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Authors	García-Cabrerizo, Rubén;Barros-Santos, Thaísa;Campos, David;Cryan, John F.
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Short Communication

The gut microbiota alone and in combination with a social stimulus regulates cocaine reward in the mouse

Rubén García-Cabrerizo [a,*], Thaísa Barros-Santos [a], David Campos [a], John F. Cryan [a,b,*]

[a] APC Microbiome Ireland, University College Cork, Cork, Ireland
[b] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

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ABSTRACT

The gut microbiota is a key factor in the maintenance of physiological homeostasis and immunity. Correlational studies have demonstrated that alterations in microbiota composition have been associated with addiction. Moreover, animal studies have confirmed a link between reward and social processes, which may be shaped by the gut microbiota thus influencing neurodevelopment and the programming of social behaviors across diverse animal species. However, whether there is an interaction between the microbiota and social reward processes in the context of drug reward remains unclear. To this end, we explored the influence of gut microbiota in regulating behaviourally conditioned responses to different rewards (cocaine and social interactions). Depletion of the intestinal microbiota resulted in differential reward responses to both drug and social stimuli with an attenuation of the former and enhancement of the latter independent of concomitant immune changes. Moreover, the combination of depleting the gut microbiota in the presence of a positive social stimulus attenuates cocaine reward. Together these data suggest that the two-pronged approach of targeting the microbiota and enhancing social behaviour could constitute a valuable component in reducing harm in drug use by altering the salient effects of cocaine.

1. Introduction

Drug addiction is characterized by a compulsive seeking of a drug despite its long-term negative consequences on health and well-being. Helping the substance-dependent individual reorient their behaviours toward non-drug-related activities has remained one of the greatest challenges for therapeutic interventions (Venniro et al., 2020). Animal research on addiction is stymied by a translational problem, and despite our understanding of circuits and molecular mechanisms of addiction, treatment options remain largely unchanged (Heilig et al., 2016). This impasse is at least partially due to limitations of animal models of addiction, which rarely incorporate social factors. The quality of the social context and the emotional valence of the social experience have a profound impact on mental health and illness, which supports the connection between the severity of substance use disorder and social interactions (Fosnocht et al., 2019). In clinical and preclinical studies, it has been reported that adverse social interactions and social isolation promote drug intake and relapse, while positive social interactions tend to be protective (Venniro et al., 2018).

The gut microbiota, is the community of microorganisms that colonize the intestinal tract, is increasingly recognized as an important modulator of brain circuits, involved in regulating brain reward functions, representing a potential target for intervention in drug addiction (García-Cabrerizo et al., 2021). Preliminary evidence indicates that abused drugs induce perturbations in the gut microbiome and that such changes could be responsible for the pathogenesis of substance use disorders (Meckel and Kiraly, 2019). Moreover, gut microbes are essential for the correct programming of social behaviours, having a major impact on the modulation of sociability and its neurobiological underpinnings across the animal kingdom (Sherwin et al., 2019) and targeting the microbiome has been shown to modulate social responses (O'Connor et al., 2021; Sen et al., 2022; Sgritta et al., 2019). However, the question remains: can the gut microbiome shape social behaviours in the context of addiction.

Here, we evaluated gut-brain interactions to understand the role of gut microbiota in the regulation of brain reward processes, demonstrating the importance of gut microbes in modulating drug and non-drug rewards and the feasibility as an alternative to drug abuse. We also sought to examine if any changes were concomitant with alterations at the level of the immune system.

* Corresponding authors.
E-mail addresses: ruben.garciacabrerizo@ucc.ie (R. García-Cabrerizo), j.cryan@ucc.ie (J.F. Cryan).

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2. Materials and methods

2.1. Animals

Seventy-five male C57BL/6 mice (6 weeks old) were purchased from Envigo (Envigo Laboratories, UK) and allowed to acclimate to the animal facility for two weeks before experiments started. Mice were initially housed in groups under specific environmental settings (21 ± 1 °C, 55 ± 10 % humidity, 12 h light/dark cycle) in standard mouse cages with *ad libitum* food and tap water until day 8 of the experimental design, when they were housed individually. All experimental procedures were conducted in accordance with the European Communities Council Directive 86/609/EEC following approval by the University College Cork Animal Ethics Experimentation Committee (AE19160/P134) and the Health Products Regulatory Authority.

