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured in the prefrontal cortex, hippocampus, amygdala, striatum and hypothalamus using HPLC coupled to electrochemical detection as previously described (Clarke et al., 2013; Desbonnet et al., 2015). Dissected brain tissue was homogenised in a mobile phase containing 2 ng/20 µl of an internal standard N-methyl 5-HT. Following this, homogenised samples were centrifuged at 14,000g for 15 min at 4°C. 20 µl of the supernatant was injected onto the HPLC system, which consisted of a SCL 10-Avp system controller, LC-10AS pump, SIL-10A autoinjector (with sample cooler maintained at 4°C), CTO-10A oven, LECD 6A electrochemical detector and an online Gastorr Degasser. A reverse-phase column (Synergi 4u MAX-RP 80A, 250 4.6 mm), maintained at 30°C, was employed in the separation (flow rate 0.9 ml/min). Characteristic retention times were determined by standard injection and were run at regular intervals during the sample analysis. Chromographs were generated using Class-VP5 software and allowed identification of the desired monoamines. Monoamine concentrations were calculated using analyte:internal standard peak height ratios and expressed as nanograms of neurotransmitter per gram of fresh tissue weight.

RNA extractions, reverse transcription and quantitative RT-PCR

The amygdala, hippocampus, prefrontal cortex and spinal cord (lower lumbar sacral portion) were rapidly dissected from individual animals and stored in RNAlater for 24h before freezing at -80°C (Desbonnet et al., 2015). Using a commercially available mirVana™ total RNA extraction kit (Thermo Fisher Scientific/Ambion), total RNA was extracted from each brain region following manufacturer’s protocol. RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Thermo Fisher Scientific/Applied Biosystems) in a G-storm thermocycler (G-storm, Surrey, UK). Taqman gene expression assays were used to determine specific gene expression levels in individual brain regions using the AB7300 system (Thermo Fisher Scientific/Applied Biosystems). Each transcript value was calculated as the average of triplicate samples from several rats per experimental condition. Values were normalized to β-actin. Gene expression was calculated using the formula 2^ΔΔct. This value
was then normalised to the vehicle group to calculate fold change. T-test was used for gene expression analysis. A p-value <0.05 was considered statistically significant.

**Serum corticosterone immunoassay**

On the first day of FST, tail blood samples were obtained from each individual animal at 4 different time points. Blood was taken immediately before the FST and at 30 min, 45 min and 90 min following the swim stress. Approximately 200 µl of blood was collected in tubes containing EDTA to avoid coagulation. The tubes were centrifuged at 3500 x g at room temperature for 15 minutes. Plasma was removed and stored at -80°C. Measurement of corticosterone levels was carried out using a commercially available ELISA kit (Corticosterone ELIA Kit, ADI-900-097, Enzo Life Sciences) according to the manufacturer’s protocol. Absorbance was read at 405nm using a plate reader (Synergy HT, BioTek Instruments, Inc.).

**DNA extraction and sequencing**

Total DNA was isolated from caecal and faecal contents and processed for analysis of microbiota composition in line with Illumina at Teagasc high throughput sequencing center. Using the Illumina platform, raw 300 bp paired-end reads were merged using Flash (Magoč and Salzberg, 2011) and quality checked using the split_libraries script from the QIIME package (version 1.80). (Caporaso et al., 2010). Following this, reads were clustered into operational taxonomical (OTUs) and chimeras removed using with the 64-bit version of USEARCH 7.0 (Edgar, 2010). Taxonomy was assigned through a blast search against the SILVA 16S specific database (version 111) (Quast et al., 2013). Alpha and beta diversities were generated within QIIME. Using weighted Unifrac distances, principal coordinate analysis (PCoA) plots were generated and visualized using v0.9.3-dev (Vázquez-Baeza et al., 2013) of the EMPPeror.

