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The gut microbiota metabolite indole increases emotional responses and adrenal medulla activity in chronically stressed male mice

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ABSTRACT

Background and aims: The gut microbiota produces metabolites that are an integral part of the metabolome and, as such, of the host physiology. Changes in gut microbiota metabolism could therefore contribute to patho-physiological processes. We showed previously that a chronic and moderate overproduction of indole from tryptophan in male individuals of the highly stress-sensitive F344 rat strain induced anxiety-like and helplessness behaviors. The aim of the present study was to extend the scope of these findings by investigating whether emotional behaviors of male mice that are moderately stress-sensitive but chronically exposed to environmental stressors would also be affected by indole.

Methods: We colonized germ-free male C3H/HeN mice with a wild-type indole-producing *Escherichia coli* strain, or with the non-indole producing mutant. Gnotobiotic mice were subjected to an unpredictable chronic mild stress procedure, then to a set of tests aimed at assessing anxiety-like (novelty and elevated plus maze tests) and depression-like behaviors (coat state, splash, nesting, tail suspension and sucrose tests). Results of the individual tests were aggregated into a common z-score to estimate the overall emotional response to chronic mild stress and chronic indole production. We also carried out biochemical and molecular analyses in gut mucosa, plasma, brain hippocampus and striatum, and adrenal glands, to examine biological correlates that are usually associated with stress, anxiety and depression.

Results: Chronic mild stress caused coat state degradation and anhedonia in both indole-producing and non-indole producing mice, but it did not influence behaviors in the other tests. Chronic indole production did not influence mice behavior when tests were considered individually, but it increased the overall emotionality z-score, specifically in mice under chronic mild stress. Interestingly, in the same mice, indole induced a dramatic increase of the expression of the adrenomedullary *Pnmt* gene, which is involved in catecholamine biosynthesis. By contrast, systemic tryptophan bioavailability, brain serotonin and dopamine levels and turnover, as well as expression of gut and brain genes involved in cytokine production and tryptophan metabolism along the serotonin and kynurenine pathways, remained similar in all mice.

Conclusions: Chronic indole production by the gut microbiota increased the vulnerability of male mice to the adverse effects of chronic mild stress on emotional behaviors. It also targeted catecholamine biosynthetic pathway of the adrenal medulla, which plays a pivotal role in body's physiological adaptation to stressful events. Future studies will aim to investigate the action mechanisms responsible for these effects.

Abbreviations: DA, dopamine; DOPAC, dihydroxyphenyl acetic acid; ELISA, enzyme-linked immunosorbent assay; GF, germ-free; 5-HIAA, 5-hydroxyindole acetic acid; HPA, hypothalamic-pituitary-adrenal; HPLC, high performance liquid chromatography; 5-HT, serotonin; HVA, homovanillic acid; MDD, major depressive disorder; RT-PCR, reverse transcription polymerase chain reaction; SPF, specific pathogen free; UCMS, unpredictable chronic mild stress

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1. Introduction

The human gut harbors a microbial ecosystem, the gut microbiota, whose collective genome encodes 500 times more genes than the human genome (Li et al., 2014). Since several years, experiments using germ-free (GF) animals, or inducing gut microbiota shifts in conventional animals with antibiotics, probiotics or prebiotics, have shown that gut microbiota influences brain and behavior, thus undoubtedly proving the existence of a gut microbiome-brain axis (Cryan and Dinan, 2012). Based on this concept, investigations were undertaken to find out if gut microbiota from people with anxiety disorder or major depressive disorder (MDD) had distinct characteristics. Differences in the diversity or relative abundances of some bacterial groups occur between patients and healthy controls, but with variance among studies (Naseribafrouei et al., 2014; Jiang et al., 2015; Zheng et al., 2016; Kelly et al., 2016; Stevens et al., 2018; Valles-Colomer et al., 2019). However, a recent, randomized, double-blind, placebo-controlled study of patients with mild to moderate symptoms of anxiety and depression showed that a 6-week consumption of the probiotic *Bifidobacterium longum* NCC3001 reduced depression scores, and responses to negative emotional stimuli in amygdala and fronto-limbic regions (Pinto-Sanchez et al., 2017). In addition, fecal microbiota transplantation experiments from patients suffering from anxiety disorder or from MDD to GF mice and rats resulted in anxiety-like and depressive-like behaviors in the recipient animals (Zheng et al., 2016; Kelly et al., 2016; De Palma et al., 2017). These behavioral disturbances were accompanied by alterations of the host's metabolome, as assessed in the serum (Zheng et al., 2016; De Palma et al., 2017), feces and brain (Zheng et al., 2016), and by dysregulated tryptophan metabolism, as indicated by an increased plasma kynurenine/tryptophan ratio (Kelly et al., 2016).

The gut microbiota metabolizes tryptophan mainly into indole through the action of the enzyme tryptophanase encoded by the *tnaA* genes. Further metabolism of indole by intestinal and hepatic xenobiotic metabolizing enzymes leads to a family of oxidized and conjugated derivatives (Lee et al., 2015). Among them, oxindole and isatin are neurodepressant molecules. Following a systemic injection, both decrease locomotor activity in rats; in addition, oxindole induces a loss of the righting reflex and a hypotension, and isatin promotes anxiety-like and depressive-like behaviors, particularly helplessness (Bhattacharya and Acharya, 1993; Carpenedo et al., 1998; Medvedev et al. 2005). Indole production may vary according to inter-individual gut microbiome differences. Indeed, by studying *in silico* the *tnaA* gene distribution within the microbiome of 203 individuals, we showed that the richness of non-redundant *tnaA* genes varied from five to 100 (Jaglin et al., 2018). This suggests that humans could be exposed to different amounts of indole, depending on their gut microbiota profile. This raises the question of whether an excessive production of indole, due to natural characteristics of an individual's gut microbiota, or to its disturbance, could contribute to the onset of anxiety and/or depression symptoms. In this line, we previously showed that an excessive production of indole in the hindgut of male rats induced anxiety-like behavior and helplessness (Jaglin et al., 2018). That study used the F344 rat strain, which is known to be genetically prone to anxiety (Sarrieau et al., 1998). In the present study, we hypothesized that an excessive production of indole by the gut microbiota may enhance the behavioral impairments induced by a chronic exposure to environmental stressors. To test this hypothesis, we exposed gnotobiotic male mice mono-associated with a wild-type *E. coli* strain naturally producing indole, or with a knockout non indole-producing mutant, to an unpredictable chronic mild stress procedure (UCMS; Yalcin et al., 2008). To avoid interference with a genetic predisposition to stress, we chose the C3H/HeN mouse strain, which is moderately stress-sensitive (Ibarguen-Vargas et al., 2008). At the end of the UCMS procedure, we assessed anxiety-like and depressive-like behaviors with several tests. We also analyzed biological pathways associated with anxiety, depression and stress. Specifically, we examined the neuroendocrine response to stress by analyzing the expression of genes encoding enzymes involved in the biosynthetic pathways of adrenal corticosterone and catecholamines. Serotonin (5-HT) and dopamine (DA) have been identified as two neurochemical systems in the brain that are unbalanced in depression and anxiety. After an UCMS procedure, many studies show a reduction in 5-HT content in hippocampus, and some of them a reduction in DA content in striatum (Hill et al. 2012). Therefore, we investigated whether indole production affects the serotonergic and dopaminergic systems in those brain areas. Numerous data have also highlighted the importance of low-grade inflammation in the pathophysiology of depression (Dantzer et al. 2008; Dooley et al., 2018). Indeed, inflammatory conditions promote the metabolism of tryptophan along the kynurenine pathway at the expense of the 5-HT pathway, which leads to neurotoxic metabolites; they also impair the production of tetrahydrobiopterin, an important cofactor for the 5-HT pathway (Vancassel et al., 2018). In addition, recent studies showed that gut microbiota disturbances may be involved in the immune mechanisms that influence mood and behavior leading to depression (Waclawiková and El Aidy, 2018). Consequently, we analyzed parameters relevant to inflammation in the mucosa of gut compartments inhabited by gut bacteria, namely the ileum and colon,