2.2. Antibiotic administration

In order to deplete the gut microbiota, a wide-spectrum of non-absorbable antibiotics (ABX) in a cocktail consisting of ampicillin (1 g/L, CAS no. 69-52-3), gentamicin (1 g/L, CAS no.1405-41-0), imipenem (0.25 g/L, CAS no. 74431-23-5) and vancomycin (0.5 g/L, CAS no. 1404-93-9) was prepared and administered via drinking water for 21 consecutive days. Antibiotics were dissolved in autoclaved water, covered in tin foil to avoid degradation of photosensitive compounds and changed every-two days. Control animals received autoclaved water, which was also changed every-two days. Liquid intake was estimated by measuring bottle weights before replenishment to ensure the correct amount of liquid intake and animals were weighed throughout the experiment to ensure body weight was maintained.

2.3. Conditioned place preference (CPP) test

The CPP apparatus (Panlab, Spain) consists of two equal sized compartments with distinct visual cues interconnected by a transparent plexiglass corridor. The general procedure consisted of three phases: habituation, conditioning and testing. During these phases, an empty circular wired mesh (10 cm high and 8 cm diameter) was introduced within each compartment to allow the social contact between animals (i.e., visual and sniffing) while preventing aggressive behavior. The behavioral naïve conspecific mice were habituated to being enclosed in the circular wired mesh for 5, 15 and 30 min on three consecutive days prior to the experiment. During the habituation phase, experimental mice were allowed to move freely between the CPP compartments for 15 min per day on 2 consecutive days. The second day (day 10), the time spent in each compartment was recorded and mice with a strong aversion (≤30 % of the session time) or preference (≥70 %) for either compartment were excluded from the study (9 animals were excluded). On days 11 and 12 mice were left undisturbed in their home cages. Animals were randomly assigned to either a saline (Sal-water n = 7; Sal-ABX n = 7), cocaine (Coc-water n = 10; Coc-ABX n = 12), social interaction (SOC-water n = 8; SOC-ABX n = 8) or cocaine + social interaction (Coc/SOC-water n = 12; Coc/SOC-ABX n = 11) group. Conditioning phase consisted of alternating four stimulus-paired conditioning sessions (cocaine or social interaction) with four vehicle-paired sessions on alternate days. Cocaine (hydrochloride salt, Sigma, St. Louise, USA) at 10 mg/kg dissolved in saline (0.9 % NaCl, 2 ml/kg) or saline, was injected intraperitoneal (i.p.) immediately before placing the mouse into the chamber. When compartments were paired with social interaction during the conditioning phase, each mouse received an i.p. saline injection and placed in the compartment in which another conspecific unfamiliar mouse (age and sex matched) was present, encapsulated in a circular wired mesh allowing some contact between them. The day after the last conditioning session, mice were placed for 15 min drug-free in the middle compartment of the CPP allowing the animal to move freely between the compartments. Place preference in the test session was expressed as a CPP score in seconds (seconds spent in the paired compartment – seconds spent in the unpaired compartment). Behavioral variables in the test session were scored using Ethovision v.16 (Noldus) by a blinded researcher. During the test session, the time spent by the mice interacting actively with the circular wired mesh in the compartments was also analyzed.

2.4. Gut homogenization and cytokine assay

Distal ileum was homogenized with PBS buffer and fetal bovine serum in the presence of protease inhibitors (Roche) in a tissue lyser FAST-PREP. The total homogenate was centrifuged at 17000g for 1 min at 4 °C. Supernatants were aspirated and stored at −80 °C. Cytokine secretion in the gut was assessed using the Proinflammatory Panel 1 (mouse) V-PLEX Kit (*Meso* Scale Discovery, cat. No. K15048D). Values under the curve fit and detection range were excluded. Concentration is expressed in pg/mg tissue.

2.5. Statistical analysis

Statistical analyses were conducted using SPSS v.28 (NY, USA). Results are expressed as mean values ± standard error of the mean (SEM) and individual symbols are shown, when appropriate, for each mouse within the bar graphs. Normal distribution for each measurement reported was evaluated with Shapiro-Wilk normality test. Changes in body weight and liquid consumption were analyzed using a two-way repeated measures ANOVA followed by a Fisher LSD test. CPP scores and proinflammatory levels of cytokines in the ileum were analyzed using three-way ANOVA followed by a Fisher's LSD post hoc test. Time actively interacting with the wired mesh was analyzed using a two-way ANOVA followed by a Fisher's LSD post hoc test. Two-tailed Student's *t*-test was used to analyze the differences between water and ABX-treated animals for colon length, spleen and cecum weight as well as to determine differences in CPP scores between Coc-water and Coc/SOC-ABX mice. The level of significance was set at $p \leq 0.05$.