**Statistics**

All data are presented as mean ± SEM and analyzed using a two-tailed t-test between vehicle and antibiotic treated rats using the statistical software package SPSS 21.0 (IBM). All data
presented was analyzed using parametric test (t-test) after both normality and homogeneity was determined (all passed normality and homogeneity test). For MWM and corticosterone 2-way ANOVA repeated measures was carried out on training data to determine significance and was corrected for multiple comparisons. All statistical analysis for microbiota sequencing analysis was carried out in R and corrections for multiple comparisons were made using the Benjammini-Hochberg method. For MWM and qPCR, averaging of individual measurements was averaged before doing groupwise statistics or ANOVA. qPCR was conducted in triplicate and averaged before appropriate statistical test. Significance was denoted with selection of a P-value of less than 0.05. For plotting all data, Graphpad Prism (v5) was used. We also conducted Grubbs method to test for any specific outliers (Grubbs, 1950). Statistical significance was indicated as follows: * indicates P<0.05, ** indicates P<0.01 and *** indicates P<0.001.
Results

**Altered gut microbiota during adulthood did not influence anxiety-related behaviors**

Antibiotic treatment had no impact on anxiety-related behaviors as examined by EPM and OF, two well-established behavioral paradigms commonly used to investigate anxiety-like behaviours in rodents (Table 1).

**Antibiotic treatment impaired aspects of hippocampal-based learning.**

Chronic antibiotic treatment resulted in impaired spatial learning as tested using the MWM. There were no visible differences in learning during the acquisition training days in the MWM (figure 2A). However, when challenged on the probe day (removal of the platform) antibiotic treated rats failed to remember where the platform was within the maze and spent less time in that quadrant (15.30±1.24 vs. 11.40±1.20, t(18)=2.250, p=0.037) (figure 2B). Probe trial was scored for the first 30 seconds. No differences in NOR task was noted between groups. Antibiotic treated rats spent the same amount of time interacting with the novel and familiar object as the vehicle treated rats (figure 2C). No difference was observed when a discrimination index was calculated between groups (figure 2D).

**Antibiotic treatment increased visceral sensitivity.**

Along with reduced spatial learning, antibiotic treated rats also displayed visceral hypersensitivity as measured by CRD. Antibiotic treatment increased threshold (42.90±4.40 vs. 58.22±3.47, t(17)=2.688, p=0.015) indicating more pressure was required to induce pain behaviors (Figure 3A). However, total pain scores demonstrated were not significantly different between groups (5.70±1.89 vs. 4.44±0.74, t(17)=0.591, p=0.561) (figure 3B). No differences were observed in somatic pain sensation following antibiotic treatment in the hot plate test (figure 3C).

**Antibiotic treated rats displayed greater depressive-like behaviors.**

Following antibiotic treatment rats displayed great depressive-like behaviors as indicated by a decreased swimming and increased immobility scores (24.30±0.66 vs. 18.20±1.07, t(18)=4.828, p=0.001 and 27.80±0.85 vs. 31.90±1.64, t(18)=2.215, p=0.039) (figure 4A). No differences between groups were noticed in climbing score or latency (data not shown) to
first immobile episode (7.80±0.71 vs. 9.90±1.15, t(18)=1.544, p=0.140 and 41.60±11.38 vs. 27.40±4.37, t(18)=1.156, p=0.259) (figure 4A). Antibiotic treated rats had a higher number of fecal pellets present at the end of the swim test (1.50±0.401 vs. 2.80±0.35, t(18)=2.414, p=0.026) (figure 4B). Treatment with antibiotics during adulthood did not affect plasma corticosterone response when compared to vehicle treated rats following the forced swim stress (figure 4C).

Analysis of gene expression in the central nervous system

Analysis of mRNA transcript levels in the hippocampus and amygdala revealed altered levels of glucocorticoid receptor (Nr3c1) (GR) and corticotrophin releasing hormone receptor 1 (Crrh1) (Figure 5A,B). Whole amygdala and hippocampus homogenates revealed reduced expression levels of both receptors mRNA transcripts after antibiotic treatment compared to vehicle treated rats. Additionally Bdnf levels were increased in the amygdala and a trend to increase in the hippocampus (Figure 5A,B) (hippocampus Crrh1, t(17)=3.345, p=0.003/GR, t(18)=2.761 p=0.013 and amygdala Crrh1, t(18)=3.404, p=0.003/GR, t(18)=2.474, p=0.023, amygdala Bdnf mRNA levels, t(17)=2.976, p=0.008). Statistically we did find a significant change in Slcla3 and TripV1 however, the fold change was low and may not reflect functional changes at the protein level (Figure 5C). Additionally we did not see any change in mRNA levels of myelin-related genes in the prefrontal cortex following chronic antibiotic treatment (Figure 5B).

