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Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour

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ABSTRACT

Background: There is growing appreciation for the importance of bacteria in shaping brain development and behaviour. Adolescence and early adulthood are crucial developmental periods during which exposure to harmful environmental factors can have a permanent impact on brain function. Such environmental factors include perturbations of the gut bacteria that may affect gut–brain communication, altering the trajectory of brain development, and increasing vulnerability to psychiatric disorders. Here we assess the effects of gut bacterial depletion from weaning onwards on adult cognitive, social and emotional behaviours and markers of gut–brain axis dysfunction in mice. *Methods:* Mice were treated with a combination of antibiotics from weaning onwards and effects on behaviours and potential gut-brain axis neuromodulators (tryptophan, monoamines, and neuropeptides) and BDNF expression were assessed in adulthood. *Results:* Antibiotic-treatment depleted and restructured gut microbiota composition of caecal contents and decreased spleen weights in adulthood. Depletion of the gut microbiota from weaning onwards reduced anxiety, induced cognitive deficits, altered dynamics of the tryptophan metabolic pathway, and significantly reduced BDNF, oxytocin and vasopressin expression in the adult brain. *Conclusions:* Microbiota depletion from weaning onwards by means of chronic treatment with antibiotics in mice impacts on anxiety and cognitive behaviours as well as key neuromodulators of gut–brain communication in a manner that is similar to that reported in germ-free mice. This model may represent a more amenable alternative for germ-free mice in the assessment of microbiota modulation of behaviour. Finally, these data suggest that despite the presence of a normal gut microbiome in early postnatal life, reduced abundance and diversity of the gut microbiota from weaning influences adult behaviours and key neuromodulators of the microbiota–gut–brain axis suggesting that dysregulation of this axis in the post-weaning period may contribute to the pathogenesis of disorders associated with altered anxiety and cognition.

1. Introduction

Studies in germ-free mice have been crucial to our growing understanding of specific health benefits conferred by the commensal microbiota on host physiology, and more recently on brain function and development (Stilling et al., 2014). Specifically, the absence of bacteria from birth not only alters immune system function and our capacity to fight infection (Hapfelmeier et al., 2010), but also interferes with normal brain function and behaviours including anxiety (Diaz Heijtz et al., 2011; Neufeld et al., 2011; Clarke et al., 2013), sociability (Desbonnet et al., 2014), and memory (Gareau et al., 2011). Clinical studies reporting altered composition of the microbiota in disorders such as depression (Naseribafrouei et al., 2014; Dinan and Cryan, 2013), irritable bowel syndrome (Kennedy et al., 2014; Bonfrate et al., 2013; Jeffery et al., 2012), and autism (Finegold et al., 2010; Finegold, 2011; Mayer et al., 2014) give further credence to the theory that equilibrium of the microbial milieu in the gut, based on the specific nature, relative abundance and diversity of the various microbe

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constituents, is essential to brain development in the host. These findings have also opened up new avenues of opportunity for the development of effective therapies not only for disorders affecting the gut (Clarke et al., 2012), but also in the treatment of psychiatric conditions (Dinan, 2013).

Adolescence and early adulthood are important periods for brain development (Sturman and Moghaddam, 2011; O'Connor and Cryan, 2014). Significant changes relating to neuronal architecture and function occur during adolescence that, from an evolutionary perspective, promote the maturation of behaviours and skills such as social and cognitive capabilities necessary to achieve independence from the secure family environment (Burnett et al., 2011). Environmental events have the capacity to shape and mould neuronal architecture and implement adaptations in brain function that in theory better equip an individual to cope with their environmental challenges. Nevertheless, aversive environmental factors experienced by an individual during this window of vulnerability can also result in maladaptive changes in brain development; the qualitative and quantitative nature of which depends on various factors (Spear, 2011). Hence, it is not surprising that many adult neuropsychiatric disorders, particularly schizophrenia, have their roots in this vulnerable period (Paus et al., 2008). The gut microbiota through associated metabolic and immune activities, afford significant advantages to the host throughout development (Selkrig et al., 2014). Recent studies have focused on the deleterious effects of early life antibiotic exposure on various health outcomes (Cho et al., 2012; Cox et al., 2014). To our knowledge, changes in brain development and behaviour as a result of gut microbiota depletion from early adolescence specifically, have not been assessed to date.

In this study we aim to examine the specific contributions of the microbiota from weaning onwards to brain development and behaviours by depleting the gut microbiota using a combination of antibiotics administered in high doses in the drinking water of mice. Thus by examining the effects of gut microbiota depletion on stress-responsivity, emotional and cognitive behaviours and neurochemical measures relevant to the microbiota–gut–brain axis (monoamines, tryptophan, kynurenine, BDNF, neuropeptides), we can assess the specific role bacteria play in brain development from weaning onwards.

2. Methods

2.1. Animals

First-generation offspring from NIH Swiss breeding pairs obtained from Harlan (UK) were used in all experiments. NIH Swiss mice were housed 4–5/cage in standard mouse cages in our barrier laboratory animal housing facility under a strict 12-h light/dark cycle. Antibiotic-treated and control mice received the same autoclaved pelleted diet (Special Diet Services, product code 801010). All mice were tested in adulthood (postnatal day 55–80). Experiments were conducted in accordance with the European Directive 86/609/EEC and the Recommendation 2007/526/65/EC, and were approved by the Animal Experimentation Ethics Committee of University College Cork.

2.2. Antibiotic treatment

To deplete the gut microbiota in adolescence [postnatal day (P) 21 onwards], a combination of antibiotics was chosen based on a previous report that this combination reduced the faecal bacterial DNA load by 400-fold while ensuring the animals' health (Reikvam et al., 2011). To avoid any confounding effects resulting from chronic stress induced by oral gavage (Branchi et al., 2005), antibiotics were administered in the drinking water and bottles were changed every second day. The dose of antibiotics was adjusted according to the mean volume of water consumed per mouse on each day. Water was autoclaved and water intake was monitored daily for the first week to adjust antibiotic dose and ensure the health of the animals, and subsequently monitored twice a week until termination of the study. The antibiotic cocktail consisted of ampicillin (1 mg/ml), vancomycin (5 mg/ml), neomycin (10 mg/ml), metronidazol (10 mg/ml), and supplemented with amphotericin-B (0.1 mg/ml). Groups consisted of non-treated control males ($n = 14$), and antibiotic-treated males ($n = 15$).