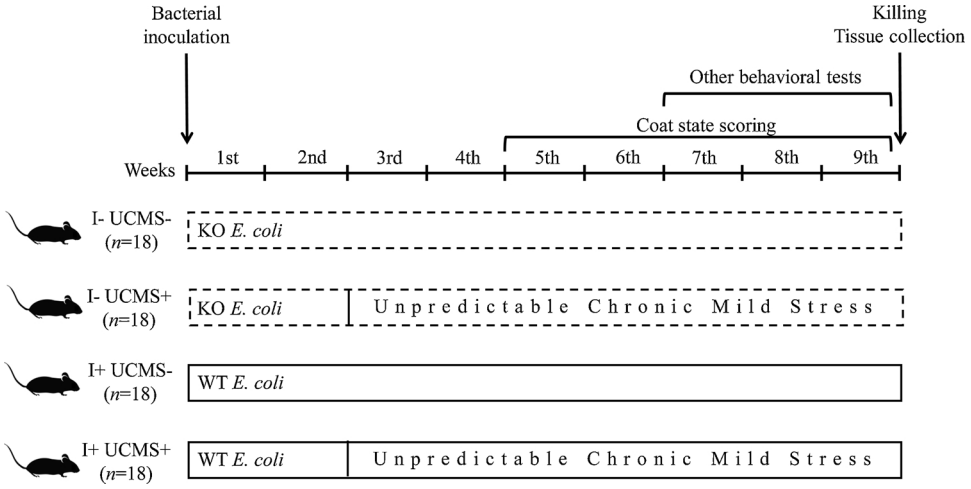

Fig. 1. Study design timeline.
I- mice (–) and I+ mice (—) were mono-associated with a non indole-producing *E. coli* strain (knockout, KO) or with an indole-producing *E. coli* strain (wild-type, WT). UCMS + mice were subjected to an unpredictable chronic mild stress procedure (UCMS) for 7 weeks, starting at the end of the 2nd week. For details on the UCMS procedure and the behavioral tests, see Material and methods section and Supplementary material and methods.

as well as in plasma and in hippocampus and striatum.

2. Materials and methods

2.1. Bacterial strains

The National Institute of Genetics (Mishima, Japan) provided the wild-type *E. coli* BW25113 strain, and the single-gene knockout JW3686 mutant invalidated for the *tnaA* gene (Baba et al., 2006). The strains were stored at −80 °C in Brain Heart Infusion broth with added glycerol (final concentration 40%). For inoculation of GF mice, they were grown in lysogeny broth overnight at 37 °C in aerobic conditions. The cultures were distributed in sterile vials and transferred in sterile conditions into the isolators.

2.2. Animals

Seventy-two 4-week old male GF mice of the C3H/HeN strain were obtained locally from the GF rodent breeding facility Anaxem (Germ-free animal facilities of the Micalis Institute, INRAE, France). They were randomly separated in four groups of 18 mice and transferred in four sterile Plexiglas isolators (Eurobioconcept, Paris, France). The isolators were ventilated with HEPA-filtered sterile air under positive pressure. They were fitted with DPTE® aseptic transfer systems (Getinge, Les Ulis, France) allowing sterile connection of containers (Getinge, Les Ulis, France) to import sterile consumables. Inside the isolators, the animals were housed in collective cages (4-5 mice/cage) containing sterile bedding made of wood shavings. They had free access to autoclaved tap water and a γ-irradiated (45 kGy) standard diet (R03; Scientific Animal Food and Engineering, Augy, France). The animal room was maintained at 20-24 °C and kept on a 12 h light/dark cycle (lights on at 7:30 a.m.).

2.3. Animal experimental design

Fig. 1 depicts the experimental design. After a 1-week acclimatization period, two groups of mice were inoculated intragastrically with the wild-type strain, and the two other groups with the mutant strain (0.2 mL culture/mouse). Thereafter, the bacterial status was monitored every 2 weeks by microscopic examination, and cultures, of freshly voided feces. The mice mono-associated with the wild-type strain or with the mutant strain were named "I+" mice and "I−" mice, respectively. Two weeks after microbial colonization, one "I−" group and one "I+" group were transferred from collective cages to individual cages, and exposed to a UCMS procedure for 7 weeks. The four groups were subsequently named: "I− UCMS−", "I− UCMS+", "I+ UCMS−", and "I+ UCMS+". Seven tests aimed at evaluating anxiety- and depressive-like behaviors were carried out in the following order: coat state assessment from the 3rd to the 7th week of the UCMS period; novelty, tail suspension, splash, nesting, sucrose consumption and elevated plus maze tests from the 5th to the 7th week of the UCMS period, with intervals of at least 2 days between the tests. At the end of the 5th week, non-stressed mice were transferred from collective cages to individual cages in preparation for the splash, nesting and sucrose consumption tests. Thereafter, all mice remained in individual cages until the end of the experiment. Fresh feces were collected from each mouse before the beginning and at the end of the UCMS period for tryptophan and indole assays. To ascertain that indole was absorbed by the intestinal mucosa and further metabolized, urine was collected in a subset of eight mice/group to measure excretion of 3-indoxylsulfate, which is the major final metabolite of indole. For this purpose, mice were placed 24 hours in metabolic cages at the beginning of the UCMS period. Fecal and urine samples were stored in cryotubes at −80 °C. All mice were killed by decapitation immediately after the last behavioral test, namely the elevated plus maze.

All procedures were carried out in accordance with the European guidelines for the care and use of laboratory animals and approved by the ethics committee of the INRAE Research Center at Jouy-en-Josas (approval reference: APAFIS#6826-2016070416487526 v2).