Long-term antibiotic treatment altered brain monoamines and plasma tryptophan levels

Treatment with antibiotics altered the levels of key monoamine neurotransmitters in various brain regions. Within the hippocampus there was a reduction in the levels of 5-HT t(18)=2.445, p=0.025) along with increased 5-HIAA/5-HT turnover t(18)=4.286, p=0.0004). Noradrenaline (NA) was significantly increased in the striatum in antibiotic treated rats t(17)=4.582, p<0.003). The DA precursor, L-DOPA and metabolite HVA were altered in the hippocampus t(18)=5.134,p<0.0001 and t(17)=4.406,p=0.0004) and prefrontal cortex (t(18)=4.988,p=0.0002 and t(17)=3.414,p=0.003). See table 2 for a full summary of all HPLC results. Tryptophan levels, the precursor to 5-HT, were increased in the plasma of antibiotic treated compared to vehicle treated rats (18568.0±824.2 vs. 21253.0±798.9,
t(17)=2.33,p=0.032) (figure 6A). There was no significant difference in kynurenine concentrations and trend to decrease in the kyn/tryptophan ratio (figure 6B,C).

Microbial diversity was affected by antibiotic treatment

Following completion of the study, microbiota was sequenced from both 4 week faecal and 3 month caecal contents of antibiotic and vehicle treated animals. Sequencing demonstrated that microbial diversity was affected by administration of antibiotic in the drinking water which was obvious from Unweighted UniFrac PCoA plot (beta-diversity) (Figure 7A,C). At the phylum level, relative abundance of detected bacteria of each animal revealed a significant decrease in abundance of Firmicutes and Bacteroidetes (Figure 7B,D, Figure 8 A-B and E-F). Subsequently following this decrease there was also an increase in antibiotic treated rats for Proteobacteria and Cyanobacteria (Figure 7B,D). At the family level the impact on Firmicutes consisted of significant decreases in Porphyromonadaceae, Prevotellaceae, Bacteroidaceae, Rikenellaceae, S24-7 and on Bacteroidetes composed of Lachnospiraceae and Ruminococcaceae for both the 4 week faecal and 3 month caecal samples (Figure 8 C-D and G-H).
Discussion

In this study, we assessed the impact of a chronic depletion of the gut microbiota during adulthood on behavioural and molecular hallmarks of altered microbiota-gut-brain axis dysfunction. Our results reveal both overlaps with domain-specific behavioural features of other microbiota-deficient rodents across earlier developmental periods as well as an overall distinct signature of antibiotic-induced microbiota depletion during adulthood. This includes an increase in depressive-like behaviours, lower visceral hypersensitivity and impaired cognition in the absence of anxiety-like behaviours. Moreover, our study once again confirms the importance an intact gut microbiota for normal tryptophan availability and the CNS serotonergic system.

One of the main findings is that the phenotype observed is different from that reported in both GF rats which have increased anxiety and higher HPA axis response to stress (Crumeyrolle-Arias et al., 2014) and the decreased anxiety in GF mice (Heijtz et al., 2011; Neufeld et al., 2011; Clarke et al., 2012). However, we do find some similarities between our antibiotic-induced microbiota depletion model and GF rats with GF rats displaying increased depressive-like behaviours with complimentary lower serotonin levels (Crumeyrolle-Arias et al., 2014). Moreover, although previous studies have employed adult antibiotic treatment regimes in mice, outcomes in rats have been less characterized (Verdú et al., 2006; Bercik et al., 2011a; Reikvam et al., 2011; Cho et al., 2012; Zhang et al., 2014; Desbonnet et al., 2015; Fröhlich et al., 2016; Möhle et al., 2016).

As expected, chronic antibiotic exposure during adulthood significantly reduced the diversity and richness of the gut microbiota with significant decreases in both *f Firmicutes* and *Bacteroidetes* which were the most abundant phylum present in vehicle treated animals, which is consistent with previous studies examining the effects of antibiotic exposure (Verdú et al., 2006; Cho et al., 2012; Zhang et al., 2014; Fröhlich et al., 2016). Additionally, changes in these specific phyla have been associated with neurodevelopmental disorders such as autism and many gastrointestinal disorders (Clemente et al., 2012; Hsiao et al., 2013). Coinciding with this dramatic reshaping of the gut microbiome, our antibiotic treated rats also displayed significant changes in depressive-like behaviour as assessed in the FST. This sustained depletion of the gut microbiota induced increased immobility and decreased swimming behaviors in these rats. This finding is consistent with recent studies in GF animals (Zheng et al., 2016) and studies which demonstrated that targeting the gut microbiota
with probiotics can have antidepressant effects (Desbonnet et al., 2010; Bravo et al., 2011; Hsiao et al., 2013b).