2.3. Body/tissue weight

To monitor the general health of the animals, body weights were recorded twice a week until commencement of the behavioural tests and also on the day of sacrifice. Post-mortem weights of spleen and adrenal glands were also measured and spleen/body and adrenal/body weight ratios were calculated for each mouse.

2.4. Behavioural assessments

Behavioural testing commenced approximately 4 weeks following the first dose of antibiotics (postnatal day 55) and where possible behaviour was scored using automated Ethovision video tracking software (XT 7.0; Noldus, The Netherlands). Details relating to the behavioural tests are provided in the Supplementary methods. Exploration and non-spatial cognition was assessed in the novel object recognition test. Anxiety was assessed using the light/dark box test by measuring time spent in the light chamber and faecal pellet excretion. The social transmission of food preference (Clipperton et al., 2008) test was adopted to assess social interaction and social memory in mice. Behavioural tests were conducted in the order above over a 2 week period in adulthood (postnatal day 55–70) with a 1 week interval between testing to minimise any stress associated with repeated testing. Antibiotic treatment was continued throughout testing (Fig. 1).

2.5. Acute restraint stress and tissue dissection

The absence of bacteria in mice is reported to increase the neuroendocrine response to acute restraint stress in adulthood (Sudo et al., 2004). Therefore, the effects of antibiotic-induced microbiota depletion on the corticosterone response to acute stress were assessed. One week after behavioural testing (postnatal day 77–80) and 30 min before sacrifice, antibiotic-treated and control mice were assigned to non-stress and stress groups such that half of each treatment group was subjected to a 30 min restraint stress to assess effects of antibiotic treatment on corticosterone response to an acute stressor. Mice were sacrificed, blood samples were taken and brains were dissected as described in the Supplementary methods.

2.6. Corticosterone assay

Serum corticosterone levels were assayed in duplicate using a Corticosterone Immunoassay Kit according to the manufacturers' instructions (Enzo Life Sciences, UK). The assay has a sensitivity of 26.99 pg/ml.

2.7. DNA extraction and high throughput DNA sequencing

Total DNA was isolated from caecal contents and processed for analysis of microbiota composition in line with 454 protocols at the Teagasc high throughput sequencing centre as described in Supplement 1. Phylum counts for each sample were extracted from

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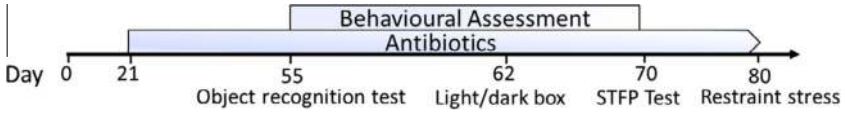

Fig. 1. Experimental timeline showing when antibiotic treatment commenced, and the order of procedures performed in adult mice.

MEtaGenome ANalyzer (MEGAN). α and β diversity indices and rarefaction curves were generated using Qiime. A phylogenetic tree was calculated using the FastTree software and the resulting principal coordinate analysis was visualised within KiNG.

2.8. Brain mRNA quantification

Dissected brain tissue was placed in RNAlater (Sigma–Aldrich, Ireland) and then stored at −70 °C. Details of RNA isolation and RT-PCR are provided in the Supplementary methods. BDNF mRNA was assayed in the hippocampus and oxytocin, corticotrophin-releasing factor (CRF), and vasopressin were assayed in the hypothalamus. Relative quantification was analysed with the 7300 system SDS software mRNA expression was measured as fold change relative to non-treated.

2.9. High performance liquid chromatography (HPLC)

The monoamines noradrenaline (NA), serotonin (5-HT) and dopamine, and metabolites of these neurotransmitters including, 5-hydroxyindole acetic acid (5-HIAA), L-3,4-dihydroxyphenylalanine (L-DOPA), and dihydroxyphenylacetic acid (DOPAC) were measured in the frontal cortex, amygdala, and hippocampus by HPLC coupled to electrochemical detection as described previously (Desbonnet et al., 2008). Concentrations were calculated as ng/g of fresh tissue weight.

Tryptophan, kynurenine and kynurenic acid concentrations were assayed in serum samples by HPLC coupled to UV and fluorescent detection as described previously (Fitzgerald et al., 2008). Concentrations were calculated as ng/ml of serum.

2.10. Statistics

Multifactorial and repeated measures analysis of variance was employed to assess group differences (main factors: antibiotic treatment, stress and time) as appropriate using SPSS 20.0. Unpaired *t*-tests were used to determine differences between treatment groups for behaviour and tissue weight ratios and pyrosequencing data. Statistically significant main effects were followed with *post hoc* comparisons using the Least Significant Difference test. A two-tailed *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Antibiotic water consumption

There was no effect of antibiotic treatment on water consumption in mice (see Fig. 2).

3.2. Body/tissue weights

Body weights did not differ between treatment groups prior to treatment at P21. Both groups gained weight from weaning (P21) through to adulthood (P75) [time: $F(9, 27) = 21.61$, $p < 0.001$]. Only in the initial period did antibiotic treatment reduce body weight relative to their non-treated counterparts

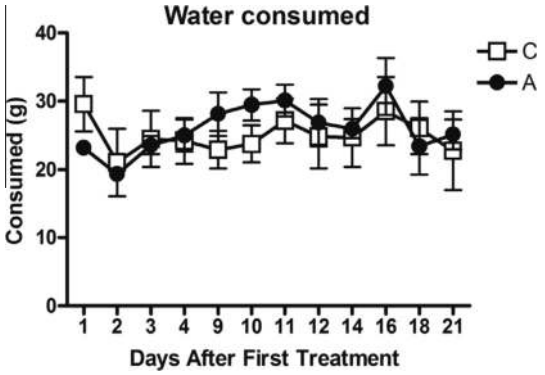

Fig. 2. Effect of antibiotic treatment on mean water consumption per mouse as measured on specific days during the first 3 weeks of treatment. Data are expressed as means ± S.E.M, $n = 14–15$ per group. Statistical analysis showed no difference in mean water consumption between control (C) and antibiotic-treated (A) groups.

