Maternal antibiotic administration during a critical developmental window has enduring neurobehavioural effects in offspring mice

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1. Introduction

As the links between gut microbiota composition and behaviour become more established through both clinical and preclinical studies, research has begun to focus on the effects of microbiota disruption during critical windows of development. To date, the impact of antibiotic administration has been assessed throughout the lifespan in animal models including perinatally [1], during early life [2], adolescence [3], adulthood [4] and in old age [5]. Of particular interest is the early postnatal period as this is a critical time for both neuronal development [6] and the initial seeding of the microbiota [7]. Indeed microbial colonization during this period has been linked to development of the hypothalamic-pituitary-adrenal (HPA) axis, influencing stress response in mice [8]. However, due to the high rates of antibiotic use in the postpartum period in both mother and infant, disturbance to the microbiota in early life is common, which has the potential to significantly impact brain and behaviour in the offspring.

Maternal antibiotics are administered to prevent postpartum infection in the mother for reasons including trauma sustained during delivery, post-surgical complications following C-section, or physiological changes that may occur during pregnancy [9]. During these treatment periods, mothers are recommended to continue breastfeeding and each of the antibiotics used maternally are considered safe for nursing infants with no reported adverse effects on the neonate being observed during early life [10,11]. Monitoring of adverse events in the offspring following maternal antibiotic administration is generally confined to the duration of treatment and a short period thereafter, neglecting to account for any potential long-term consequences. Maternal antibiotic use has however, been linked to the development of asthma in offspring [12], and this link is present whether antibiotics were administered before or during pregnancy, or in the weeks following birth [12]. Furthermore, the fact that antibiotics administered to the mother can...
reach breast milk and potentially exert a direct impact on the offspring’s microbiota must be considered. This potential for disruption to the microbiota during a critical window may have significant impact later in life and these effects require more detailed analysis.

Antibiotic exposure during early-life, however, is not solely as a result of maternal antibiotic administration as antibiotics are also the most common class of drug that is administered to infants directly [13], with oral penicillins particularly prevalent [13]. While antibiotic use among children may be critical to maintain health during this vulnerable period, there are also associated risks. Since it is known that the microbiota can influence host metabolic activity [14], disruption of its composition during this period can impact the host by either promoting weight gain [15] or by stunting growth [16], thereby having lasting effects on body weight throughout the lifespan [16]. Links between childhood antibiotic use and development of IBD [17] and asthma [18] have also been made. Antibiotic use in the first year of life has been linked to neurocognitive outcomes later in childhood, with more behavioural difficulties and more symptoms of depression at follow-up [19]. Antibiotics also serve as a valuable tool to modulate the microbiota in preclinical models that assess behaviour. Compared to other modes of gut microbiota modulation, such as the germ-free mouse, antibiotics offer far greater flexibility over the extent to which they disrupt the microbiota. They can be administered at any stage of the lifespan, either acutely [20] to mimic a short course as seen clinically, or in cocktails administered over prolonged periods to effectively eradicate the microbiota [21,22].

Animal models of maternal antibiotic administration in the perinatal period have been shown to have long-term effects on offspring metabolic activity [23] and immune system development [24], with the pre-weaning period having been identified as pivotal for these changes in immune response [25]. Indeed, a “critical window” has been suggested in immune system development, during which disruption, if it occurs, may lead to predisposition to a number of diseases that are initiated by an abnormal mucosal immune response [26]. Furthermore, antibiotic administration during this window has been shown to induce changes in brain and behaviour. When dams were administered penicillin V from one week prior to delivery until the end of the weaning period, alterations were observed in anxiety in the offspring, as well as changes in sociability, social novelty and aggression [1]. These changes were associated with alterations in microbiota composition and some could be prevented with co-administration of the probiotic Lactobacillus rhamnosus JB-1 [1]. This study highlights the potential for antibiotic administration during this critical window to have long-term effects on brain and behaviour and provides the basis for the selection of the single antibiotic used in our study.

As well as effects on behaviour, administration of antibiotics directly to rodent offspring has shown to have an influence on the brain, including alterations that impact synaptic plasticity in these animals. Aspects of brain physiology including Bdnf expression [27], NMDA receptor subunit expression [27] neurogenesis [28] and electrophysiological recordings [22] have each been shown to be altered following antibiotic administration. Such changes in the brain may provide clues towards a mechanism for the behavioural disruptions observed following antibiotic administration. The widespread alterations that are observed across different animals, strains and antibiotic cocktails in the above studies demonstrates the powerful effects that antibiotics can have on the brain and on behaviour. Our study aims to shed further light on these findings by assessing changes in offspring behaviour following administration of either a single antibiotic or a cocktail of antibiotics to mothers for one week, beginning one day following the birth of their offspring. The results of these investigations may prove to be clinically relevant to how maternal infections in the perinatal period are treated in the future.