The alteration in depressive-like behaviours also tallies with the reduction in serotonin in the CNS. We specifically found a decrease in levels of hippocampal 5-HT and 5-HT/5HIAA turnover following antibiotic treatment during adulthood. Studies in GF rodents have also shown changes with regards to hippocampal 5-HT with a decrease reported in GF rats and increases in mice (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014). We also investigated expression levels of the 5-HT transporter and receptors within the hippocampus to further probe this system in our model but found no changes in gene expression. This is in line with a previous study in GF mice that showed a change in 5-HT levels but no change in expression of serotonin transporter in the hippocampus (Clarke et al., 2013). Additionally we also found changes in the levels of dopamine precursor HVA and metabolite L-DOPA in the prefrontal cortex and hippocampus with changes in HVA/DA turnover in the striatum. These changes in dopamine turnover are in line with previous studies that also highlight a dysregulation in the synthesis and degradation of this monoamine (Heijtz et al., 2011; Crumeyrolle-Arias et al., 2014). Although inconsistencies in the direction of changes observed following microbiota deficiency on monoamine levels are apparent in these studies, all highlight that absence or depletion of the gut microbiota deeply affect neurotransmitter systems.

The most consistent finding in relation to behavioral changes in GF rodents is their altered anxiety-like phenotype (Heijtz et al., 2011; Neufeld et al., 2011; Clarke et al., 2012). Additionally, previous studies have also highlighted that depletion of the gut microbiota via antibiotics during adolescence can also alter anxiety in mice as tested by light/dark box (Desbonnet et al., 2015). Thus it is of interest that we observed no change in anxiety in two well-validated behavioural screens, the open field or elevated plus maze in antibiotic treated animals. This may be related to the timing or duration of microbiota depletion while the diverging findings in GF rats which display increased anxiety as compared to mice also needs to be considered. Ultimately GF rodents lack any exposure to microbiota during all key neurodevelopmental stages and colonization post-weaning of GF animals has the capacity to reverse the altered anxiety (Clarke et al., 2012) and HPA axis responses (Sudo et al., 2004). Therefore these data agree with the concept of critical windows during development and adolescence for microbial influence over anxiety circuits (Foster and McVey Neufeld, 2013; Borre et al., 2014; McVey Neufeld et al., 2016). However, transient gut-microbiota manipulations such as infection, antibiotics, probiotics do impact anxiety during adulthood.
(Bercik et al. 2011; Bercik et al. 2010; Bravo et al. 2011; Messaoudi et al. 2010; Lyte et al. 2006; Goehler et al. 2007).

In agreement with the lack of impact on anxiety-like behaviours, corticosterone levels were also not altered in our study. Interestingly we found a significant decrease in GR mRNA levels in the amygdala and hippocampus of antibiotic-treated rats. This was also seen in GF rats where they displayed an increase in mRNA levels of CRF in the hypothalamus along with a decrease in GR in the hippocampus (Crumeyrolle-Arias et al., 2014).

Although the current study demonstrates that these transient microbiota-deficient animals have increased circulating tryptophan levels, this is similar but less marked in magnitude to the changes seen in GF mice (Clarke et al., 2013) or in animals who were microbiota-deficient in adolescence (Desbonnet et al., 2015). Indeed, we have only observed a trend to reduction in the kyn/trp ratio, an index of metabolism along the kynurenine pathway. This suggests that alternative metabolic fates for tryptophan, such as bacterial indole production (Lee et al., 2015) are more disrupted following microbiota-depletion during adulthood. This also makes sense in the context of our normal HPA axis responses, a well-known stimulus for tryptophan 2,3-dioxygenase-mediated metabolism along the kynurenine pathway (O’Mahony et al. 2014a; O’Mahony et al. 2014b). Future studies should assess indole production and microbial metabolite-driven local 5-HT production (Reigstad et al., 2015; Yano et al., 2015). This increase in tryptophan availability, albeit less marked than GF animals, is in contrast to a reduction in 5-HT in the hippocampus. This appears to be driven by an increased serotonin turnover as indicated by the 5-HIAA/5HT ratio as we did not observe any alteration in serotonin-related gene expression for TPH and the precise stimulus for this increase is serotonin turnover is unclear.