[time × antibiotic: $F(9, 27) = 3.77$, $p < 0.001$, Supplementary Fig. 1A], particularly on P34 ($p < 0.01$) and P37 ($p < 0.05$).

Postmortem spleen/body weight ratios were reduced following antibiotic treatment [$t = 4.2$, $p < 0.0005$, Supplementary Fig. 1B]. There were no significant effects of antibiotic treatment on postmortem adrenal/body weight ratios.

3.3. Novel object recognition test

Both groups habituated to the testing apparatus over the 3 sessions on day 1. Total exploration of objects did not differ as a result of antibiotic treatment (Fig. 3A). Antibiotic treatment diminished the ability of mice to discriminate between a novel and a familiar object in this test [$t = 3.4$, $p < 0.05$, Fig. 3B].

3.4. Light/dark box test

Antibiotic-treated mice excreted more faecal pellets relative to non-treated mice [$t = 2.3$, $p < 0.05$, Fig. 3C]. Antibiotic treatment significantly increased time spent by the mouse in the light chamber [$t = 2.6$, $p < 0.05$, Fig. 3D]. Latency to enter the light chamber and transitions between light and dark chambers were not altered by antibiotic treatment.

3.5. Social transmission of food preference test

The effects of antibiotic treatment on food preference differed when tested immediately after the social interaction period and 24 h later [antibiotic × time: $F(1, 27) = 5.08$, $p < 0.05$]. Mice treated with antibiotics exhibited a similar preference for the cued food to non-treated mice when tested immediately after social interaction (Fig. 3E), but showed a reduced preference for the cued food when tested 24 h later [$t = 2.5$, $p < 0.05$, Fig. 3F].

3.6. Corticosterone

The experience of an acute restraint stress (30 min) prior to sacrifice induced an increase in serum corticosterone concentrations [$F(1, 27) = 6.18$, $p < 0.05$]. There was no difference in corticosterone concentrations between antibiotic-treated and non-treated mice following stress or at baseline (data not shown).

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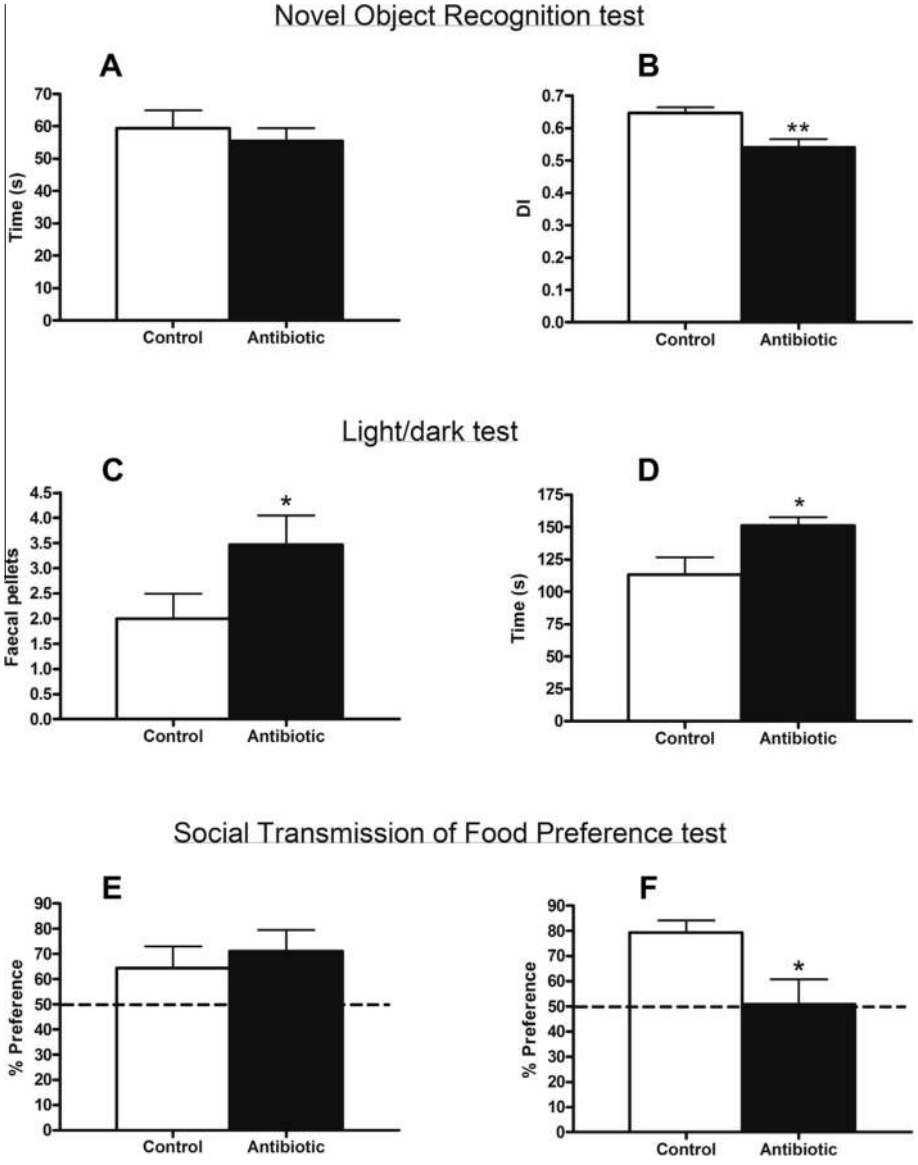

Fig. 3. Effect of antibiotic treatment from weaning on adult behaviour including time spent exploring objects (A) and discrimination index (B) during the test trial of the novel object recognition test; faecal pellet excretion (C) and time spent in the light chamber (D) during the light–dark test; and preference for the cued food immediately after social interaction with the demonstrator mouse (E) and preference for the cued food 24 h after social interaction with the demonstrator mouse (F) in the social transmission of food preference test. Groups consisted of non-treated control ($n = 14$), antibiotic-treated ($n = 15$). Data are expressed as means ± S.E.M. Statistical differences between groups were determined using Unpaired t-tests; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control mice.