2.4. Unpredictable chronic mild stress procedure

We used the UCMS procedure described by Yalcin et al. (2008). The "I− UCMS+" and "I+ UCMS+" mice were subjected for 7 weeks to different types of mild stressors, including disturbed bedding (change or removal of sawdust, wet sawdust, 8 marbles placed on the sawdust surface), cage exchange (mice were placed in cages emptied of fellow mice), cage tilting at 45°, crowding, restraint in a drilled Falcon® tube, water or food deprivation, altered length and time of light/dark cycle. The stressors were applied in a semi-random manner, and the sequence was changed every week to prevent the mice from getting accustomed to it (Table 1).

2.5. Behavioral tests

Behavioral test procedures are detailed in the Supplementary Material and Methods. Within a day, the tests were performed between 10:00 a.m. and 04:00 p.m. All were carried out inside the isolators, except for the elevated plus maze, whose device is too large to be placed in an isolator. Therefore, this test was carried out at the end of the experiment, in a dedicated conventional room. Mice were removed from the isolators and housed in a conventional room for 1-2 h to allow them to adapt prior to the test (Crumeyrolle-Arias et al., 2014).

Tests were videotaped, and two independent observers, whose results were averaged, analyzed videos blindly. We then applied z-normalization across data obtained in the seven behavioral tests, as described previously by Guilloux et al. (2011). The z-scores indicate how many standard deviations (s) an observation (X) is above or below the mean of a reference group (m), *i.e.* $Z = (X - m) / s$. "I− UCMS−" mice were chosen as the reference group. Z-scores for behavioral data were first computed within each test, then across the tests for equal weighting of the seven tests that constituted the final emotionality z-score.

Table 1
Nature, duration and succession of the stressors applied during a typical week of the unpredictable chronic mild stress (UCMS) procedure. The UCMS procedure lasted 7 weeks. The order of the stressors varied each week, except for the weekend light/dark stressor.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday Sunday
09:00 a.m.		cage tilting				
10:00 a.m.	marbles on sawdust				crowding	
11:00 a.m.						
12:00 a.m.				wet sawdust		
01:00 p.m.				sawdust removed	cage tilting	
02:00 p.m.			cage exchange			
03:00 p.m.		restraint		new sawdust		
04:00 p.m.						
Overnight	water deprivation	light on		food deprivation		light on for a short time

2.6. Euthanasia and fluid and tissue collection

Mice were killed by decapitation after 7 weeks of UCMS, immediately after the elevated plus maze test (Fig. 1). The truncal blood was collected in a tube coated with an anticoagulant solution; after centrifugation (3000 g, 20 min, 4 °C), the plasma was aliquoted into cryotubes and frozen at −80 °C. The anticoagulant agent was sodium EDTA 0.5 M for corticosterone assay (8 mice/group), and heparin for tryptophan and kynurenine assays (10 mice/group). Brains were quickly removed from the craniums, and the hippocampus and striatum were dissected out on ice, weighed, frozen in dry ice, then stored at −80 °C for monoamine and gene expression analysis, in the right and left hemispheres, respectively. The adrenal glands were collected, weighed, and transferred into cryotubes filled with RNA*later*™ stabilization solution (Invitrogen, Carlsbad, CA, USA). The intestinal tract was removed and dissected on ice; the small intestine and the colon were gently flushed with PBS (pH 7.4) to remove residual digestive contents, 1-cm long tissue fragments were excised from the ileum and the upper part of the colon, and transferred into cryotubes filled with RNA*later*™ stabilization solution. After 24 h at room temperature, the cryotubes containing the adrenal glands and the intestinal tissues were stored at −80 °C until gene expression analysis.

2.7. Biochemical analyses

Fecal tryptophan and indole, as well as urinary 3-indoxylsulfate, were analyzed by HPLC with fluorescence detection as described in Jaglin et al. (2018). For tryptophan analysis in plasma, thawed samples were added with 10 % (v/v) perchloric acid 4 N to precipitate proteins. After centrifugation (8000 g, 15 min, 4 °C), supernatants were collected and analyzed by HPLC with fluorescence detection in the same conditions as those used for fecal samples. Plasma kynurenine was measured by a method of competitive enzyme linked immunoassay, using the IDK® kynurenine high sensitive ELISA kit (Immundiagnostik, Bensheim, Germany). Plasma total corticosterone was measured with an in-house radioimmunoassay, using a highly specific antibody provided by H. Vaudry (University of Rouen, France), as previously described (Richard et al., 2010). Plasma free corticosterone was measured by isotopic dilution and plasma ultrafiltration using a Centrifree filter device (YM membranes 30 K, Millipore) as in Richard et al. (2010). The percentage of free corticosterone was calculated as the ratio of [cpm in the filtrate (free corticosterone)]/[cpm in the filtrate + cpm in the retentate (bound corticosterone)]. Free corticosterone concentration was then calculated as the percentage of free corticosterone multiplied by total corticosterone concentration. Contents of 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA), DA and its metabolites dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA), were measured in the hippocampus and the striatum by HPLC coupled with electrochemical detection, as described previously (Crumeyrolle-Arias et al., 2014).

2.8. Quantification of mRNA expression levels by real-time RT-PCR

Real-time RT-PCR was used to quantify the mRNA of genes (i) encoding enzymes of the corticosterone (*Hsd3b, Cyp21a1* and *Cyp11b1*) and catecholamine (*Th*, *Dbh*, *Pnmt*) biosynthetic pathways in the adrenal glands, (ii) encoding cytokines (*Il1b*, *Il6, Il10, Ifng*, *Tnfa*) or involved in the 5-HT (*Tph1, Tph2*) and kynurenine pathways (*Ido1*, *Kmo*, *Aadat*) in the brain and gut mucosa, and (iii) involved in the tetrahydrobiopterin pathway (*Gch1*) in the brain. Methodological details are reported in the Supplementary Material and Methods.

2.9. Statistical analyses

Fecal concentrations of bacterial cells and tryptophan, and urinary concentrations of 3-indoxylsulfate, were compared between "I−" and "I+" mice, using an unpaired *t*-test for normally distributed data with equal group variances, and a Mann-Whitney test otherwise. The effect of UCMS on fecal indole concentration was determined by comparing "I + UCMS−" and "I+ UCMS+" groups, before and after the UCMS procedure, with a two-way ANOVA for repeated measurements, followed by the Bonferroni pairwise multiple comparison test. The effects of indole production, UCMS and their interaction on behavior, and gut, blood, brain, and adrenal glands parameters were determined by comparing the four groups "I− UCMS−", "I− UCMS+", "I+ UCMS−", and "I+ UCMS+". Two-way ANOVA, followed by the Bonferroni pairwise multiple comparison test, was used for normally distributed data with equal group variances. The Kruskal-Wallis test, followed by the Dunn's pairwise multiple comparison test, was used otherwise.

Normally distributed data with equal group variances were expressed as means ± standard errors of the means. Non-normally distributed data, or with unequal group variances, were expressed as medians (interquartile ranges). The level of significance was set at $P < 0.05$. Calculations were performed with the GraphPad Prism software (version 7.03, La Jolla, CA, USA).