In our current study, we demonstrated that antibiotic treatment resulted in reduced visceral hypersensitivity as detected by CRD. This is somewhat contradictory to previous findings that showed decreased in pain threshold as measured by CRD following antibiotic treatment during early life. (O’Mahony et al. 2014a). However, this could be explained by the very different windows for antibiotic exposure in these studies. Contrary to our findings, a previous study did show that administration to an antibiotic cocktail increased visceral hypersensitivity which could be ameliorated by exposure to prebiotics (Verdú et al., 2006). Coinciding with this change in visceral sensitivity, we did not see a change in somatic pain following hot plate test. We also noted a decrease in Crhr1 mRNA levels in both the
hippocampus and amygdala of antibiotic treated rodents. \textit{Crhr1} in the CNS is implicated in stress-related alterations of both anxiety and depressive behaviours along with autonomic and visceral functions (Moloney et al., 2015b; Taché, 2015). Interestingly, \textit{Crhr1} signaling mediates hypersensitivity to colorectal distension in animal models such as stress during early-life, prolonged psychological stress and even chronic anxiety. Intra-hippocampal administration of an antagonist to \textit{Crhr1} attenuates visceral perception and when CRH is administered to the central nucleus, increases in abdominal contraction response to CRD have been noted indicating that \textit{Crhr1} in the amygdala may mediate changes in visceral sensitivity. However, we did not see a change in noradrenaline which has been hypothesized to be implicated in the sensitization of visceral nociception by CRH acting on its receptor \textit{Crhr1} in these studies. Interestingly, previous work that studied microbial depletion with vancomycin in early life only impacted visceral pain with no changes in cognition or anxiety as tested by MWM, open field and EPM (O’Mahony et al. 2014b).

Previous studies have highlighted the decline in cognition in relation to microbiota deficient models when tested in the NOR (Gareau et al., 2010; Fröhlich et al., 2016) but a decline in spatial memory has not been observed in other studies. This highlights again that timing of depletion can have varying effects on behavioral outcome as we see a subtle profile of cognitive impairment following microbiota-depletion during adulthood which is apparent in the MWM but not the NOR. However, the type of antibiotic and the length of treatment must be considered when comparing different antibiotic depletion studies.

CNS transcriptional regulation has emerged as a prominent feature of the gut microbiota (Stilling et al. 2014b; Luczynski et al. 2016). GF studies have highlighted that expression of BDNF is highly dysregulated in these animals. Many studies have shown changes in expression in both GF and antibiotic studies (Gareau et al. 2010; Sudo et al. 2004; Desbonnet et al. 2015; Hoban et al. 2016; Stilling et al. 2015; Fröhlich et al. 2016; Bercik et al. 2011). Here we see an increase and trend towards increased BDNF expression in the amygdala and hippocampus respectively. The majority of studies in GF mice show a decrease in various brain regions (Arentsen et al. 2015; Sudo et al 2004) however, others have shown increases (Neufeld et al., 2011; Stilling et al., 2015; Hoban et al., 2016). Regardless it is clear that dysregulated BDNF levels appear to be a hallmark of disturbed microbiota-gut brain axis. Recently we have shown a link between the ability of the microbiota to influence myelin gene expression and subsequently myelination (Hoban et al., 2016) in GF mice. However, when depleting the gut microbiota via antibiotics we did not see changes in prefrontal cortex
myelin gene expression. This could be due to timing of depletion. However, a recent study showing that in situations of demyelination, depleting the gut microbiota via antibiotics blunts the demyelination in these animals which could account for the absence of changes in our study (Gacias et al., 2016).

Future studies should additionally consider measuring levels of sex hormones including testosterone post antibiotic treatment. A previous study conducted fecal microbiota transplantation form male to female mice resulted in an increase in testosterone in females (Markle et al., 2013). Since it is apparent that the gut microbiota influences hormonal levels and testosterone has been shown to influence BDNF expression this measure could underlie some of our observed molecular changes (Allen et al., 2015; Purves-Tyson et al., 2015). Furthermore, given that there are marked sex differences in many of the parameters assessed future studies should also include both males and females; indeed previous work has shown sex-dependent differences in relation to the gut microbiota (Clarke et al., 2013; Kundakovic et al., 2013; Bolnick et al., 2014; Moloney et al., 2015a; Hoban et al., 2016).