3.7. High throughput DNA sequencing

Microbiota diversity of caecal contents was affected by antibiotic treatment (Supplementary Fig. 2); antibiotics reduced the number of observed bacterial species [$F(1, 25) = 26.47$, $p < 0.0001$, Fig. 4A], the phylogenetic diversity [$F(1, 25) = 29.54$, $p < 0.0001$, Fig. 4B] and species richness (Chao1) [$F(1, 25) = 34.56$, $p < 0.0001$, Fig. 4C] in caecal contents of mice. There was a significant interaction between stress and antibiotic treatment; while acute stress increased bacterial species number and diversity measures in the non-treated mice, it further reduced the number of observed bacterial species [stress × antibiotic: $F(1, 25) = 6.98$, $p < 0.02$], phylogenetic diversity [stress × antibiotic: $F(1, 25) = 7.11$, $p < 0.02$], and species richness (Chao1) [stress × antibiotic: $F(1, 25) = 4.62$, $p < 0.05$] in antibiotic-treated mice. Shannon diversity data revealed a similar reduction in biodiversity as a result of antibiotic treatment [$F(1, 25) = 63.84$, $p < 0.0001$, Fig. 4D]. The Simpson diversity data, which takes into account the number of species present as well as the relative abundance of each species, showed a similar pattern as a result of antibiotic treatment; antibiotics reduced this diversity measure [$F(1, 25) = 27.43$, $p < 0.0001$, Fig. 4E] but was not affected by stress.

Analyses of the relative abundance of detected bacteria within each sample at a phylum level revealed a decrease in the abundance of Firmicutes [$F(1, 25) = 9.9$, $p < 0.005$] and Bacteroidetes [$F(1, 25) = 39.9$, $p < 0.0001$] and an increased abundance of Proteobacteria [$F(1, 25) = 48.0$, $p < 0.0001$] and Cyanobacteria [$F(1, 25) = 30.8$, $p < 0.0001$] in antibiotic treated mice (Fig. 5). Consistent with the decreases in Firmicutes and Bacteroidetes, at the family level antibiotic treatment reduced relative abundances of Prevotellaceae [$t = 6.3$, $p < 0.0001$], Rikenellaceae [$t = 5.7$, $p < 0.0001$] and Incertae Sedis XI [$t = 4.2$, $p < 0.0005$; Supplementary Fig. 3].

The effect of acute stress on phylum Bacteroidetes differed between control and non-treated groups [antibiotic × stress: Bacteroidetes, $F(1, 25) = 4.2$, $p < 0.05$, Fig. 6A]. There was no effect

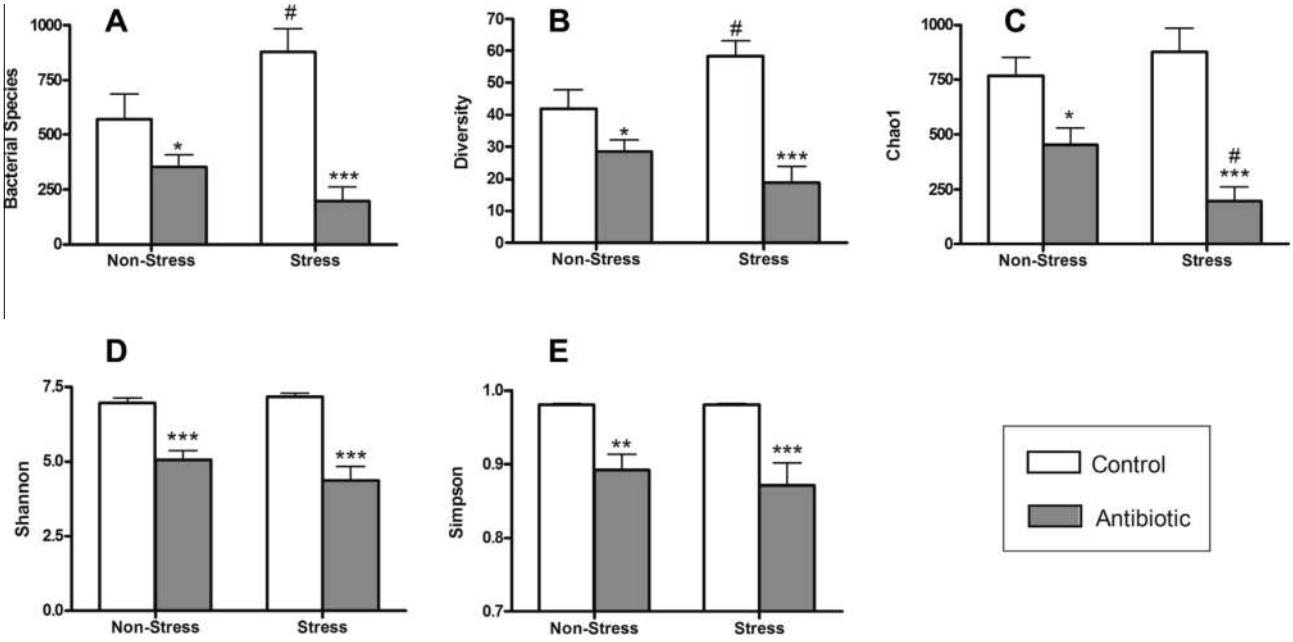

Fig. 4. Effects of antibiotic treatment from weaning and acute restraint stress on bacterial diversity and richness measures in caecal contents of male mice on conclusion of experiments; including number of observed bacterial species (A), phylogenetic diversity (B), Chao1 (C), Shannon (D), and Simpson (E). Data are expressed as means ± S.E.M, $n = 7–8$ per group. Statistical differences between groups were determined using GLM analysis with Least Significant Difference *post hoc* test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$ (antibiotic effect), $^{\#}p < 0.05$ (stress effect).