2. Materials and methods

Study Design. One day following the birth of the pups weaning mothers were divided into three groups: Vehicle, Penicillin V or antibiotic cocktail, with interventions administered for 7 days in drinking water. Early life behavioural tests were carried out at postnatal day 9 (Ultrasound Vocalization, USV) and 10 (Homing test, HT). Adult behavioural tests were then undertaken beginning at 8 weeks of age following a washout period. Tests were undertaken in the following sequence: Marble burying test (MBT); Elevated Plus Maze (EPM); Three Chamber test (3CT); Novel Object Recognition (NOR); Carmine Red Test (CRT); Light-dark box (LDB); Aversive Open field (AOF) and Forced Swim test (FST). Animals were culled at the end of the experiment and relevant tissues harvested for analysis. N numbers for behavioural tests: Vehicle n = 13, penicillin V n = 11, antibiotic cocktail n = 15

2.1. Animals

All animal experiments were approved by the animal experimentation ethics committee at University College Cork (UCC), and by the health products regulatory authority (HPRA) of Ireland in accordance with EU directive 2010/63/EU. 8-week-old female and male C57BL/6 were obtained from Envigo laboratories, UK. Breeding began after 2 weeks of acclimatization to the holding room. Conspecifics were purchased from Envigo and allowed to acclimatise in the room along with the other mice for two weeks before undertaking the three-chamber test. Animals were kept under a strict 12:12 h light-dark cycle and in a temperature-controlled room (20 ± 1 °C, 55.5 % humidity), with food and water supplied ad libitum. Male offspring were weaned at P21 and housed in groups of 3–4 mice per cage, siblings from the same litter were kept together after weaning meaning that they each received the same treatment and stopped mixing of the microbiota from coprophagy, there were 8–10 pups in each litter with 3–4 males per litter. Groups consisted of offspring from 14 independent litters. In addition to animals purchased for breeding, 8-week-old C57BL/6 mice were purchased from Envigo laboratories, UK, for use as conspecifics in the three-chamber test of sociability.

2.2. Antibiotic administration

In order to deplete the maternal gut microbiota, dams were administered antibiotics in drinking water for seven days, beginning one day after the birth of the pups (postnatal day 1, P1). Dams were exposed to either a single antibiotic – Penicillin V, at a dose of 31 mg/kg/day (as per [1], or a cocktail of antibiotics (consisting of Ampicillin 1 mg/mL; Vancomycin 0.5 mg/mL; metronidazole 1 mg/mL; ciprofloxacin 0.2 mg/mL and Imipenem 0.25 mg/mL), or water. Antibiotics were
dissolved in autoclaved water and changed every two days. Control animals received just autoclaved water, which was also changed every two days. Liquid intake was estimated by measuring bottle weights before replenishment. Antibiotics were available in drinking water ad libitum for the duration of administration. Bottles were changed every 48 h throughout this period, and consumption of water in each cage was monitored to ensure that there were not significant differences in antibiotic consumption between treatment groups. Maternal weights were taken daily during the antibiotic administration period to ensure that excessive weight loss did not occur. Cages were cleaned every second day during treatment (as well as in control groups) to prevent the re-establishment of a normal microbiota through coprophagy.

2.3. Behavioural testing

2.3.1. Isolation-induced ultrasonic vocalisation tests (USV)

Isolation-induced ultrasonic vocalisations (USV) are produced by mouse pups during the first two weeks of life when separated from littermates [30]. At P9 pups were removed from the home cage and habituated in a room away from their mother and littermates for ten minutes. During the experiment, animals were placed in a clean, plastic container, within a sound-attenuating chamber. Ultrasonic vocalisations were monitored using an ultrasonic-sensitive microphone – a bat detector (US mini-2 bat detector, Summit, Birmingham, USA) tuned into the range of 60–80 kHz – suspended above the isolated pup for three minutes, the number and total duration of calls were noted.

2.3.2. Homing test

The homing test (HT) evaluates the tendency of pups to recognize the nest of their mother and siblings at P10 and was carried out as described previously [30]. The floor of a clean mouse cage was subdivided into three areas by wire mesh dividers, one of which was uniformly covered with wood shavings from the home cage, thus containing familiar odour stimuli. The opposite space was covered with wood shavings from the nest of their mother and siblings at P10 and was carried out as described [31]. Cleaned cages were lined with a 5-cm layer of chipped wood shavings from the home cage, thus containing familiar odour stimuli.
being returned to their home cage. Apparatus was cleaned with 10 % ethanol and allowed to dry between experiments. Recordings were made with a camera placed above the apparatus and scoring was undertaken manually, assessing the time spent in each chamber, the number of transitions between chambers, and the number of exploratory rearings made by each animal.

2.3.8. Forced swim test (FST)

In this test mice were gently placed in a cylinder containing water (23–25 °C) (Temperature monitoring is essential as alterations may impact performance in the test [36]) at a height of 17 cm. Animals were left in the water for 6 min with activity being recorded by a camera positioned overhead. Immobility time was scored for the last 4 of the 6 min. Following removal from the cylinder, animals were dried gently and placed in a separate cage for recovery. This has previously been described in our lab [37].