Conclusion

In conclusion, our data confirms that multiple aspects of behavior continue to be dictated by the composition of the gut microbiota during adulthood. The current study suggests that long-term exposure to antibiotics in adulthood represents a period in which disturbance of the gut bacteria along with dysfunctional microbiota-gut-brain axis signaling can impact both brain and behavior. The data generated from this study demonstrates that chronic antibiotic treatment in rats represents a useful approach for investigating the impact of prolonged disturbance of the gut microbiota in adulthood. To our knowledge this study is among the first to demonstrate the impact of microbiota depletion in adulthood on both rat behaviour and brain neurochemistry and serves as a counterpoint to GF rodent-based studies which have significant drawbacks and limitations due to the extensive physiological systems critically impacted as a result of a lifetime without signals from the gut microbiota.
Figure Legends

Figure 1. Experimental time line. (A) Graphic depiction of experimental treatment including time and duration of antibiotic treatment pre- and post-commencement of behavioral testing. Elevated plus maze (EPM), open field (OF), novel object recognition (NOR), colorectal distension (CRD), forced swim test (FST) hot plate, Morris water maze (MWM). (B) Individual animal weights during the first 6 weeks of antibiotic treatment before behavioral testing commenced. Day 0 weights highlighted in blue depict initial animal weights the day before antibiotic treatment started. p<0.05*, p<0.01**

Figure 2. Effect of antibiotic treatment on hippocampal-based behavioral tests. No difference during training days (A) in time to find platform in antibiotic treated rats during MWM however, on probe day antibiotic treatment resulted in reduced time spent in the quadrant (B) that originally contained the platform. In NOR no difference in interaction time with novel or familiar object (C) was observed following antibiotic treatment nor was there any difference in ability to discriminate between objects (D). Bar graph data is represented as mean ± S.E.M (n=10 per group). * p<0.05 respectively compared to vehicle treated rats.

Figure 3. Antibiotic treatment altered responses to visceral pain but not somatic sensitivity. Higher mmHg was required to display notable pain behaviors (A) however; no change in total pain behaviors was noted in antibiotic treated rats (B) compared to vehicle controls. There was no difference in somatic pain sensitivity between groups following hot plate test (C). Data is represented as mean ± S.E.M. * p<0.05 respectively compared to vehicle treated rats.

Figure 4. Antibiotic treatment effect on depressive-like behaviours and corticosterone response to swim stress. (A) Antibiotic treatment significantly reduced the frequency of swimming episodes and increased the number of times to display immobility. No significant effect was noted in climbing episodes. (B) There were no differences noted in latency to first immobile phase. (C) Antibiotic treated rats had higher fecal output during the 6min forced swim trail. (D) No difference in corticosterone response to swim stress was observed in antibiotic treated rats. * indicates p<0.05 and *** indicated P<0.001 compared to vehicle treated rats.

Figure 5. Antibiotic treatment alters mRNA expression levels of stress related genes in the hippocampus and amygdala. Antibiotic treated rats displayed decreased Crhrl and Nr3cl (Glucocorticoid receptor, GR) gene expression in the hippocampus and amygdala (A,B). Within the amygdala, Bdnf mRNA levels were increased in antibiotic treated rats (B). No changes in myelin-related were detected in the prefrontal cortex (B) Pain-related genes were investigated in the spinal cord (lower lumbar sacral) (C). Statistically we did find a significant change in Slcla3 and TripV1. Bar graphs indicate average values in n=10 per group after b-actin normalization relative to average control levels (p<0.05 * and p<0.01**). Data graphed as ± SEM.
Figure 6. Plasma levels of L-Tryptophan and Kynurenine following antibiotic treatment. 
(A) Antibiotic treated rats had increased Tryptophan levels compared to vehicle treated animals. 
(B) No effect of antibiotic was noted in kynurenine levels nor ratio between kynurenine and tryptophan 
(C) in blood plasma. Data is represented as mean ± S.E.M. * p<0.05 respectively compared to VEH treated rats.

Figure 7. Antibiotic treatment altered faecal and caecal microbial contents. (A) Unweighted Principal Component Analysis (PCoA) of 4 week microbial contents of antibiotic and vehicle treated rats (beta diversity). (B) A doughnut plot representing the distribution at the Phylum level for the 4 week faecal samples. Each segment represents percentages of total reads for the individual phylum. The outer plot represents the vehicle treated phylum whereas the inner plot is antibiotic treated. Each segment is colour coded to represent individual phylum and is accompanied by a colour coded legend. (C,D) represents the 3 month cecal microbial content and phylum distribution.