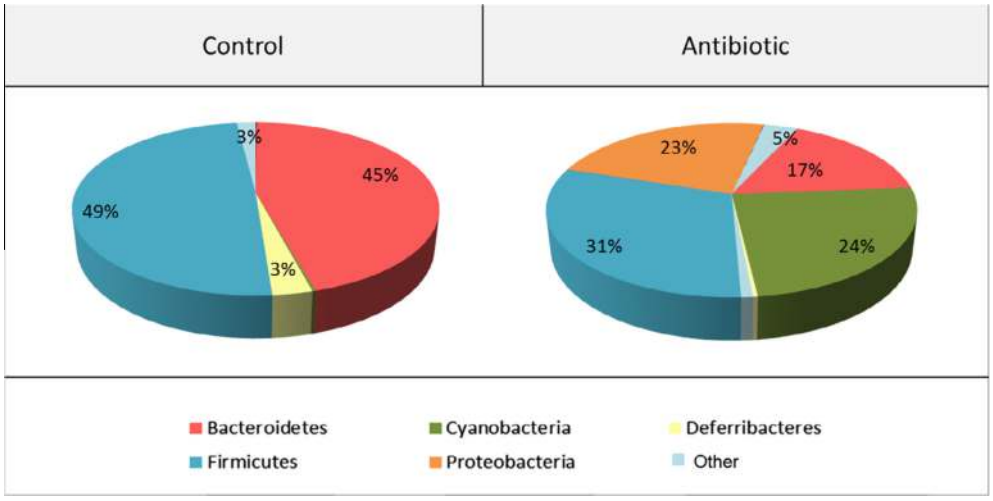

Fig. 5. Effects of antibiotic treatment during adolescence on microbiota distribution at a phylum level in caecal contents of mice on conclusion of experiments. The pie charts represent percentage of total reads for the corresponding colour coded phylum ($n = 14–15$ per group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of stress on the relative abundance of other bacterial phyla. At a family level, acute stress increased Rikenellaceae in non-treated mice [stress × antibiotic: $F(1, 25) = 4.5$, $p < 0.05$] whereas stress had no effect on Rikenellaceae in antibiotic-treated mice (Fig. 6B).

3.8. Serum tryptophan and metabolites

Concentrations of tryptophan, kynurenine, and both the kynurenic acid/kynurenine and kynurenine/tryptophan ratios were measured in serum samples of antibiotic-treated and non-treated males. Antibiotic treatment increased tryptophan [$F(1, 25) = 16.4$, $p < 0.0005$, Fig. 7A] and reduced kynurenine levels [$F(1, 25) = 41.3$, $p < 0.0001$, Fig. 7B] relative to controls. The kynurenic acid/kynurenine ratio significantly increased [$F(1, 25) = 10.7$, $p < 0.005$, Fig. 7C] and the kynurenine/tryptophan ratio decreased [$F(1, 25) = 73.0$, $p < 0.0001$, Fig. 7D] as a result of antibiotic treatment. Acute stress reduced tryptophan concentrations [$F(1, 25) = 17.5$, $p < 0.0005$] and increased the kynurenine/tryptophan ratio [$F(1, 25) = 9.0$, $p < 0.01$] equally in control and antibiotic treated mice; antibiotic treatment did not significantly alter stress effects on tryptophan or its metabolic pathway.

3.9. Hippocampus BDNF

Antibiotic treatment reduced BDNF mRNA expression in the hippocampus [$F(1, 25) = 5.13$, $p < 0.05$, Fig. 8A]. There was no effect of acute stress on hippocampus BDNF levels.

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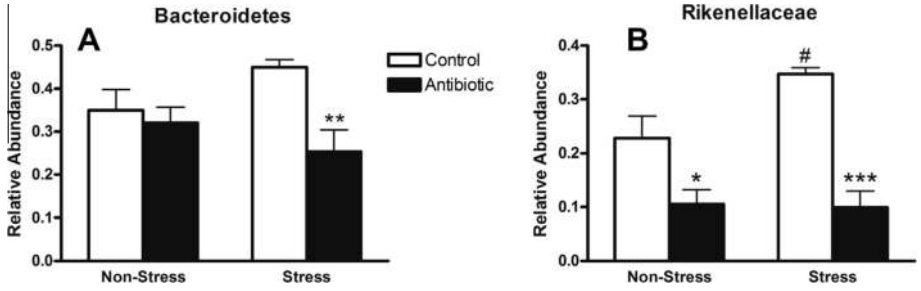

Fig. 6. Effects of acute stress in control and antibiotic-treated mice on relative expression of Bacteroidetes at a phylum level (A) and *Rikenellaceae* at a family level (B) in caecum contents of mice on conclusion of experiments. Data are expressed as means ± S.E.M, $n = 7–8$ per group. Statistical differences between groups were determined using GLM analysis with Least Significant Difference *post hoc* test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.01$ (antibiotic effect); $^{\#}p < 0.05$ (stress effect).