2.4. Whole intestinal transit (Carmine red test - CRT)

A test mouse was administered with carmine dye by oral gavage; the latency for the excretion of the first red-coloured faecal pellet was recorded.

2.5. Murine HPA axis response

Blood samples were taken to assess the HPA response to a mild acute stress (i.e: FST) in adulthood. Samples were obtained from the tail at 30, 60, 90 and 120 min following the forced swim test, fresh plasma was isolated from whole blood by centrifuging samples at 3000 rpm for 6 min. Following removal from the cylinder, animals were dried gently and placed in a separate cage for recovery. This has previously been described in our lab [37].

2.6. RNA isolation, synthesis of cDNA and qPCR analysis

Total RNA was isolated from the hippocampus (HIP) using the GenElute Mammalian Total RNA minprep kit, as per manufacturer’s instructions (Sigma-Aldrich). RNA concentration was quantified using the ND-1000 spectrophotometer (Nanodrop). Following RNA extraction equal amounts of RNA were reverse transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies, Carlsbad, CA). All cDNA was stored at -20 °C until time of analysis. Total corticosterone was measured according to manufacturer’s protocol. A corticosterone ELISA kit was used to determine plasma concentrations (Enzo Life Sciences, Farmingdale). Plasma dilution was 1:50.

2.7. Statistical analysis

Data distribution was checked by Kolmogorov-Smirnov test and variances were compared using Levene’s test. For parametric data, a Paired Student t-test, a One-way ANOVA, Two-way ANOVA and two-way repeated measures ANOVA followed by Bonferroni post-hoc was applied accordingly to the protocol adopted. For nonparametric data, a Kruskal-Wallis test followed by U-Mann Whitney was used. All statistical analyses were carried out using IBM SPSS Statistics 22.0 for Windows software package. Extreme outliers and technical outliers were excluded when values are 2 x Standard Deviation from the mean. F values, P values are presented in the text of the results section.

3. Results

3.1. The effects of antibiotic administration on maternal body weight

Mothers exposed to the antibiotic cocktail one day following the birth of their pups experienced weight-loss in the days after initiation of treatment. (Fig. 1A) (Repeated-measured two-way ANOVA, F (14, 84) = 3.516, P = 0.0002 (interaction between treatment and time), F (7, 84) = 35.89, P < 0.0001 (time), F (2, 12) = 2.283, P = 0.1445 (treatment). Post hoc assessment found a significant difference in body weight between vehicle and cocktail groups (P < 0.05) at P1-4 only, and between cocktail and Penicillin V group at P1 only (P < 0.05) after which no differences were observed between the groups.

Offspring weight was observed throughout the course of the experiment, however no differences were observed (Fig. 1B) (repeated measured two-way ANOVA: F (2, 37) = 2.128: P = 0.1334 (treatment), F (16, 296) = 1.720: P = 0.0422 (interaction between treatment and time).

Table 1

| Primer Details for PCR.|
|-------------------------|
| **Bdnf** | L- AGTCTCCAGGACAGCAA R- TGCAACCAGAATATGA AAGCT CAG GTAATTA |
| **GluN2a** | L- TACCTCCAGGCGTGAAACA R- AAGTCCGGTCTGTCA CCAT GAAGC |
| **GluN2b** | L- CCCAGTCTAATGCTGA ATAGGGT R- GGAAGTGTAGATGGCAGCA CAGCA |
| **Psd-95** | L- GTCCCTTGAGAATGTT CTATGGT R- AACTCTGGTGACCTTC GT |
| **CamKII** | L- TCTTTTCCGATCTCTGT GC R- GTGCTTAAGAGTGTAGTG AGTG |
| **Fos** | L- CACGGCTTCTACTACC ATTCC R- ACAATCTGGGCAAAAG |

**Fig. 1.** Maternal and offspring weights. (A) Significant maternal weight loss was observed between antibiotic cocktail group and control from treatment day 1 to treatment day 4. There was also significant weight-loss in the cocktail group compared to the penicillin V group on day 1. (B) No differences were observed in body weight between the offspring groups. Data expressed as mean ± SEM. Repeated measures two-way ANOVA with Bonferroni post-hoc test [Vehicle n = 5, Penicillin V, n = 5, cocktail n = 5]. *p < 0.05 between control and cocktail group. &p < 0.05 between Penicillin V and cocktail group.
3.2. Early-life behaviours are altered following antibiotic administration

As the disruption to the maternal microbiota through the administration of antibiotics in the drinking water occurs during a critical point of development in early life, the effects at this early stage are measured by observing behaviour in the days following cessation of antibiotics to the mothers. It was observed that in early-life (P9) there was no difference in either the total number of vocalisations (Fig. 2a) (F(2,36) = 0.9887, P = 0.3877) or the total duration of vocalisations (Fig. 2b) (F(2,36) = 2.413, P = 0.111) between any of the groups. Offspring attachment to maternal bedding was also analysed, with the time spent in maternal bedding being significantly reduced in animals whose mother had received a cocktail of antibiotics compared to those whose mothers received vehicle (Fig. 2c) (F(2,36) = 2.287, P < 0.005). Indicating that these animals may be exhibiting early social recognition and maternal attachment deficits compared to the other groups tested.