3. Results

3.1. Validation of the mouse model mono-associated with a bacterial strain expressing or deficient for the tryptophanase activity

We ascertained that both wild-type and mutant *E. coli* strains colonized the gut of the gnotobiotic mice at the same level by measuring their fecal concentrations. This was indeed the case as the fecal concentrations of the wild-type strain in the "I+" mice, and of the mutant strain in the "I−" mice, were 10.3 ± 0.1 and 10.4 ± 0.1 $\log_{10}$ CFU/g wet feces, respectively (unpaired *t*-test; $P > 0.05$; $n = 35$ mice/group).

We also checked that the "I−" mice did not produce indole, while the "I+" mice did, by measuring the indole and tryptophan concentrations in the feces. As expected, the fecal concentration of indole was null in the "I−" mice, while it reached 56.1 ± 2.4 nmol/g wet feces in the "I+" mice ($n = 36$ mice/group). Accordingly, the fecal concentration of tryptophan was much higher in the "I−" mice than in the "I+" mice: 298.5 (91.2) *vs.* 157.4 (43.2) nmol/g wet feces, respectively (Mann-Whitney test; $P < 0.0001$; $n = 36$ mice/group).

The UCMS procedure had no effect on the fecal concentrations of indole: these were 60.4 ± 3.3 and 51.8 ± 3.2 nmol/g wet feces before starting the UCMS procedure, and 54.3 ± 2.5 and 49.1 ± 3.0 nmol/g wet feces at the end of the UCMS procedure, in the "I+ UCMS−" and "I + UCMS+" groups, respectively (2-way ANOVA for repeated measurements followed by a Bonferroni multiple comparison test; $P > 0.05$). These concentrations were of the same order of magnitude as those measured elsewhere in conventional mice fed the same standard diet (Mir et al., unpublished results).

The indole produced in the hindgut by the wild-type strain resulted in the circulation of mammalian indole derivatives in the body, as shown by the presence of 3-indoxylsulfate in the urine of the "I+" mice. 3-Indoxylsulfate concentration was 108.2 (35.9) nmol/mL in the "I+" mice while it was null in the "I−" mice ($n = 8$ mice/group).

3.2. Indole increased the adverse effect of chronic mild stress on emotional behaviors

The mice must have passed all the tests to be taken into account in the calculation of the emotionality z-score. Therefore, if a mouse did not meet the criteria needed to validate a test, it was removed from all the tests. It was so in two tests. In the sucrose consumption test, the mice must drink at both bottles and, in the elevated plus maze, the number of visits in at least one arm should be ≥ 3 (Pellow et al., 1985). Based on those criteria, 12 out of the 18 "I− UCMS−" mice, 10 out of the 18 "I− UCMS+" mice, 11 out of the 18 "I+ UCMS−" mice and 11

out of the 18 "I+ UCMS+" mice were retained. The results of the parameters measured in each test, as well as the z-scores computed from the results of each test, are illustrated in Fig. 2.

Regarding the coat state assessment, the weekly scores of each animal were averaged between the 3rd and the 7th week of UCMS, and the average score was taken as an index of a depression-like state induction (Yalcin et al., 2008). This index, and the resulting coat state z-score, were different among groups (Kruskal-Wallis test; $P < 0.0001$). Both were greater in "I–UCMS+ mice than in "I–UCMS−" mice ($P < 0.01$), and in "I+UCMS+" mice than in "I+UCMS−" mice ($P < 0.001$).

In the novelty test, the latency time to go to the unknown object was similar in all groups. In contrast, the number of crossed squares differed among groups (Kruskal-Wallis test; $P < 0.01$), with "I+UCMS+" mice crossing more squares than "I+UCMS−" mice ($P < 0.05$), which indicates their locomotor activity was increased, while there was no difference between "I–UCMS+" and "I–UCMS−" mice. The z-scores computed from those two parameters differed among groups (Kruskal-Wallis test; $P < 0.05$), but pairwise comparisons did not show significant differences. In the tail suspension test, the immobility time and the computed z-score were similar in all groups. This was the same with the nesting test, in which the nest quality score and the resulting z-score were similar in all groups, and with the splash test, in which the latency time before the first grooming and the groomings' overall time, as well as the resulting z-score, were similar in all groups. In the sucrose consumption test, the sucrose consumption and the computed z-score differed among groups (Kruskal-Wallis test; $P < 0.001$): pairwise comparisons indicated a lower sucrose consumption, *i.e.* an anhedonic behavior, in all chronically stressed mice, whether their gut microbiota produced indole ("I+ UCMS+" mice *vs* "I+ UCMS−" mice, $P < 0.01$), or not ("I–UCMS+" mice*vs* "I− UCMS−" mice, $P < 0.05$). Finally, in the elevated plus maze test, all groups spent the same time and paid the same number of visits to the aversive open arms, compared to the time spent and the number of visits in both closed and open arms; consequently, z-scores computed from these data were similar in all groups.

Z-scores of the seven tests assessing anxiety-like and depression-like behaviors were collectively integrated to lead to an overall emotionality z-score. This one differed among groups (Kruskal-Wallis test; $P < 0.01$), and pairwise comparisons indicated that it was similar between "I–UCMS–" and "I–UCMS+" mice, while it was greater in "I+UCMS+" mice than in "I+UCMS−" mice ($P < 0.01$; Fig. 3). Overall, these findings indicate that the production of indole by the gut microbiota increased the adverse effects of chronic mild stress on emotional behaviors.