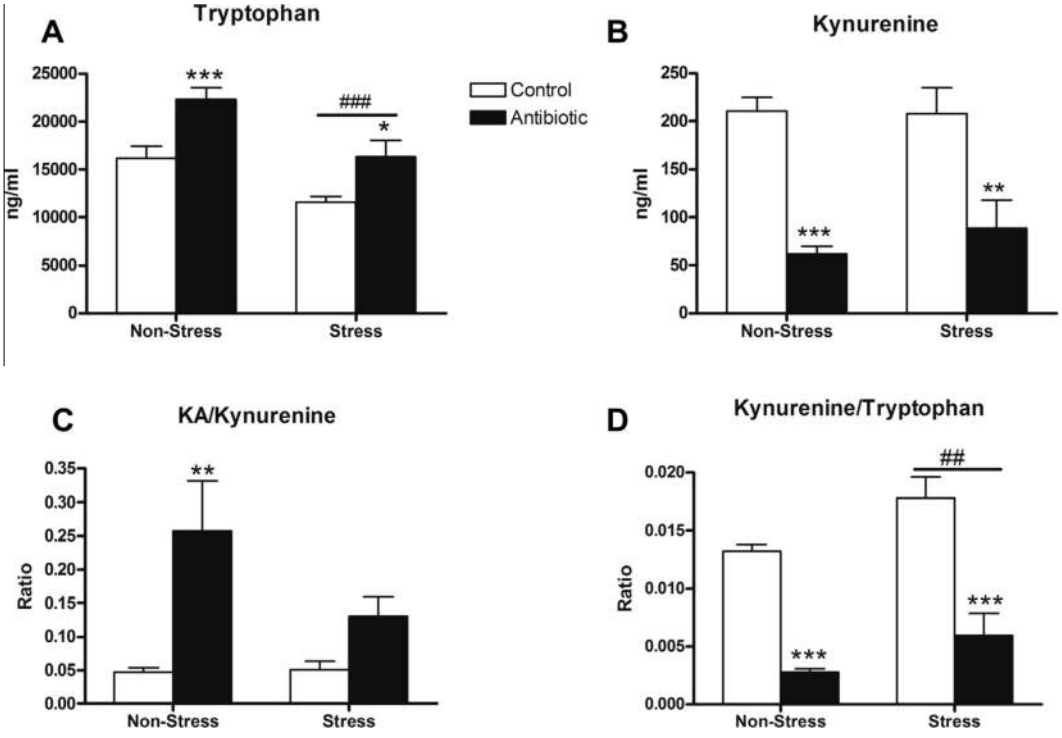

Fig. 7. Effect of antibiotic treatment from weaning and acute restraint stress on concentrations of tryptophan (A), kynurenine (B), the kynurenic acid (KA)/kynurenine ratio (C) and the kynurenine/tryptophan ratio (D) in serum at time of sacrifice. Data are expressed as means ± S.E.M, $n = 7–8$ per group. Statistical differences between groups were determined using GLM multifactor analysis with Least Significant Difference *post hoc* test; $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0001$ (antibiotic effect); $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (stress effect).

3.10. Hypothalamus neuropeptides

Antibiotic treatment had no effect on CRF mRNA expression (Fig. 8B) but significantly reduced oxytocin mRNA [antibiotic: $F(1, 25) = 6.28$, $p < 0.02$, Fig. 8C] and vasopressin mRNA [antibiotic: $F(1, 25) = 37.78$, $p < 0.0001$, Fig. 8D] expression in the hypothalamus. There was no effect of acute stress on mRNA expression of these neuropeptides in either antibiotic-treated or non-treated mice.

3.11. Brain monoamines

3.11.1. Hippocampus

Antibiotic treatment increased noradrenaline [$F(1, 25) = 7.96$, $p < 0.01$] and 5-HIAA [$F(1, 25) = 21.75$, $p < 0.0001$] levels; this increase in 5-HIAA was specific to non-stressed mice [antibiotic × stress: $F(1, 25) = 4.76$, $p < 0.05$, Table 1]. Levels of 5-HIAA [$F(1, 25) = 26.95$, $p < 0.0001$] and the 5-HIAA/serotonin ratio [$F(1, 25) = 13.26$, $p < 0.002$] were elevated in acutely-stressed mice. There was no effect of antibiotics on serotonin or the 5-HIAA/serotonin ratio in the hippocampus.

3.11.2. Prefrontal cortex

Acute stress induced an increase in 5-HIAA [$F(1, 25) = 9.71$, $p < 0.005$] and the 5-HIAA/serotonin ratio [$F(1, 25) = 24.1$, $p < 0.0001$] in both vehicle and antibiotic-treated groups (Table 1). Antibiotic treatment had no effect on concentrations of serotonin, dopamine or their metabolites in this brain area in male mice.

3.11.3. Amygdala

Antibiotic treatment increased levels of L-DOPA [$F(1, 25) = 25.49$, $p < 0.0001$] and HVA [$F(1, 25) = 8.36$, $p < 0.005$] in the amygdala. There was no effect of antibiotic treatment on serotonin, noradrenaline or dopamine concentrations. Acute stress also increased HVA [$F(1, 25) = 5.50$, $p < 0.05$], particularly in the antibiotic-treated group ($p < 0.05$).

Table 1
Effects of antibiotic treatment during adolescence and acute restraint stress on concentrations of monoamines and monoamine metabolites in the hippocampus, prefrontal cortex (Cx) and amygdala. Data are expressed as means ± S.E.M, $n = 7–8$ per group. Statistical differences between groups were determined using GLM multifactor analysis with Least Significant Difference *post hoc* test; $^{*}p < 0.01$, $^{**}p < 0.0001$ (antibiotic effect); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (stress effect).

	Control		Antibiotic	
	Non-Stress	Stress	Non-Stress	Stress
Hippocampus				
Noradrenaline	831 ± 52	801 ± 82	894 ± 51*	1111 ± 76*
Dopamine	67 ± 12	82 ± 10	81 ± 7	103 ± 8
Serotonin	656 ± 68	699 ± 38	834 ± 77	721 ± 33
5-HIAA	367 ± 43	665 ± 46##	548 ± 50	684 ± 45
5-HIAA/5-HT	0.58 ± 0.07	0.84 ± 0.13	0.67 ± 0.05	0.95 ± 0.04#
Prefrontal Cx				
Dopamine	758 ± 235	1195 ± 372	787 ± 380	1115 ± 504
Serotonin	901 ± 110	1272 ± 158	1048 ± 52	1015 ± 152
5-HIAA	334 ± 89	500 ± 36	320 ± 23	486 ± 48
5-HIAA/5-HT	0.27 ± 0.03	0.42 ± 0.05#	0.31 ± 0.02	0.49 ± 0.02##
Amygdala				
Noradrenaline	389 ± 62	508 ± 72	538 ± 42	571 ± 62
Dopamine	926 ± 226	761 ± 253	1138 ± 352	1619 ± 480
Serotonin	1170 ± 206	1118 ± 141	1098 ± 136	1129 ± 65
L-DOPA	154 ± 21	157 ± 24	244 ± 14**	283 ± 27**
HVA	310 ± 53	371 ± 56	405 ± 44*	636 ± 98*##

4. Discussion

Since the seminal discovery of the first antibiotic in 1928 the ability to eliminate bacteria and treat infection has undoubtedly improved disease morbidity and human mortality (Fleming, 1929). Yet the growing appreciation of the health benefits conferred by non-pathogenic bacterial populations in our external and internal environments has led us to re-evaluate the conventional view that all bacteria are harmful (Ubeda and Pamer, 2012). The findings of the current study support this viewpoint and suggest that significant bacterial depletion from early adolescence and restructuring of the depleted microbiota populations in the gut can alter brain development and behaviour.