3.3. Anxiety-like behaviours are altered by early-life maternal antibiotic administration

When mice are exposed to an unfamiliar environment, the increased potential for threat means that there may be an associated increase in anxiety. It is possible to measure this in rodents via several behavioural tests. In the marble burying test, increased levels of marble burying correspond to an increased in repetitive behaviours and is regarded as neophobic behaviour [39]. Here, the offspring of mothers administered both low-dose penicillin V, or an antibiotic cocktail buried a significantly greater number of marbles (Fig. 3) (F(2,36) = 2.287, P =< 0.005). Indicating that these animals may be exhibiting early social recognition and maternal attachment deficits compared to the other groups tested.

Fig. 2. Early-life behavioural alterations following maternal antibiotic administration. (A) & (B) early-life maternal antibiotic administration had no impact on either total number or duration of vocalisations (c) early-life administration of an antibiotic cocktail to mothers caused a significant reduction in the time spent in maternal bedding arena compared to a group where the mother received only water. Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n = 13, penicillin V n = 11, antibiotic cocktail n = 15]. $p <0.01 between vehicle and cocktail groups.

Fig. 3. Maternal antibiotic treatment leads to increased anxiety-like behaviour in the marble-burying test. Offspring from mothers in both of the treatment groups buried a significantly greater number of marbles than offspring in the control group. Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n = 13, penicillin V n = 11, antibiotic cocktail n = 15]. *p < 0.05 between vehicle and Penicillin V, $p < 0.05 between vehicle and cocktail.
treatments (Fig. 4c) (F(2,29) = 0.9291, P = 0.9152). The number of head dips were similarly unaffected (Fig. 4d) (F(2,29) = 0.166, P = 0.4311).

The light-dark box test acts as another measure for rodent anxiety, taking into account the natural aversion of mice to brightly illuminated areas as well as their spontaneous exploratory behaviour in novel environments [42]. Here, the offspring of mice treated with an antibiotic cocktail (but not penicillin V) spent significantly less time in the dark than the control group (Fig. 5a) (F(2,36) = 0.01592, P < 0.05). The number of transitions (Fig. 5b) (F(2,36) = 7.585, P = 0.0956), however, and the number of exploratory rearings (Fig. 5c) (F(2,36) = 0.08713, P = 0.9167) were unaffected.

3.4. Antidepressant-sensitive behaviour remains unaffected

The mouse FST is used as a measure of antidepressant-sensitive behaviour in animals and regularly serves to determine the efficacy of novel antidepressant compounds [43]. It has also been used as a measure of the effect of microbiota manipulation on these behaviours [4,22]. Antibiotic administration has been shown to increase immobility time in the FST in rodent models [4,22]. These effects were not observed in either of the antibiotic treatment groups. (Fig. 6) (F(2,36) = 0.5641, P = 0.2293).

3.5. Maternal penicillin administration induces deficits in social recognition

The three-chamber test measures various aspects of social behaviour in rodents. Social preference is assessed by giving mice the choice of interacting with either a novel mouse, or an object. When given the choice, all groups exhibited normal social behaviour, (Fig. 7a) (Two-way ANOVA, F(2,66) = 7.123, P = 0.0016 (treatment), F(2,66) = 0.7064, P = 0.4971 (interaction between treatment and mouse/object)). Post-hoc tests confirm that they spend more time with the mouse than the object (Vehicle object vs Vehicle mouse: P < 0.001, Penicillin V object Vs Penicillin V mouse: P < 0.001, Cocktail object vs cocktail mouse: P < 0.001).

When the test was repeated, with the object being replaced with an unfamiliar mouse, the test can be used to measure social recognition. In this case, different effects are seen following antibiotic treatments (Fig. 7b) (Two-way ANOVA F(2,62) = 0.2414 P = 0.7863 (treatment), F(2,62) = 3.661, P = 0.0314 (interaction between treatment and mouse/object)). Post-hoc tests show a greater amount of time spent with the novel mouse in the vehicle group (P < 0.001) and the antibiotic cocktail group (P < 0.01) but not in the penicillin V group.

3.6. Maternal antibiotic cocktail administration induces cognitive deficits in offspring

The novel object recognition test measures hippocampal-dependent
memory in rodents. Harnessing the inherent preference of mice for novelty, this test determines the memory of previously encountered objects in these animals. Differences were observed between the groups (Fig. 8a) (Two-way ANOVA, $F(2, 72) = 2.087$, $P = 0.1315$ (treatment), $F(2, 72) = 4.141$, $P = 0.0198$ (interaction between treatment and Novel/familiar object), while post hoc comparison confirmed that while the vehicle treated group ($P < 0.05$) and the penicillin V treated group ($P < 0.05$) spent a greater time with the novel over the familiar object, this was not the case in the cocktail-treated group. Furthermore, when the percentage time spent with the novel object is assessed, there is a significant decrease in the interaction time in the antibiotic cocktail group only (Fig. 8b) (One Way ANOVA $F(2,36) = 0.4085$, $P < 0.05$).