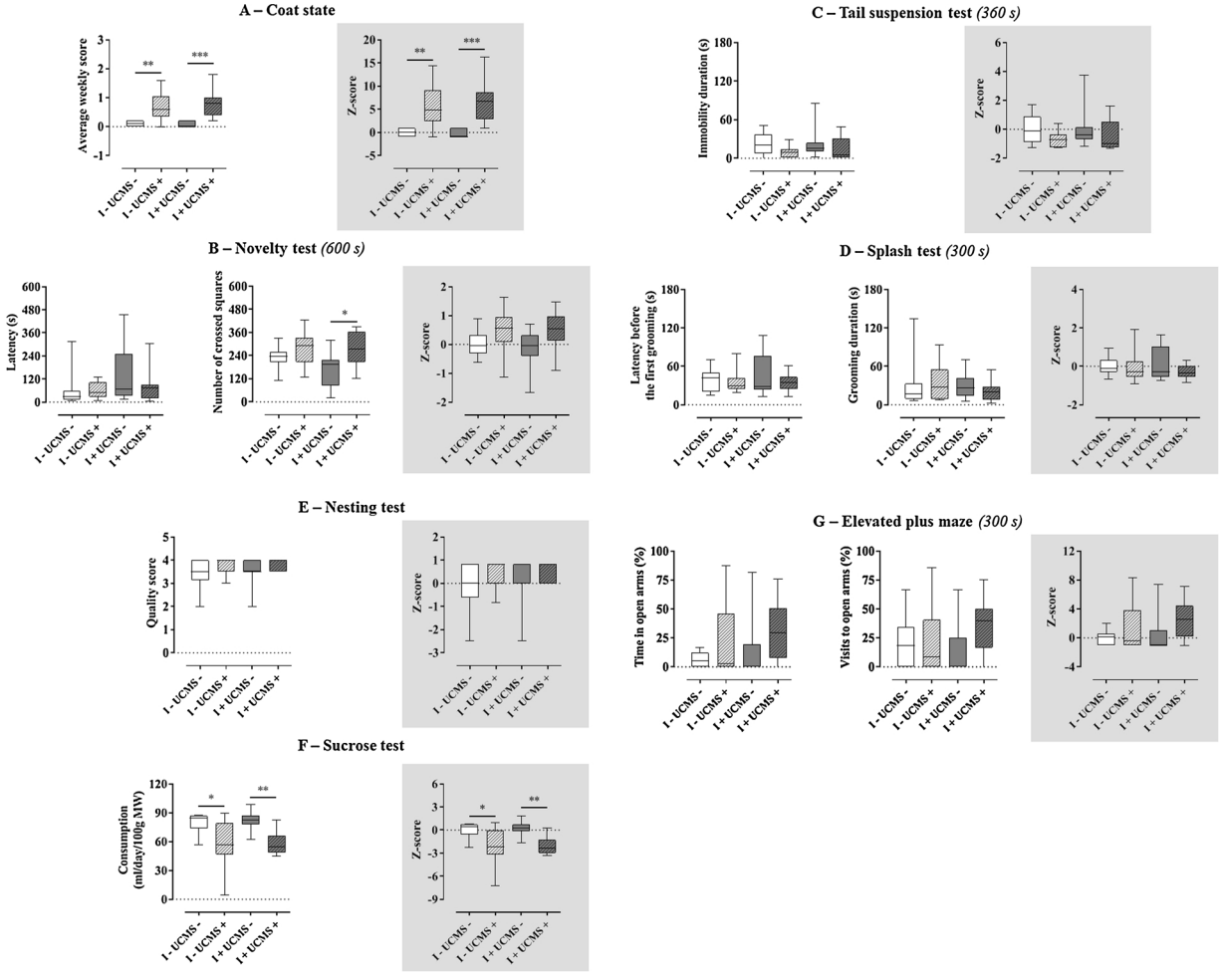

Fig. 2. Results of behavioral tests in gnotobiotic mice whose gut microbiota produces indole ("I+") or not ("I−"), and which have been exposed to unpredictable chronic mild stress ("UCMS+") or not ("UCMS−") for 7 weeks.
Coat state was scored once a week from the 3rd to the 7th week of UCMS, and other tests were carried out between the 5th and the 7th week of UCMS. Within each test, a z-score was computed from behavioral data as described in the Material and methods section. Data are expressed as medians (interquartile ranges). *P < 0.05, **P < 0.01, ***P < 0.001 between "I−UCMS−" and "I−UCMS+" mice or between "I+UCMS−" and "I+UCMS+" mice (n = 10-12 mice/group; Kruskal-Wallis test followed by a Dunn's multiple comparison test). For details on the UCMS procedure and the behavioral tests, see Material and methods section and Supplementary material and methods. MW: metabolic weight (body weight$^{0.75}$, which reflects the weight of metabolically active tissue).

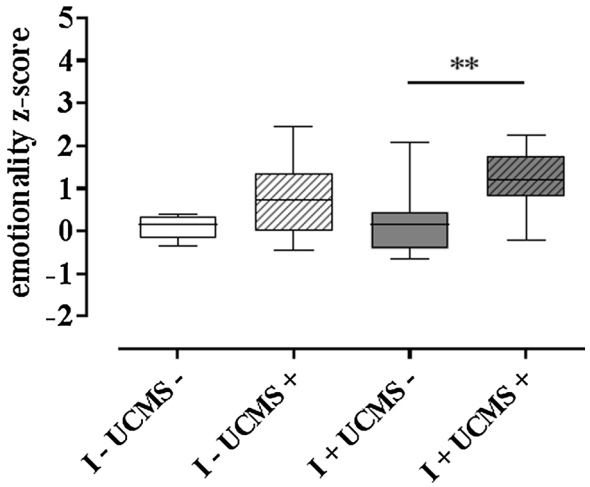

Fig. 3. Emotionality z-score in gnotobiotic mice whose gut microbiota produces indole ("I+") or not ("I−"), and which have been exposed to unpredictable chronic mild stress ("UCMS+") or not ("UCMS−") for 7 weeks.
Coat state was scored once a week from the 3rd to the 7th week of UCMS and the other behavioral tests were carried out from the 5th to the 7th week of UCMS. Z-scores were computed from the behavioral data within each test, then combined into an overall emotionality z-score as described in the Material and Methods section. Data are medians and interquartile ranges (n = 10-12 mice/group). The overall emotionality z-score increased specifically in "I+ UCMS+" mice vs "I+ UCMS−" mice (**P < 0.01; Kruskal-Wallis test followed by a Dunn's multiple comparison test). For details on the UCMS procedure and the behavioral tests, see Material and methods section and Supplementary material and methods.

3.3. Indole modified the catecholamine biosynthetic pathway in chronically stressed mice

The adrenal glands being a major endocrine tissue involved in stress response, we asked whether indole could influence the biosynthesis of stress hormones. Following exposure to the 7-week UCMS procedure, mice were killed immediately after being subjected to the elevated plus maze test. We weighed the adrenal glands and found no difference among groups (2-way ANOVA; $P > 0.05$; n = 18 mice/group; data not shown).

We measured the expression of genes involved in stress-related hormone biosynthesis, *i.e.* corticosterone produced from the zona fasciculata of the adrenal cortex, and catecholamines produced by the adrenal medulla. In the cortex, comparison of the four groups of mice did not show any significant change of the expression of genes encoding 3β-hydroxysteroid dehydrogenase (*Hsd3b*), 21α-hydroxylase (*Cyp21a1*), and 11β-hydroxylase (*Cyp11b1*), either by indole or by the chronic stress (Fig. 4). These results are consistent with hormone assays, from which plasma corticosterone concentrations did not differ among the four groups of mice (**Table S2**). Regarding catecholamine biosynthesis, we assayed the expression of genes encoding tyrosine hydroxylase (*Th*), dopamine β-hydroxylase (*Dbh*), and phenylethanolamine N-methyltransferase (*Pnmt*) (Fig. 4). Exposure to the chronic mild stress led to a significant 5-fold increase of the expression of the *Pnmt* gene in "I+" mice but not in "I−" mice (Kruskal-Wallis test followed by the Dunn's multiple comparison test; $P < 0.0001$; n = 16-18 mice/group). No effect of indole or chronic stress was observed on the expression of the two other genes.