Figure 8. Impact of antibiotic treatment on bacterial Phylum and Family. (A-H) Effect of antibiotic treatment on relative abundance of both Bacteroidetes and Firmicutes at both the phylum and family level in faecal (A-D) and caecal (E-H) samples. Data is represented as mean ±S.E.M. For statistical analysis corrections for multiple comparisons were made using the Benjammani-Hochberg method. ***P<0.001.

Table 1. Antibiotic treatment did not affect anxiety-related behaviors. Locomotion was unaffected for both groups as total distance moved in the arena was not different. Antibiotic treated rats did not move or spend more time in the center of the open field arena nor did they enter the center portion of the arena more when compared to vehicle treated rats. In EPM, antibiotic treated rats did not spend more or less time in the open and closed arms nor did they transition differently to the open or closed arm then their vehicle treated counterparts. Data is presented as mean ± S.E.M (n=10 per group).

Table 2. Summary of the effect of antibiotics on brain monoamine levels. Concentrations (ng/g of tissue) of noradrenalin (NA), dopamine (DA) and their precursor L-3,4-dihydroxyphenylalanine (L-DOPA), its metabolite homovanillic acid (HVA), and serotonin (5-HT), its metabolite 5-hydroxyindole acetic acid (5-HIAA) and both DA and 5-HT turnover in the prefrontal cortex, hippocampus, amygdala, hypothalamus and striatum of vehicle and antibiotic treated rats. Data is expressed as mean ± S.E.M. Within each row, *p<0.05 and ***p<0.001 vs same brain areas in antibiotic treated rats. NA: not available
Acknowledgments

We thank Dr. Gerard M Moloney and Mr. Patrick Fitzgerald for technical assistance with animal husbandry, tissue extraction, RNA extraction and qRT-PCR. The APC Microbiome Institute is a research centre funded by Science Foundation Ireland (SFI), through the Irish Government’s National Development Plan (Grant Number 12/RC/2273). TGD and JFC are also supported by the Irish Health Research Board, the Dept. of Agriculture, Food & the Marine and Enterprise Ireland. T.G.D., F.S. and J.F.C. are principal investigators in the APC Microbiome Institute, University College Cork. G.C. is a faculty member of the APC Microbiome Institute. The APC Microbiome Institute has conducted research funded by many Pharmaceutical & Food Companies. T.G.D. has been an invited speaker at meetings organized by Servier, Lundbeck, Janssen, and AstraZeneca and has received research funding from Mead Johnson, Cremo, Suntory Wellness, Nutricia and 4D Pharma. J.F.C. has been an invited speaker at meetings organized by Mead Johnson, Yakult, Alkermes, and Janssen and has received research funding from Mead Johnson, Cremo, Suntory Wellness, Nutricia and 4D Pharma.
References


Stilling RM, Bordenstein SR, Dinan TG, Cryan JF (2014a) Friends with social benefits: host-


Table 1. Effect of antibiotics on behavioural response in OF and EPM.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle n=10</th>
<th>Antibiotic n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open Field (OF)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locomotion (total Distance) (cm)</td>
<td>5904.0±355.2</td>
<td>5572.0±603.0</td>
</tr>
<tr>
<td>Distance in Center (cm)</td>
<td>9.27±3.34</td>
<td>8.14±2.29</td>
</tr>
<tr>
<td>Time in Center (s)</td>
<td>82.92±26.77</td>
<td>125.20±24.68</td>
</tr>
<tr>
<td>No. Transition to Center</td>
<td>2.89±0.96</td>
<td>2.66±0.72</td>
</tr>
<tr>
<td><strong>Elevated Plus Maze (EPM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time in Closed arm (s)</td>
<td>81.90±21.06</td>
<td>56.10±13.32</td>
</tr>
<tr>
<td>Time in Open arm (s)</td>
<td>167.10±22.34</td>
<td>117.30±18.52</td>
</tr>
<tr>
<td>Time in Center (s)</td>
<td>51.00±9.07</td>
<td>66.60±9.82</td>
</tr>
<tr>
<td>No. Transition to Open arm</td>
<td>4.90±1.29</td>
<td>4.40±0.93</td>
</tr>
<tr>
<td>No. Transition to Closed arm</td>
<td>9.70±0.97</td>
<td>9.30±0.77</td>
</tr>
</tbody>
</table>

No significant effect of antibiotic exposure on anxiety-related behaviours was observed.
Table 2. Effect of antibiotics on brain monoamine levels.