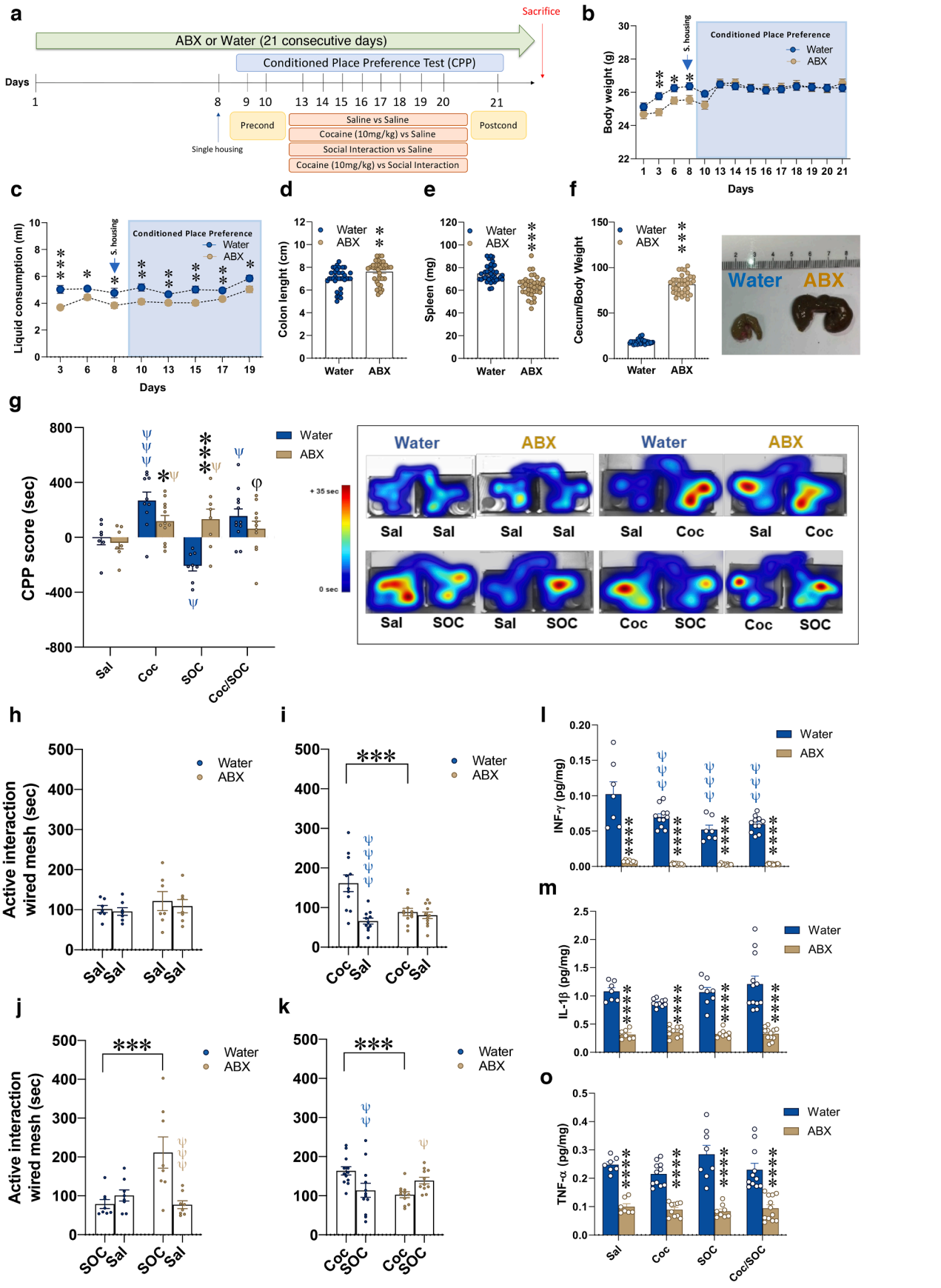
3. Results

3.1. Effects of depleting the gut microbiota with a cocktail of non-absorbable ABX on biometric measures and fluid intake