In this study antibiotic treatment from weaning induced significant diminution of microbial populations and taxonomic diversity in the adult mouse gut, which is consistent with previous reports using similar antibiotic regimens in adult mice (Cho et al., 2012; Reikvam et al., 2011; Verdu et al., 2006; Bercik et al., 2011; Zhang et al., 2014). Analysis of remaining gut bacteria in antibiotic-treated mice revealed a significant restructuring of the microbial community characterised by a decrease in the richness of bacterial species. Although the germ-free mouse construct provides an optimal means of assessing long-term effects of the absence of bacteria on brain development, it is not without its limitations; in particular behavioural assessment of these mice is restricted due to required housing of the mice in sterile isolator units to maintain germ-free conditions. Thus, treatment with the current regimen of high-doses antibiotics may provide a more amenable and cost-effective model for investigations of bacterial depletion effects on behaviour. These effects of antibiotics on biodiversity and richness of the gut microbiome are complemented by analyses at a phylum level showing a decreased relative abundance of Firmicutes and Bacteroidetes and increased abundance of Cyanobacteria and Proteobacteria. Considering that specific probiotic treatments can ameliorate depressive-like behaviours (Desbonnet et al., 2010; Bravo et al., 2011), anxiety and communication deficits (Hsiao et al., 2014) in rodents, it is not surprising that the compositional differences in the gut microbiome between antibiotic-treated and non-treated mice is also reflected in the varied effects of antibiotics on behaviour in the current study. Acute stress had opposing effects on the number of bacterial species and bacterial diversity measures in non-treated and antibiotic-treated mice, reducing these measures in the latter group. The relative abundance of bacteria in phylum Bacteroidetes decreased in antibiotic-treated mice following stress exposure, whereas at a family level, stress selectively

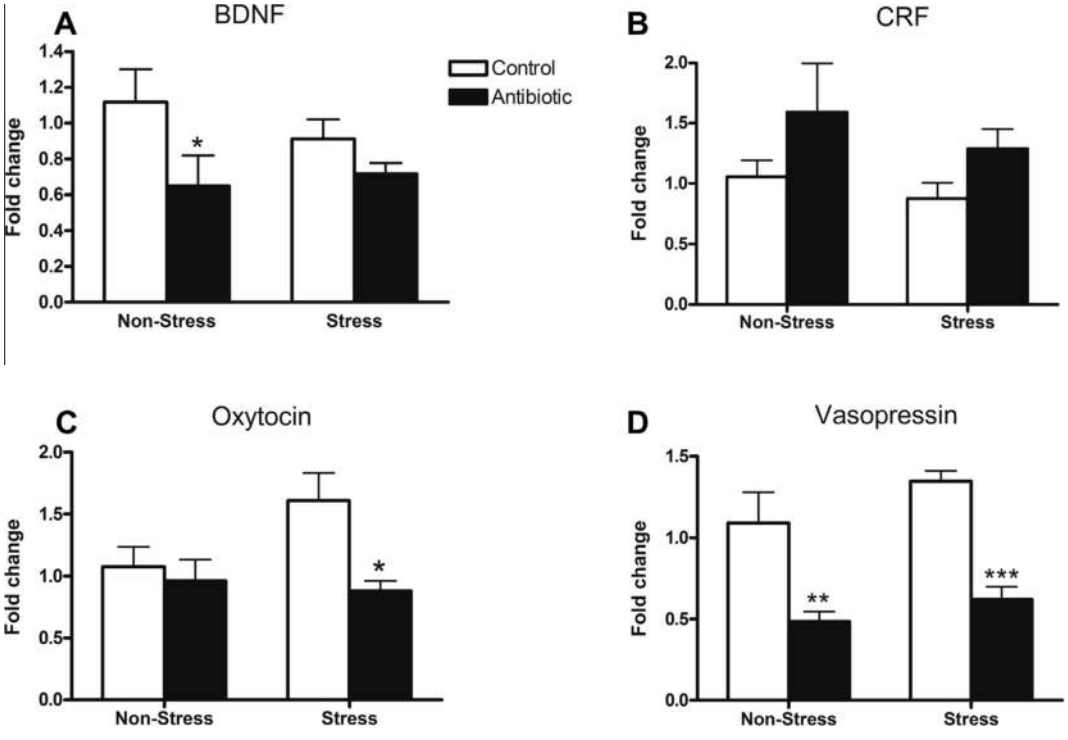

Fig. 8. Effects of antibiotic treatment from weaning and acute restraint stress on mRNA expression of BDNF in the hippocampus (A), and corticotrophin-releasing factor (CRF) (B), oxytocin (C) and vasopressin mRNA (D) in the hypothalamus. Data are expressed as means ± S.E.M, $n = 7–8$ per group. Statistical differences between groups were determined using GLM analysis with Least Significant Difference *post hoc* test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$ (antibiotic effect).

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increased Rikenellaceae in non-treated control mice. Changes in the relative abundance of Bacteroidetes have been previously reported in response to chronic psychosocial stress exposure in mice (Bailey et al., 2011) and exposure to as little as 2 h of a social stressor is sufficient to significantly affect other populations of the colonic microbiota (Galley et al., 2014) providing further evidence that stress is an important factor affecting the composition of the gut microbiota.