3.7. Plasma corticosterone levels are unaffected

Plasma corticosterone acts as a measure of HPA axis activation following an acute stressor. Increased levels of circulating corticosterone following a stressor indicate that there is an abnormal response to the event. Here, no differences were observed in HPA axis response between any of the groups (Fig. 9) (Repeated-measures Two-way ANOVA: $F(2, 28) = 1.182$, $P = 0.3217$ (Treatment), $F(8, 112) = 1.724$, $P = 0.1005$ (interaction between treatment and time).

3.8. Physiological alterations

As well as behavioural assessments, physiological differences were measured both during the experiment, as well as following the completion of behavioural assessments. Intestinal transit was assessed using the inert dye carmine red (Fig. 10a) ($F(2,33) = 0.6576$, $P = 0.0903$). Previous studies have shown that this measure can be affected by gut microbiota composition [44]; however, we did not observe any differences in transit time in antibiotic treated animals. In addition to this, fat masses (Fig. 10b) ($F(2,36) = 0.7018$, $P = 0.0784$) (Fig. 10c) ($F(2,36) = 0.4632$, $P = 0.2240$) (Fig. 10d) ($F(2,36) = 0.8245$, $P = 0.0761$) (Fig. 10e) ($F(2,36) = 1.755$, $P = 0.3141$) and cecum weight (Fig. 10f) ($F(2,36) = 2.106$, $P = 0.1160$) were assessed when the animals were culled. The only difference that was observed between the groups was spleen weight, which was significantly increased in the group treated with an antibiotic cocktail (Fig. 10g) ($F(2,36) = 2.637$, $P < 0.05$).
3.9. Hippocampal PCR analysis

To determine whether any of the treatment groups were able to affect the expression of plasticity related genes in the hippocampus, expression measured in the hippocampus. A reduction in hippocampal expression of \textit{Bdnf} was observed in the penicillin V group compared to control (Fig. 11a) ($F_{(2,21)} = 1.577$, $P = 0.0393$). None of the other genes were affected, however including \textit{GluN2A} (Fig. 11b) ($F_{(2,22)} = 0.01173$, $P = 0.9883$), \textit{GluN2B} (Fig. 11c) ($F_{(2,22)} = 0.04879$, $P = 0.9525$), \textit{PSD-95} (Fig. 11D) ($F_{(2,21)} = 0.6620$, $P = 0.5262$), \textit{CamKii} (Fig. 11D) ($F_{(2,21)} = 0.4272$, $P = 0.6579$), \textit{fos} (Fig. 11E) ($F_{(2,22)} = 0.9823$, $P = 0.3903$).

4. Discussion

The increase in maternal antibiotic use in the days and weeks following delivery, primarily driven by an increase in rates of caesarean section [45] has led to a source of gut microbiota disruption that, until this point has been relatively understudied. There are clinical reports linking maternal antibiotic use with an increase in the prevalence of asthma later in life [12], as well as showing that direct antibiotic administration in early life is linked to metabolic disorders [12], IBD [17], asthma [18] and negative neurocognitive outcomes later in childhood [19]. Furthermore, preclinical studies have linked perinatal maternal antibiotic administration and effects on behaviour later in life [1].

Here, we assessed the effects of administration of either a single antibiotic (low-dose penicillin) or a cocktail of five antibiotics (designed to ablate a large portion of the existing gut microbiota community) to
nursing dams. This allowed us to compare a clinically relevant dose of an antibiotic to a much more comprehensive disruption of gut microbiota composition. Effects of maternal antibiotic administration on behaviour were observed both in early life as well as in adulthood, with the antibiotic cocktail generally leading to more pronounced behavioural effects. Post-mortem hippocampal mRNA expression of genes related to behaviour and synaptic plasticity was also undertaken, with maternal penicillin exposure in early life leading to a significant reduction in hippocampal Bdnf in adulthood. As it has been shown that pre-weaning exposure of pups to antibiotics via the mother leads to metabolic [23], immune [24] and behavioural changes [1] we administered our antibiotic interventions to dams from the day following birth of the pups (P1). Ingested penicillin V is found in the breast milk [46] and as such is able to exert direct effects over the microbiota composition of the offspring while they are nursing.