First, we examined the effects of depleting the microbiome with a cocktail of non-absorbable ABX (Fig. 1a) in adult C57BL/6 male mice. The ABX intervention was well tolerated despite inducing an initial reduction in body weight that was eventually regained (Fig. 1b, Two-way ANOVA repeated measures significant main effect of Time $F_{3.932, 282.4} = 67.3$; $p = 0.0001$ and a two-way interaction ABX × Time $F_{13,936} = 13.9$; $p = 0.0001$). Mice also showed a significant reduction in ABX liquid intake (Fig. 1c, main effects of Time $F_{3.315, 153.4} = 8.3$; $p = 0.0001$ and ABX $F_{1,80} = 29$; $p = 0.0001$), suggesting a certain aversive taste effect, although they show a constant intake, ensuring an effect of ABX on the gut microbiota over the duration of the experiment. ABX administration has been associated with changes in physiological readouts of the gastrointestinal tract. An increase in colon length (Fig. 1d, two-tailed *t*-test: $p < 0.001$; $t = 2.740$) and cecum weight (Fig. 1f, $p < 0.0001$; $t = 37.7$) was observed as well as a decrease in spleen after ABX administration (Fig. 1e, $p < 0.0001$; $t = 5.672$).

3.2. Depleting the gut microbiota alters behaviourally conditioned responses to different rewards

To evaluate if alterations in the gut microbiota affected the behavioral rewarding effects of different stimuli (cocaine and social interactions) we used a conditioned place preference (CPP) test in mice exposed to ABX or water for 21 consecutive days. As seen in Fig. 1g, a three-way ANOVA revealed a significant effect of cocaine ($F_{1,67} = 21.8$,



(caption on next page)

Fig. 1. Effects of ABX on physiological measures and brain reward responses in mice. a) Experimental design. b) Changes in body weight (g) and c) liquid consumption (ml) in water and ABX-treated mice. d) Increase in colon length (cm) in animals exposed to ABX. e) Decrease in spleen weight (mg) in animals exposed to ABX. f) Increase in caecum weight and size in animals exposed to ABX. g) Left: CPP scores in water or ABX-exposed mice for different reward stimuli. Right: representative heatmaps illustrating the time spent in each compartment during the CPP test. h-k) Time spent interacting with the circular wired mesh during CPP test. l-o) Level of proinflammatory cytokines in ileum. Data represents mean ± SEM of each measurement for each group. Two-way ANOVA repeated measures followed by Fisher LSD test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs water when comparing the effects of ABX along the time. Three-way ANOVA followed by fisher LSD: * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ vs water exposed animals, Ψ $p < 0.05$, ΨΨΨ $p < 0.001$ vs Sal-water (blue) or Sal-ABX (yellow) in CPP test and proinflammatory levels of cytokines. Two-way ANOVA followed by fisher LSD: *** $p < 0.001$ vs water-exposed animals, Ψ $p < 0.05$, ΨΨ $p < 0.01$, ΨΨΨ $p < 0.001$ vs stimulus paired in water (blue) or ABX (yellow) exposed animals in the interaction with the circular wired mesh during CPP test. Two-tail Student's t tests for pair comparisons of physiological measures between water and ABX exposed mice ** $p < 0.01$, *** $p < 0.001$ vs water or for comparison in CPP test φ $p < 0.05$ vs Coc-water. Sal: saline; Coc: cocaine, SOC: social interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$p = 0.0001$), a two-way interaction ABX × Cocaine ($F_{1,67} = 12.4$; $p = 0.0008$); ABX × Sociability ($F_{1,67} = 7.8$, $p = 0.007$) and a three-way interaction ABX × Cocaine × Sociability ($F_{1,67} = 4.1$; $p = 0.05$). Specifically, we observed that ABX-exposed mice showed a reduction in cocaine CPP score compared to cocaine water-exposed mice (Coc-water vs Coc-ABX; $^{*}p < 0.05$). Despite this reduction in cocaine-associated reward effects in mice with a depleted gut microbiota, some degree of cocaine conditioning was observed when compared to ABX exposed saline-conditioned animals (Sal-ABX vs Coc-ABX; $^{\Psi}p < 0.05$). On the other hand, when evaluating the rewarding effects of a natural stimulus such as social interactions, we observed that ABX-treated mice showed an increased CPP score for social stimuli when compared to social water-exposed mice (SOC-water vs SOC-ABX; $^{***}p < 0.001$). In addition, increased social CPP scores were observed in ABX-exposed when compared to ABX saline-conditioned mice (Sal-ABX vs SOC-ABX; $^{\Psi}p < 0.05$), an effect that was not observed between social and saline water-exposed mice, showing a decreased social CPP indicating aversiveness to the stimulus (Sal-water vs SOC-water; $^{\Psi}p < 0.05$). In contrast, no significant differences in cocaine preference were observed when mice were conditioned concurrently for cocaine in one compartment and social interactions in another. However, a significant increase in cocaine CPP score was observed in Coc/SOC water-exposed mice compared to Sal-water (Sal-water vs Coc/SOC-water; $^{\Psi}p < 0.05$), an effect not observed in ABX-exposed mice (Sal-ABX vs Coc/SOC-ABX; n.s.), demonstrating a reduction in cocaine preference in animals with depleted gut microbiota in combination with the presence of social stimuli. Furthermore, a reduction in cocaine preference in microbiota-depleted mice with social stimuli was observed when compared to Coc-water mice (Coc-water vs Coc/SOC-ABX; $^{\varphi}p < 0.05$), suggesting that microbiota depletion in combination with the presence of social stimuli is capable of blocking the drug rewarding effects exerted by cocaine in the CPP test.