Antibiotic-mediated depletion of the gut microbiota induced significant changes in concentrations of neuromodulatory substances such as tryptophan and kynurenine peripherally in serum, and the serotonin metabolite 5-HIAA in brain areas relevant to the regulation of core behaviours associated with brain–gut dysregulation. Decreases in gene expression of the growth factor BDNF in the hippocampus, and the neuropeptides vasopressin and oxytocin in the hypothalamus also provide insight into the possible mechanisms and systems involved in microbiota modulation of brain development during adolescence. These biological changes are paralleled by behavioural changes including reduced anxiety, non-spatial memory deficits and impaired performance in the social transmission of food preference test in males. The finding of enlarged caeca and reduced spleen weights in the present study parallel the anatomical observations reported previously in germ free mice (Smith et al., 2007) and antibiotic-treated mice (Reikvam et al., 2011), suggesting that these effects are attributable to the reduced gut microbial numbers. Previous studies have established that the effects of long-term treatment with antibiotics are not confined to peripheral organs and systems but also affect brain processes including communication (Rotschafer et al., 2012) and anxiety (Bercik et al., 2011). This effect of antibiotics on anxiety is not likely to be mediated by any other mechanism aside from its effects as an anti-microbial, as similar behavioural changes are reported in GF mice in which bacteria are absent from birth (Diaz Heijtz et al., 2011; Neufeld et al., 2011), particularly in males (Clarke et al., 2013). Interestingly, the reduced anxiety in GF mice is normalised when these mice are colonised by bacteria after weaning and subsequently assessed in adulthood (Clarke et al., 2013), suggesting adolescence is a crucial period of microbiota modulation of brain circuitry subserving anxiety.

Antibiotic treatment from early adolescence also affected cognitive function. Despite spending equal amounts of time exploring objects during the first test session in a familiar arena, antibiotic-treated mice exhibited reduced capacity to discriminate between a novel and a familiar object during the test session. Similar deficits are reported in GF mice in the presence and absence of stress (Gareau et al., 2011). Performance in the STFP test, as assessed by preference for a novel food encountered by a cage-mate immediately following, and 24 h after, a social interaction session with that cage-mate, not only informs on the quality of social behaviours in a mouse but also assesses the ability to retain information obtained through this interaction over a 24 h period. Although preference for the novel food encountered by a cage-mate is increased in a similar manner in control and antibiotic-treated mice immediately after the social interaction, preference for this same food is no longer present 24 h later. These data suggest that although the social interactions are of comparable quality in antibiotic-treated and non-treated mice, the capacity to retain this information 24 h later is diminished in the antibiotic-treated group. These cognitive impairments are paralleled by decreased BDNF mRNA in the hippocampus of antibiotic-treated mice, a finding that correlates with previous reports in GF mice (Diaz Heijtz et al., 2011; Clarke et al., 2013; Gareau et al., 2011). The neuroprotective role of BDNF in the brain, and the association between altered levels in the hippocampus and learning and memory performance is well established (Mizuno et al., 2000; Tyler et al., 2002; Baj et al., 2013).

It is still unclear how compositional changes in the gut microbiota induce changes in brain expression of BDNF, or indeed, in anxiety and cognition. Similar decreases in Firmicutes (Finegold et al., 2010; De Angelis et al., 2013) and Bacteroidetes (Finegold et al., 2010) have been reported in faecal samples of children with autism, a neurodevelopmental disorder associated with significant social and cognitive impairments. It is likely that neurotransmitters common to both the gut and brain have some role to play in microbiota-mediated changes in brain function. Altered concentrations of the serotonin metabolite, 5-HIAA, in the hippocampus and serum levels of the serotonin-precursor, tryptophan, in antibiotic-treated mice give further credence to the potential involvement of serotonin in microbiota–gut–brain axis communication.

The role of vasopressin and oxytocin in social memory are well established (Gabor et al., 2012; Takayanagi et al., 2005; Skuse et al., 2014). Interestingly, both oxytocin and vasopressin mRNA levels were significantly reduced in the hypothalamus of antibiotic-treated mice suggesting that gut microbes interact, directly or indirectly, to modulate the activity of these neuropeptides, and through this mechanism, potentially influence programming of cognitive and social behaviours in the brain. This association between gut microbes and sociability has recently been demonstrated in germ-free mice, where absence of microbiota from birth resulted in impaired sociability and social memory when assessed in adulthood (Desbonnet et al., 2014). Although the current experiments revealed no deficiency in the quality of social exchange in the STFP test, it is possible that other aspects of the social behavioural repertoire that could not be assessed in the STFP test may have been affected by antibiotic treatment from early adolescence. Considering that social and cognitive deficits represent core pathological features of neurodevelopmental disorders, it is not surprising that evidence implicating microbiota in the pathophysiology of these disorders has emerged in recent years (Hsiao et al., 2014; de Theije et al., 2014).

In conclusion, these data suggest that adolescence and early adulthood represent critical periods during which perturbations to the gut microbiota and dysregulation of microbiota–gut–brain axis communication can significantly impact on brain development and behaviour resulting in altered cognitive and anxious phenotypes in adulthood. Although the precise mechanisms by which the gut microbiota mediate changes in the brain have yet to be elucidated, our findings indicate that neuromodulators that traverse the microbiota–gut–brain axis including noradrenaline, tryptophan and the neuropeptides, oxytocin and vasopressin are potentially important in shaping behaviours such as anxiety and cognition. These data also indicate that chronic high-dose antibiotic treatment in mice represents a useful model for assessing the importance of gut microbiota during distinct stages of early life on brain development and behaviour in the absence of any disruption to the immediate postnatal gut microbiome, an unavoidable feature of the germ-free mouse construct. Further studies focussing on the effects of bacterial depletion during more defined periods of early brain development on specific dimensions of the affected behaviours and their neuronal correlates are required to identify those periods of particular vulnerability for the microbiota–gut–brain axis and its potential relevance to neurodevelopmental disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2015.04.004.

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