3.4. Neither indole nor chronic mild stress affected the serotonergic and dopaminergic systems in the hippocampus and striatum

As the serotonergic and dopaminergic systems are usually reported

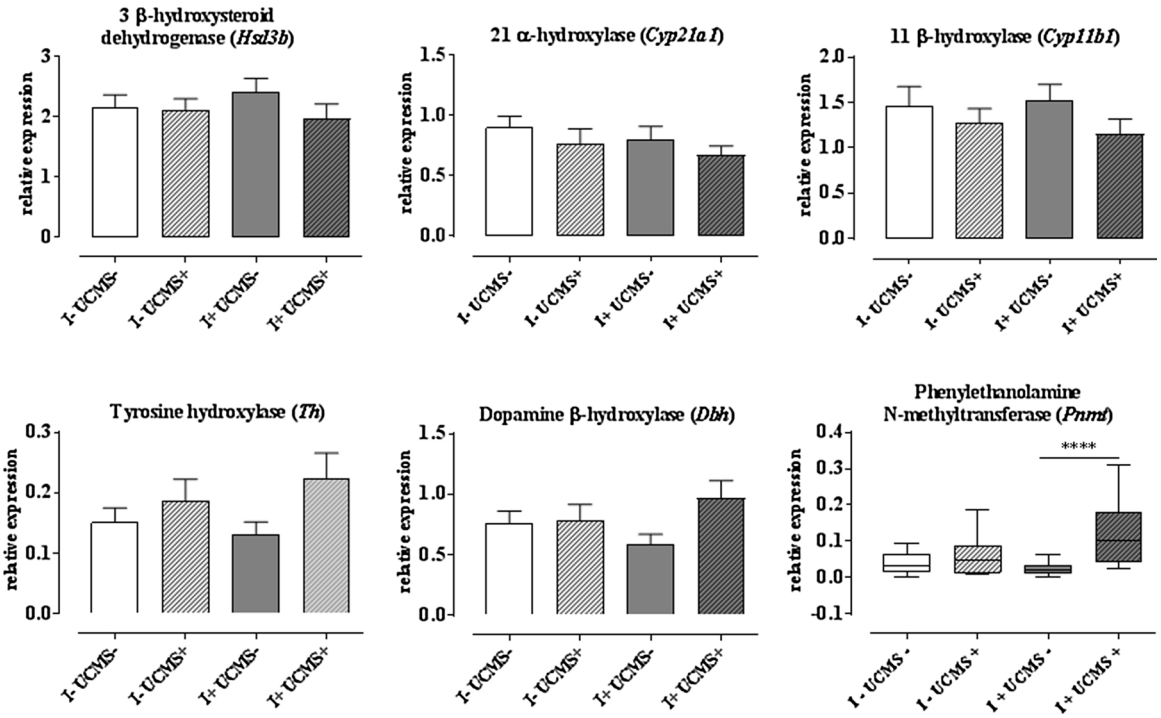

Fig. 4. Expression level of genes encoding enzymes of the corticosterone (upper panel) and catecholamine (lower panel) pathways in the adrenal glands of gnotobiotic mice whose gut microbiota produces indole ("I+") or not ("I−"), and which have been exposed to unpredictable chronic mild stress ("UCMS+") or not ("UCMS−") for 7 weeks.
Expression levels were analyzed by mRNA quantification using RT-PCR, as described in the Supplementary material and methods. Data are medians and interquartile ranges (*Pnmt*; n = 16-18 mice/group), or means and SEM (all other genes; n = 16-18 mice/group). Expression of the *Pnmt* gene increased specifically in "I+ UCMS+" mice *vs* "I+ UCMS−" mice (****P < 0.0001; Kruskal-Wallis test followed by a Dunn's multiple comparison test).

to be unbalanced after chronic stress and in mood disorders, we analyzed whether indole and the UCMS procedure would affect them in the gnotobiotic mice. The concentrations of 5-HT and its metabolite 5-HIAA, and the 5-HIAA/5-HT ratio that reflects 5-HT turnover, were similar in the four groups of mice, either in the hippocampus or in the striatum (Table 2). The concentrations of DA and its metabolites DOPAC and HVA were virtually null in the hippocampus, regardless of the mice group; in the striatum, concentrations of those molecules, as well as the DOPAC/DA and HVA/DA ratios that reflect the turnover of DA, were similar in the four groups of mice (Table 2).

3.5. Cytokine gene expression in brain and gut were not substantially affected by indole or chronic mild stress

As low-grade inflammation is pointed out in the pathophysiology of mood disorders, we looked for an effect of indole and/or chronic stress on cytokine expression in hippocampus and striatum, and in the gut mucosa of the ileum and the colon. In hippocampus and striatum, we measured the gene expression of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ: amplicons were detected beyond the 38th amplification cycle only, indicating that those genes were not or very lowly expressed (data not shown). In the ileum and colon, we measured the gene expression of the pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ, and of the anti-inflammatory cytokine IL-10. Despite a pre-amplification step, we could not detect amplicons of the *Ifng* and *Il10* genes, either in the ileum or in the colon. The *Il1b* gene expression was similar in the four groups of mice, both in the ileum and in the colon. With regard to the *Tnfα* gene, its expression was significantly 2-fold decreased in the colon of "I+ UCMS+" mice compared with "I+ UCMS−" mice (Kruskal-Wallis test followed by the Dunn's multiple comparison test; $P < 0.01$; $n = 10$ mice/group; data not shown).

3.6. Neither indole nor chronic mild stress affected the tryptophan metabolism pathways in gut, systemic circulation and brain

We measured the expression of the genes encoding the tryptophan hydroxylase, the rate-limiting enzyme of the 5-HT pathway, in the ileal and colonic mucosa (*Tph1* gene), and in hippocampus and striatum (*Tph2* gene). No difference occurred between the four groups of mice (data not shown). We also measured in hippocampus and striatum the expression of the *Gch1* gene, which encodes an enzyme responsible for the production of tetrahydrobiopterin, an important cofactor of tryptophan hydroxylase. There again, there was no difference between the four groups of mice (data not shown). Overall, these data indicate that the 5-HT pathway was not affected by indole or by chronic mild stress.

With regard to the kynurenine pathway, neither indole nor chronic stress modified the expression of the *Ido1* gene, in the ileal and colonic mucosa as well as in hippocampus and striatum (data not shown). We analyzed in the latter ones the expression of the *Kmo* and *Aadat* genes, involved respectively in the production of neurotoxic and neuroprotectant derivatives from kynurenine. The *Kmo* gene was not or very lowly expressed (amplicons detected beyond the 38th amplification cycle), and the expression level of the *Aadat* gene was similar in the four groups of mice (data not shown). Chronic stress slightly increased the tryptophan and kynurenine plasma concentrations in both "I+" and "I−" mice (2-way ANOVA; $P < 0.01$; $n = 10$ mice/group; **Figure S1**), but the kynurenine/tryptophan ratio was similar in the four groups of mice. Overall, these data suggest no impact of indole or chronic mild stress on the gut, systemic and brain conversion of tryptophan to kynurenine.