During the postconditioning sessions, the active interaction time with the circular wired mesh inside the compartments was also evaluated. The results showed in cocaine-conditioned mice an increased interaction with the circular wired mesh associated with the cocaine compartment in animals exposed to water, an effect that was not observed in animals treated with ABX (Fig. 1i), reaffirming that the microbiota plays an important role in drug reward responses (effect of cocaine $F_{1,40} = 16.3$; $p = 0.0002$; ABX $F_{1,40} = 5.2$; $p = 0.03$; and an interaction cocaine × ABX $F_{1,40} = 11.7$; $p = 0.002$). In this regard, we also observed an increase in the active interactions associated with social stimuli in the ABX-exposed animals (effect of social interaction $F_{1,28} = 6.1$; $p = 0.02$; ABX $F_{1,28} = 5.7$; $p = 0.02$; and an interaction social interaction × ABX $F_{1,28} = 11.8$; $p = 0.002$), suggesting an increase in the rewarding effects of social stimuli in the microbiota-depleted mice (Fig. 1j). Finally, when both stimuli were conditioned concurrently an increase in interaction with the cocaine-associated wired mesh was observed in animals exposed to water, an effect that was not observed in the ABX-treated mice where they showed an increase in the interaction time with the social wired mesh when compared with the cocaine (interaction cocaine/social interaction × ABX $F_{1,42} = 12.4$; $p = 0.001$), suggesting a decrease in the rewarding effects of the drug and an increase in the social rewarding effects (Fig. 1k).

3.3. ABX administration reduces intestinal inflammation in ileum

Alterations in gut microbiota are associated with intestinal inflammation, increasing cytokine production which might act in the central nervous system. It has been suggested that increases in proinflammatory cytokines might promote drug addictive behaviors as well as social withdrawal (Eisenberger et al., 2017; Hofford et al., 2019). We observed that the administration of ABX reduced the level of proinflammatory cytokines (IFN-γ, IL-1β and TNF-α) in the ileum (Fig. 1l-o). Specifically, a three-way ANOVA showed a significant effect of ABX ($F_{1,62} = 255.4$; $p = 0.0001$) and Sociability ($F_{1,62} = 14.5$; $p = 0.0003$), a two-way interaction ABX × Sociability ($F_{1,62} = 10.9$; $p = 0.002$); Sociability × Cocaine ($F_{1,62} = 6.8$; $p = 0.01$), and a three-way interaction ABX × Cocaine × Sociability ($F_{1,62} = 4.6$; $p = 0.04$) on IFN-γ levels (Fig. 1l). On the other hand, three-way ANOVA only detected a significant effect of ABX ($F_{1,65} = 171.1$; $p = 0.0001$ and $F_{1,65} = 163.5$; $p = 0.0001$) for IL-1β and TNF-α respectively (Fig. 1m–o).

4. Discussion

These results indicate that the gut microbiota plays an important role in modulating reward processes. Depletion of the gut microbiota with an antibiotic cocktail attenuated cocaine place preference while enhancing preference for social stimuli. Moreover, the combination of depleting the gut microbiota in the presence of social stimuli was able to attenuate cocaine reward and may constitute an important component in altering drug effects. These effects occur independent from the microbiota-depletion effects on the immune system.