Previous studies using perinatal antibiotic administration have shown an alteration of the gut microbiota composition [1,16,23] that they suggest as the mechanism behind behavioural and physiological alterations. The risk of penicillin V exerting a direct effect on the brain is very low due to its negligible penetration of the blood-brain barrier [47] as well as its rapid renal clearance [47] therefore accumulation in the serum should not occur. Within the cocktail group, direct effects of metronidazole on the brain are possible. It crosses the blood-brain barrier [48] and it is known to enter breast milk [49], in our study, we did not assess the effect of antibiotic treatment on maternal milk nutrient composition. In order to control for this, in subsequent studies it may be worthwhile to administer an intraperitoneal injection of antibiotics as a control, as has been undertaken in other antibiotic studies [50]. In our study, however, behavioural testing took place a minimum of seven weeks following the completion of the course of antibiotics; therefore, any residual toxic effects of the drugs are regarded as highly unlikely.

An additional factor that may lead to alterations in behaviour in adulthood is maternal behaviour and care during the weaning period. While maternal care is a factor known to alter behaviour later in life [51] it was not monitored in our study. Care was taken to determine that no abnormalities were observed in nests, and that no cannibalism occurred. These factors were not altered between groups, suggesting that maternal antibiotic administration did not affect maternal care, a finding consistent with previous studies [1,8]. The weight of the mothers in the antibiotic cocktail group decreased in the early days following the introduction of the antibiotic cocktail to their drinking water, compared to the other two treatment groups. This observed weight-loss may be due to the aversive taste of metronidazole. Previous studies have shown that this antibiotic, in particular, is associated with reduced fluid consumption [52] and additional weight loss compared to other antibiotics [52]. Furthermore, bacterial depletion in the gut is known to cause diarrhoea, which is associated with weight loss [53]. By the end of the antibiotic intervention the maternal bodyweight had recovered such that there

![Fig. 10. Intestinal motility and tissue weights following maternal antibiotic administration. (a) intestinal motility was unaltered in both treatment groups compared to control. Tissue weights following the animal culls were also generally unaffected for (b) epididymal fat, (c) subcutaneous fat, (d) mesenteric fat, (e) cecum weight, (f) brown fat. (g) spleen weight was significantly increased following maternal administration of an antibiotic cocktail. Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n = 13, penicillin V n = 11, antibiotic cocktail n = 15]. $p < 0.05$ between vehicle and cocktail groups.](image-url)
were no differences between treatment groups and controls. The initial behavioural tests took place in the days following cessation of maternal antibiotics to determine whether exposure to antibiotics up to this point caused altered behavioural development. Ultrasonic vocalizations are used to assess social development in early life, with different characteristics of the calls being determinants of various social paradigms [54]. Previous research in which microbiota from high-fat-diet-fed mice was transplanted to mothers prior to breeding and the USV characteristics of the offspring measured found that these mice vocalized less upon maternal separation than pups from control dams [55]. In our study however, no differences were observed either in the duration, or number of vocalizations. This may be due to the fact that in the previous study dysbiosis was present in the mothers prior to breeding, and in our study, dysbiosis only occurred in the days following the birth of the pups.

The second test of early-life behaviour undertaken analysed attachment to maternal bedding at P10. The premise of this test is that recognition of maternal odours is vital for mother-offspring pairing through facilitating the establishment of social behaviours. Mouse pups at P10 have demonstrated the ability to recognize pertinent social stimuli and move towards their mother’s nest when removed from it [56]. We observed the animals whose mothers received an antibiotic cocktail spent less time in the maternal bedding than the control offspring. This pairing between mother and offspring is important for the development of social behaviours [57]. Interestingly, it is the penicillin group which displayed a disruption in social preference in the three-chamber test in adulthood. And, although these two tests measure different aspects of sociability, it may be that the differential effects on

**Fig. 11.** Hippocampal mRNA expression of synaptic plasticity genes. (a) BDNF (B) GluN2A (C) GluN2B (D) PSD95 (E) CamKII (F) fos. Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Fishers LSD. [vehicle n = 8, penicillin V n = 10, antibiotic cocktail n = 7]. *p < 0.05 between vehicle and Penicillin V groups.
the microbiota in early life have different effects on development of sociability. It should also be noted that pup locomotion was not assessed in these experiments, and this may also be a factor in the differences observed. Another factor that may play a role is the alteration in odour cues in the bedding of cocktail-treated mothers. Such scents involved in recognition have been linked to the presence of fermentative bacteria [58]. As such, ablation of the microbiota can modulate the levels of these bacteria and therefore the intensity of the odour cues.