4. Discussion

We report here the first application of a chronic mild stress procedure in an animal model harboring a simplified gut microbiota. This procedure was reliably effective, as behavioral endpoints reported in conventional mice (Yalcin et al., 2008; Hill et al., 2012) were achieved in both groups of stressed gnotobiotic mice: the coat state deteriorated progressively, reflecting a decline in self-grooming behavior, and the sucrose consumption was reduced, reflecting an anhedonic state. Interestingly, in an unrelated study using the same UCMS procedure in germ-free *vs.* conventional C3H/HeN male mice, we noticed that the coat state deteriorated in stressed conventional mice, as expected, but not in stressed germ-free mice (Heberden et al., unpublished results). Taken together, these results suggest that germ-free male mice of the C3H/HeN strain are less susceptible to chronic mild stress than conventional counterparts, and that colonization with *E. coli* tends to restore a typical susceptibility level.

While the chronic mild stress caused a coat state degradation and an anhedonia in the gnotobiotic mice, regardless of their simplified gut

Table 2
Brain concentrations (pmol/mg wet tissue) of serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA), and of dopamine (DA) and metabolites (dihydroxyphenylacetic acid, DOPAC; homovanillic acid, HVA) in gnotobiotic mice whose gut microbiota produces indole ("I+") or not ("I−"), and which have been exposed to unpredictable chronic mild stress ("UCMS+") or not ("UCMS−") for 7 weeks. Data are expressed as medians (interquartile ranges). For all molecules, no difference occurred between the 4 groups of mice (n = 9-10 mice/group; Kruskal-Wallis test; $P > 0.05$). For details on the UCMS procedure, see Material and methods section.

	Mice			
	"I− UCMS−"	"I− UCMS+"	"I+ UCMS−"	"I+ UCMS+"
Hippocampus				
5-HT	2.7 (0.5)	2.4 (0.7)	2.1 (0.7)	2.1 (0.8)
5-HIAA	2.3 (0.6)	2.3 (0.9)	2.1 (0.5)	1.9 (0.8)
ratio 5-HIAA/5-HT	*0.90 (0.23)*	*1.08 (0.52)*	*1.00 (0.20)*	*0.95 (0.35)*
DA	0.0 (0.8)	0.0 (0.2)	0.1 (0.7)	0.0 (0.1)
DOPAC	0.0 (0.3)	0.0 (0.1)	0.0 (0.2)	0.0 (0.1)
HVA	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.1)
ratio DOPAC/DA	-	-	-	-
ratio HVA/DA	-	-	-	-
Striatum				
5-HT	2.1 (1.7)	2.7 (1.1)	3.2 (3.4)	3.1 (1.1)
5-HIAA	0.6 (3.3)	0.4 (0.2)	0.6 (2.7)	0.5 (1.2)
ratio 5-HIAA/5-HT	*0.23 (3.58)*	*0.15 (0.09)*	*0.16 (3.42)*	*0.18 (0.86)*
DA	64.7 (33.0)	61.2 (31.3)	65.9 (25.2)	57.3 (27.0)
DOPAC	7.7 (5.7)	8.5 (2.7)	8.7 (5.7)	7.6 (6.1)
HVA	2.4 (2.9)	2.5 (2.3)	3.1 (2.9)	3.3 (4.1)
ratio DOPAC/DA	*0.14 (0.13)*	*0.15 (0.12)*	*0.15 (0.07)*	*0.14 (0.08)*
ratio HVA/DA	*0.07 (0.08)*	*0.04 (0.10)*	*0.04 (0.05)*	*0.05 (0.07)*

microbiota ability to produce indole, it did not modify behaviors in the other tests. Similarly, indole production did not significantly influence the behavior of mice, when tests were considered individually. To study the impact of chronic stress and indole on the emotional behavior of mice, taken as a whole, we integrated behavioral data in an overall emotionality z-score. Guilloux et al. (2011) developed this procedure by analogy with the symptom rating scales used in humans to aggregate sets of convergent symptoms. This integration of behavioral parameters revealed that indole production by the simplified gut microbiota intensified the overall emotional response to chronic mild stress. Interestingly, indole production did not modify the emotional behavior of the non-stressed mice, contrarily to what we had shown previously in rats (Jaglin et al., 2018). Such a discrepancy could be due to animal species and strain specificities. Indole metabolism in the liver, which leads to neuroactive indole derivatives such as isatin or oxindole, may be different in C3H/HeN mice *vs.* F344 rats. Alternatively, as C3H/HeN mice are genetically less prone to stress than F344 rats (Sarrieau et al., 1998; Ibarguen-Vargas et al., 2008), their brain sensitivity to the deleterious effects of indole derivatives may be lower. In any case, this difference could not be due to a weaker exposure to indole. Indeed, the indole-producing *E. coli* strain settled in the digestive tract of the C3H/HeN mice at the same, or even slightly higher level than in F344 rats (Jaglin et al., 2018). The fecal indole concentrations were also slightly higher in the mono-associated mice than in the mono-associated rats (Jaglin et al., 2018). Interestingly, the fecal indole concentrations of the mono-associated rats were 4-fold greater than in conventional rats fed on the same standard diet, reflecting an overproduction of indole (Jaglin et al., 2018). On the opposite, in the present study, the fecal indole concentrations of the mono-associated mice were in the same order of magnitude as those of conventional mice of an unrelated study, fed on the same standard diet (Mir et al., unpublished results). This means that, contrarily to what we observed in rats, the mice mono-associated with the indole-producing *E. coli* strain did not exhibit an overproduction of indole, compared to mice harboring a complex microbiota. Such a discrepancy between rats and mice could be due to a different gut microbiota composition (Nagpal et al., 2018), potentially leading to a different prevalence of the genes encoding the tryptophanase enzyme in the rat and mouse metagenomes.

Next, we looked for the effect of indole production on the biological correlates that are usually associated with emotional behaviors (Hill et al., 2012; Vancassel et al., 2018). First, we did not observe inflammation in stressed mice, whether they were colonized with the wild-type or the mutant strain. The genes encoding cytokines in gut mucosa and brain were either expressed at similar levels in all groups of mice, or too poorly expressed to allow comparisons. Low expression of some genes likely resulted from an insufficient maturation of the immune system of the mice due to their association with a single bacterial species. Indeed, gnotobiotic animals, though highly valuable systems to understand microbiota-host communication, cannot fully recapitulate the developmental complexity of the immune system (Rooks and Garrett, 2016). In this regard, Sudo et al. (2004) showed that mono-association of GF male mice with an enteropathogenic strain of *E. coli* substantially increased plasma concentrations of the pro-inflammatory cytokines IL-6 and IL-1β. On the opposite, this increase was minimal in male mice mono-associated with the mutant strain lacking its virulence factor (in that case the translocated intimin receptor, which allows *E. coli* cells to adhere to the intestinal epithelium). In the present study, we used non-pathogenic *E. coli* strains and their effect on cytokines was in the same line as the one reported by Sudo et al. (2004). Second, the catabolism of tryptophan to indole in the digestive tract could have decreased tryptophan bioavailability in the body: this was not the case since the plasma concentration of tryptophan was the same in indole producers and non-producers. Furthermore, indole production and chronic mild stress did not seem to affect tryptophan metabolism along the 5-HT and kynurenine pathways. Indeed, the plasma kynurenine/tryptophan ratio, the expression of the gene encoding tryptophan hydroxylase, which is the rate-limiting enzyme of the 5-HT pathway, and the expression of kynurenine pathway genes, both in gut mucosa and brain (hippocampus and striatum), were the same in all mice groups. Eventually, indole production and chronic mild stress did not influence the brain serotonergic and dopaminergic systems, as assessed in the hippocampus and striatum by measuring 5-HT and DA levels and turnovers. In their review on the effect of chronic mild stress on neurobiological variables, Hill et al. (2012) report that neurochemistry findings vary among studies, likely due to variations in the chronic mild stress procedure, including stressor type and procedure duration, in the housing conditions, etc. Therefore, additional analyses targeting the transporters, receptors and enzymes metabolizing these monoamines would refine the present results and help make a stronger conclusion on the effect of chronic mild stress and indole.