<table>
<thead>
<tr>
<th></th>
<th>Prefrontal Cortex</th>
<th>Hippocampus</th>
<th>Amygdala</th>
<th>Hypothalamus</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle (n=10)</td>
<td>Antibiotic (n=10)</td>
<td>Vehicle (n=10)</td>
<td>Antibiotic (n=10)</td>
<td>Vehicle (n=10)</td>
</tr>
<tr>
<td>LDOPA</td>
<td>204.5±17.4</td>
<td>412.6±30.2***</td>
<td>296.4±46.5</td>
<td>764.1±78.3***</td>
<td>NA</td>
</tr>
<tr>
<td>DA</td>
<td>61.9±7.7</td>
<td>92.6±23.7</td>
<td>NA</td>
<td>NA</td>
<td>61.3±17.1</td>
</tr>
<tr>
<td>HVA</td>
<td>195.9±28.9</td>
<td>348.8±34.6**</td>
<td>366.9±46.5</td>
<td>118.3±29.1***</td>
<td>140.5±25.8</td>
</tr>
<tr>
<td>5-HT</td>
<td>671.3±33.4</td>
<td>641.1±15.3</td>
<td>505.8±28.6</td>
<td>403.0±30.8*</td>
<td>580.3±54.4</td>
</tr>
<tr>
<td>HVA/DA</td>
<td>3.42±1.0</td>
<td>4.156±0.835</td>
<td>NA</td>
<td>NA</td>
<td>2.129±0.609</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>0.643±0.025</td>
<td>0.627±0.029</td>
<td>1.034±0.038</td>
<td>1.373±0.069**</td>
<td>0.741±0.039</td>
</tr>
</tbody>
</table>
A

10 Weeks Old

Antibiotic treatment

Week 1

Week 6

**

Week 7

Week 8

Week 10

Week 11

Week 12

Week 13

EPM

OF

NOR

CRD

FST (with Stress Bleeds)

Hot Plate

MWM

Tissue Collection (7 days of no behaviour before culls)

B

0 3 6 9 12 15 18 21 24 27 30 33 36 39 42

0

50

250

300

350

400

Weight (g)

Days

Vehicle n=10

Antibiotic n=10

**

*
**Forced Swim Test Behaviours**

- **Vehicle n=10**
- **Antibiotic n=10**

<table>
<thead>
<tr>
<th>Behaviours</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobility</td>
<td>25 (Vehicle) 30 (Antibiotic)</td>
</tr>
<tr>
<td>Climbing</td>
<td>5 (Vehicle) 10 (Antibiotic)</td>
</tr>
<tr>
<td>Swimming</td>
<td>15 (Vehicle) 20 (Antibiotic)</td>
</tr>
</tbody>
</table>

**Stress Induced Faecal Output**

- **Number of fecal pellets**
  - Vehicle: 1.5 ± 0.5
  - Antibiotic: 3.5 ± 0.5

**Stress Induced Corticosterone**

- **Corticosterone (ng/ml Serum)**
  - Vehicle: 200 ± 50
  - Antibiotic: 300 ± 50

---

*Significant difference (p < 0.05) between groups.*
### A

**Hippocampus**
- Stress Related Genes
- Serotonin Related Genes

### B

**Amygdala**
- Stress Related Genes

**Prefrontal Cortex**
- Myelin Related Genes

### C

**Spinal Cord**
- Pain Related Genes

---

- **Slcla3**, **Slcla2**, **mGluR7**, **mGluR4**, **mGlur1**, **mGlur5**, **Grin1**, **Grin2b**, **TripV1**, **Tlr4**
- **Nr3c1**, **Nr3c2**, **Crhr1**, **Crhr2**, **Bdnf**, **Tph2**, **Slc6a4**, **Htr2b**, **Htr1a**, **Htr2c**
- **Nr3c1**, **Nr3c2**, **Crhr1**, **Crhr2**, **Bdnf**, **Mog**, **Mag**, **Plp1**, **Mobp**, **Mbp**
- **Slc6a4**, **Htr1a**, **Htr2b**, **Tlr4**

#### Relative mRNA levels (AU)

- **Vehicle**
- **Antibiotic**

---

**p=0.050**
Highlights

- Chronic antibiotic treatment affects visceral pain and CNS monoamine levels
- Distinct behavioural profile after microbiota depletion
- No change in anxiety and HPA axis