When behavioural tests were carried out in adulthood, numerous differences were observed between the antibiotic-treated groups and the control group in which the mothers received water only. Anxiety-related behaviour was particularly affected. Both groups in which the dams received antibiotics in the drinking water displayed increased levels of anxiety-related behaviour in the MBT, with an increase in the number of marbles buried. Similarly, in the EPM, where both antibiotic groups had significantly fewer entries to the open arms of the maze. Previous antibiotic studies have produced variable results in tests of anxiety. In rats, perinatal antibiotic administration to the dam led to increased anxiety-like behaviour in the EPM [59] with an antibiotic study in zebrafish also producing an increase in anxiety-like behaviour [60]. Antibiotic studies in mice, however, have reported reductions in anxiety-like behaviour [1,3]. In a further study in which antibiotic treatment caused anxiety-like behaviour [20], when testing was repeated following a two-week washout period, anxiety-like behaviour returned to normal. The changes seen in anxiety levels a number of weeks following the cessation of antibiotics, as in our study suggests that early-life bacterial colonisation is pivotal in the establishment of these behaviours. Similarly, in germ-free mice where alterations in anxiety are observed, this behaviour was normalised when mice were colonised by bacteria after weaning and subsequently assessed in adulthood, suggesting adolescence as an additional critical period of microbiota modulation of brain circuitry linked to anxiety, such as the amygdala [61]. These results suggest the existence of multiple windows during which the microbiota may impact on normal neuronal development.

In the light-dark test, we observed that the antibiotic cocktail group spent significantly less time in the dark area than the control, indicating an anxiolytic effect of the antibiotic cocktail. This finding is more in line with what was observed in other studies assessing the impact of antibiotic administration on anxiety [1,3]. It does not correspond, however, with what we observed in the other tests of anxiety. This may be explained by the fact that each of the tests for anxiety utilized, measure different aspects of anxiety-related behaviour [62]. The LDB test is a measure of bright-space anxiety while the EPM, which is carried out under red light, is more of a measure of open-space anxiety [42]. In the FST there were no observed differences in the immobility time between any of the groups. This differs from previous studies in which antibiotic administration led to an increased immobility time in the test, and an increase in antidepressant-sensitive behaviours [4,22]. In each of these cases, there was a direct administration of the antibiotics to the study animals, potentially leading to a more significant disruption of the microbiota. A greater susceptibility to change during adulthood suggests that microbiota-based interventions may be of greater benefit in depressive-like conditions than disorders of anxiety in adults.

The link between microbiota and social behaviour is gaining much attention across species [63]. Indeed, the absence of a microbiota from birth corresponds with impaired sociability and social memory in adulthood [64], in germ-free mice. Moreover, recolonization of germ-free mice with the intestinal microbiota is able to reverse such effects. In our current study, maternal antibiotic treatment did not induce any alteration in the amount of time the animals spent interacting with a mouse over an inanimate object, with all groups exhibiting a significant preference for the mouse. When presented with a novel and familiar mouse, however, the penicillin-treated group did not display a preference between either mouse, indicating an impairment in social recognition mirroring the germ-free recombinant situation. The fact that the single antibiotic had this effect, but the cocktail did not is of note.

While this corresponds with a recent paper in which perinatal administration of penicillin V caused impairments in social novelty but not sociability [1], this paper only assessed the effects of a single antibiotic compared to control. Previous studies in which antibiotic cocktails were administered has led to conflicting results, with both a lack of effect on social behaviour [50] as well as disruptions in the ability of mice to distinguish social novelty [65] being observed. Overall, these results highlight the complexity of social behaviour. It may be that the specific bacteria targeted by penicillin V may exert a greater impact on brain areas regulating social development; however, this would require a more detailed temporal analysis of microbiota composition throughout early life in future studies.

Cognitive deficits are one of the most commonly observed behavioural deficits following antibiotic administration in rodents [32,27,28]. In our study, we also found that while the control and single antibiotic groups spent significantly more time exploring the novel object than the familiar, this was not the case in the antibiotic cocktail group. Furthermore, when the percentage time spent with the novel object was assessed, the cocktail group performed worse than the single antibiotic group. This finding potentially links the level of cognitive disruption to the extent of microbiota disruption. Further studies should aim to probe some of the mechanisms underpinning these changes through assessing alterations in protein expression; for example, cognitive disruption following antibiotics has been linked to altered Bdnf [27] and microglial expression in the hippocampus [22].

When physiological measurements between the two groups were assessed, few differences were observed. No differences in fat weights between groups were observed, indicating that the metabolic alterations seen in other instances of perinatal antibiotic administration [23] were not present in our study. Additionally, intestinal motility, which has been shown to be altered following antibiotic administration due to the impact on enteric nervous system signalling [66] is unaffected in our study. No differences were observed in plasma corticosterone expression in response to a stressor, corresponding with the results of previous antibiotic research [2,3] The spleen weight of the offspring of cocktail-treated offspring was increased compared to those whose mother received vehicle. As this is an aspect that has been shown to be altered in other rodent antibiotic models [67], it may be a factor worthy of investigation in future rodent antibiotic studies.

Post-mortem analysis of hippocampal tissue found that the offspring of the penicillin V treated mothers had a significant reduction in hippocampal Bdnf compared to vehicle. This result is perhaps unsurprising as this effect has previously been observed following antibiotic treatment [3,27,65]. What is noteworthy, however is that the same effect was not observed in the antibiotic cocktail group. Again, this may be linked to the extent of antibiotic transmission from dam to pup and therefore the antibiotic exposure. This is an aspect of the study that would require further scrutiny in future antibiotic investigations. When other plasticity-related genes were assessed no differences were observed between the groups. Interestingly, in one of the studies in which hippocampal Bdnf was shown to be reduced by antibiotic treatment [27] no effect of treatment was seen on the expression of Grin2b, mirroring our results. It is clear that plasticity related genes can be differentially affected by microbiota modulation and further investigation of these differences may be key to understanding observed behavioural changes.