Overall, the aggravating effect of indole on the intensity of the overall emotional response to chronic mild stress does not seem linked to an inflammatory process, a modification of monoaminergic systems, or a disruption of tryptophan metabolism along the 5-HT and kynurenine pathways. Interestingly, this result agrees with that obtained by Bercik et al. (2011) in a different mouse model. Indeed, a shift in gut microbiota induced by an antibiotic treatment in conventional male mice modified their anxiety-like behavior, independently of inflammatory activity or of changes in monoaminergic neurotransmitter levels, at least in the gut mucosa. In the present study, indole could have acted on brain areas involved in emotional behaviors through its neurodepressant oxidized derivatives, including oxindole and isatin. Both are known to be able to cross the blood-brain barrier (Carpenedo et al., 1998; Medvedev et al., 2005; Jaglin et al., 2018), and isatin was shown to interact with 5-HT_3 and D_2 receptors (Bhattacharya and Acharya, 1993). However, we did not try to measure them in the mice brains because our previous study in rats showed that they were only present at trace levels (Jaglin et al., 2018). In the future, using labelled tryptophan or indole will probably be necessary to identify and quantify indole derivatives that reach the brain (King et al., 1966). A concurrent or alternative action mechanism of indole could be a vagus nerve stimulation, as suggested in our previous work in rats, in which an acute indole overproduction in the gut led to an increase of c-Fos protein expression in the hindbrain dorsal vagal complex (Jaglin et al., 2018). Subdiaphragmatic vagal deafferentiation experiments should allow ascertaining this communication route. This method has been used successfully to show the role of the vagus nerve in the anxiolytic activity of the probiotic strain *Lactobacillus rhamnosus* JB-1 (Bravo et al., 2011) or, on the contrary, the lack of involvement of this route in the effect of gut microbiota on fear extinction learning (Chu et al., 2019).

As observed in our previous study with rats (Jaglin et al., 2018), indole did not influence the plasma corticosterone concentration, in either stressed mice or non-stressed mice. This lack of effect was supported by a similar expression level of the corticosterone biosynthetic pathway genes in the adrenal cortex of all mice. In contrast, the combination of indole production and chronic mild stress modified the catecholamine biosynthetic pathway in the adrenal medulla, by specifically upregulating the expression of the gene encoding the PNMT enzyme, which catalyzes the demethylation of norepinephrine to epinephrine. To the best of our knowledge, the effect of gut microbiota on the sympathetic-adrenomedullary axis has been less investigated than its effect on the hypothalamic-pituitary-adrenal (HPA) axis. Recently, Giri et al. (2019) showed that C57BL/6N GF male mice had lower urinary epinephrine concentrations than conventional counterparts, both in basal state and after an acute hypoglycemic stress. This difference was accompanied by a deregulation of the expression of several adrenal genes, including the *Pnmt* gene: this was downregulated in stressed GF mice, compared with non-stressed GF mice and stressed conventional mice. In contrast, Vodička et al. (2018) showed that the *Pnmt* gene was upregulated in BALB/c GF male mice compared with conventional counterparts, in basal state as after a chronic psychosocial stress. Discrepancy between those two studies could arise from the different

mouse strains, but also from the different types of stressors. The latter hypothesis may be supported by analogy with the work of Sudo et al. (2004) on the HPA axis. Indeed, these authors showed that an acute restraint stress induced a greater HPA axis response in GF male mice as compared to conventional ones, while an ether stimulus led to a similar response in both types of mice.

Anyhow, our results indicate that indole may be one of the microbial metabolites contributing to the regulatory effect of gut microbiota on the catecholamine biosynthetic pathway in the adrenal medulla. The involved mechanisms are unknown and, for the moment, beyond the scope of the present work. However, some hypotheses, not mutually exclusive, can be advanced. Indole signaling along the gut-brain axis may lead ultimately to an activation of the sympathetic nervous system, which conveys the brain influence over the adrenal medulla (Dum et al., 2016). However, in this case, one might expect a stimulation of the transcription of all genes of the catecholamine biosynthetic pathway. Alternatively, indole derivatives released in the bloodstream could target the adrenal medullary tissue and act directly, in a specific fashion, upon gene expression and enzyme activity. In this regard, another aromatic amino acid derivative, *p*-cresol, was shown to inactivate dopamine β-hydroxylase, which catalyzes the metabolism of dopamine into norepinephrine, *in vitro* (Goodhart et al., 1987). More recently, Patel et al. (2005) showed that a major microbial metabolite, butyrate, specifically regulated the transcription of the tyrosine hydroxylase gene in PC12 cell cultures, via a newly identified butyrate response element located upstream of the gene.

5. Conclusion

In conclusion, we showed that chronic indole production by the gut microbiota increased the vulnerability of male mice to the adverse effects of chronic mild stress on emotional behaviors. Interestingly, this occurred without any low-grade inflammation or any change in the host tryptophan metabolism pathways, which suggests other courses of action. This study also unveiled a new target, *i.e.* the adrenal medullary tissue. By affecting the catecholamine biosynthetic pathway of the adrenal medulla, indole could become a novel regulatory factor of the adrenomedullary function and, as such, play a role in body's physiological adaptation to stressful events. Thus, future studies will aim to characterize underlying mechanisms, *e.g.* a possible action of indole on the adrenal medulla stimulus-secretion coupling. The animal model we used was a simplified gut microbiota model, designed to study the specific effects of the bacterial transformation of tryptophan into indole. Other tryptophan metabolism pathways exist in the gut microbiota, which are implemented by bacterial species other than *E. coli*, and produce other tryptophan derivatives such as indole-propionic acid (Parthasarathy et al., 2018). Several studies showed that this metabolite, as well as mammalian-microbial co-metabolites of indole such as 3-indoxylsulfate, reduce brain inflammation by modulating microglia and astrocyte activity (Rothhammer et al., 2016; Rothhammer et al., 2018). Thus, a same chemical group of bacterial metabolites can have beneficial or harmful effects on the brain. The overall result is likely to be driven by both the profile and the quantity of these metabolites, which depend on gut microbiota composition and dietary habits, and by the physiological and environmental context of the host.

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Declaration of Competing Interest

All authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2020.104750.

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