We observed that depletion of the gut microbiota attenuated the rewarding effects to non-natural stimuli such as cocaine and increased social rewards. These results indicate that gut microbes are essential for inducing a large part, but not all of the cocaine rewarding effects, suggesting that gut microbiota is able to modulate drug reward responses. Previous studies demonstrated differential effects of depleting the gut microbiota on drug rewarding effects. Specifically, it was observed that altering the gut microbiota with antibiotics administered orally increased conditioning and sensitization to sub-threshold doses of cocaine in mice (Kiraly et al., 2016). On the contrary, other studies are in line with the results observed in the present work. In particular, it was reported that oral antibiotics decreased the conditioning response to cocaine (Lee et al., 2018), and reduced conditioning formation and sensitization to morphine (Hofford et al., 2021). The discrepancies observed may be due in part to the different compositions of the antibiotic cocktails used, as well as the duration of the interventions, which may play an important role in the drug-induced reward responses. Although this is not the first study to evaluate the role of the gut microbiota in the reinforcing effects of drugs, it is to our knowledge the first to assess its role in reward towards social stimuli, demonstrating a differential effect on natural stimuli enhancing social interactions in mice with a depleted gut microbiota. Social interactions with conspecifics are often described as rewarding and capable of inducing CPP in

rodents, but this process has been described as sensitive to a variety of parameters such as age, strain, context, time and the quality of the experience (Cann et al., 2020). The aversion to social interactions observed in water-exposed animals may be due in large part to the fact that social interactions are not as reinforcing in adult mice as in other rodent species. In addition, the receptivity of the conspecifics and the prior time of isolation of the experimental animals may play a key role in the reinforcing effects of social interactions. The potential reinforcing value of positive social interactions has been described as a protective factor reducing the risk of drug addiction (El Rawas et al., 2020), although other studies indicate that cocaine is able to reduce motivation for social interactions (Liu et al., 2016), and that negative social interactions facilitate the development and maintenance of drug abuse (Han et al., 2015). The results obtained show that social interactions alone were not able to reduce cocaine conditioning in our experimental parameters. The lack of social conditioning observed in the animals exposed to water could be the reason for not observing a reduction in cocaine conditioning when both stimuli were applied concurrently, although it should be noted that this trend towards an aversion did not lead to an increase in cocaine conditioning *per se*. Reductions in cocaine conditioning responses were only observed in animals exposed to antibiotics and social interactions, whereas the reduction of the effects of antibiotics to drug conditioning and the increase to social interactions showed that both approaches constitute important components that buffer the effects of the drug.

Inflammatory cytokines can modulate neural activity of dopaminergic neurons of the reward pathways, altering neurotransmitter signalling and reuptake (Felger and Miller, 2012). Previous work demonstrated that cocaine interacts with toll-like receptor 4 inducing proinflammatory signals necessary for the rewarding effects of cocaine (Northcutt et al., 2015). In addition, the administration of anti-inflammatory drugs reduced the elevated levels of proinflammatory cytokines in brain reward areas, attenuating reward responses to cocaine (Ferrer-Pérez et al., 2018). Moreover, recent evidence reveals that the immune system, and more specifically inflammatory processes, are also powerful regulators of neural circuits supporting social behavior (Eisenberger et al., 2017). Sub-threshold doses of TNF-α and IL-1β administered peripherally or intracerebroventricularly reduced social behavior in mice (Bluthé et al., 1994). IL-1 brain receptors have been described as necessary and sufficient to drive alterations in social interactions after IL-1β administration (Liu et al., 2019). In line with this, IFN-γ has also been implicated in modulations of disorders characterized by social dysfunction, suggesting a co-evolutionary link between social behavior and immune response driven by IFN-γ signalling (Filiano et al., 2016). Such a generalized reduction in proinflammatory cytokines in the ileum could be responsible for the decrease in the rewarding effects of cocaine as well as the increase in the rewarding effects of social interactions observed in ABX-exposed animals, which may indicate that the microbiota may modulate brain reward responses via the immune system (Hofford et al., 2019). Future studies should investigate how specific alterations in the gut microbiome may modulate specific reward and social circuits in the brain that may underpin the development of addictive disorders via immune system interactions. Such studies would be in line with the concept that there are overlapping effects of abused drugs, inflammation, and microbiome alterations on fronto-limbic circuitry (Carbia et al., 2021).

5. Conclusions

Taken together, the data presented provide important new evidence demonstrating the role of the gut microbiota in modulating brain reward responses and the importance of the social stimuli in countering drug-associated effects. Indeed, depleting the gut microbiota reduces the reward responses to non-natural rewards such as cocaine and increases social rewards. Moreover, the presence of a social stimulus in combination with antibiotics were able to reduce the preference for cocaine, suggesting that targeting the gut microbiota could constitute a valuable therapeutic approach for drug reward-related conditions alone or in combination with social factors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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