It seems clear therefore that maternal administration of antibiotics in the early postnatal period can alter behaviour both in early life, as well as in adulthood. Based on our data, it appears that more significant disruption of the microbiota (through administration of a cocktail of antibiotics, rather than a single antibiotic) leads to more pronounced effects on behaviour. This study brings a degree of novelty to the field as it compares the administration of two different modes of bacterial knockdown under identical conditions and, while many of the effects are mirrored between the groups, there are also a number of differences. This highlights a key issue in the field to date. The enormous variation among preclinical studies in the specific antibiotics that are
administered, the route of administration, the age and duration of administration, and the age at which behavioural tests are undertaken. Each one of these factors is pivotal to the effects on brain and behaviour. As such, we may agree that disruption of the microbiota through antibiotic administration can modulate brain and behaviour, future studies should place a much greater emphasis on how the other factors may influence outcomes.

When we compare the overall effect of a low-dose antibiotic with a severe disruption of the gut microbiota using a cocktail of antibiotics, we see differing results between treatments along with outcomes that are convergent across treatments. In pregnant dams receiving the antibiotic cocktail, we see significant weight loss, emphasising the severe effects of a higher dose of multiple antibiotics, and a potential reduction in metabolic capacity due to a larger scale microbial ablation. While in their pups we see a deficit in grooming behaviour, a reduction in time spent in the dark in the light-dark box, and a reduced interaction time with a novel object, changes in behaviour that are exclusive to this treatment group. In mice receiving Penicillin V we observe changes in social recognition and Bedn expression in the hippocampus, changes that are not evident in mice receiving the antibiotic cocktail. Furthermore, we see outcomes that are similar across both treatments, with an increase in the number of marbles buried, and fewer entries into the open arms in the EPM present in both treatment groups. Both antibiotic treatments target bacteria but differ in the extent and specificity of the microbes they target. Penicillin V is only effective against gram-positive bacteria and is less effective against anaerobes than other members of the Penicillin family. The antibiotic cocktail used in this study kills both gram-positive and gram-negative bacteria and kills both aerobic and anaerobic bacteria. The differences in outcome between antimicrobial treatments suggest that depleting different components of the microbiota has both dependent and independent mechanisms and should form the basis of future research.

The results of this study also raise a number of interesting questions that may be assessed in subsequent or follow-up studies in the future. For example, would maternal antibiotic administration during gestation as well as after the birth of the pups exacerbate the effects in the pups? What would be the long-term behavioural impacts of multiple, shorter courses of antibiotics? This scenario would mimic more closely the observed clinical situation. A further addition to this study would be to determine whether the addition of a microbiota-modulating treatment could serve to reduce the level of behavioural alterations. Previous studies have found that the addition of probiotics to antibiotic-treated animals can reverse behavioural [1] as well as inflammatory, biochemical, and electrophysiological alterations [22]. This treatment would also be of great practical use to mothers offering a safe method to prevent any long-term consequences of this treatment. One limitation of our study is that we only used male mice, we feel that the primary goal of these experiments was to understand the effect of maternal antibiotics on offspring at a key time during development, and we felt that looking at male or female mice was equally valid. Given that the male brain is particularly vulnerable to microbiota-based insults in early life we felt that as a translational model of the effect of early life antibiotic administration on behaviour that we would most likely see an effect in males.

These data provide compelling information regarding the use of antibiotics in early life. While it is not simple to extrapolate data obtained in rodent models to humans, these data support the notion that there are potential long-term detrimental consequences of maternal antibiotic use in the perinatal period. The effects observed in the antibiotic cocktail group tend to be more severe, and this is a dose that is unlikely to ever be used in a clinical setting. The use of a single, low-dose penicillin does have significant negative effects on anxiety and social behaviour in later life. These results in combination with those observed in the antibiotic-treated group suggest that disruption of the microbiota during a critical period in early life can have long-term consequences on neurodevelopment and the prevalence of psychiatric disorders later in life.

CRediT authorship contribution statement

Rory O’Connor: Formal analysis, Investigation, Writing - original draft, Data curation. Gerard M. Moloney: Formal analysis, Investigation, Writing - original draft. Christine Fulling: Formal analysis, Investigation. Ken O’Riordan: Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Project administration. Pat Fitzgerald: Methodology. Harriet Schellekens: Conceptualization, Funding acquisition. Timothy G. Dinan: Conceptualization, Project administration, Funding acquisition. John F. Cryan: Conceptualization, Methodology, Writing - review & editing, Project administration, Project administration, Funding acquisition.

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