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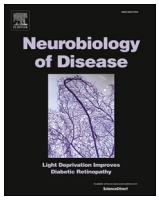

Indole induces anxiety-like behaviour in mice mediated by brainstem locus coeruleus activation

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ABSTRACT

The gut microbiota produces metabolites that enrich the host metabolome and play a part in host physiology, including brain functions. Yet the biological mediators of this gut-brain signal transduction remain largely unknown. In this study, the possible role of the gut microbiota metabolite indole, originating from tryptophan, was investigated. Oral administration of indole to simulate microbial overproduction of this compound in the gut consistently led to impaired locomotion and anxiety-like behaviour in both C3H/HeN and C57BL/6J mice. By employing c-Fos protein expression mapping in mice, we observed a noticeable increase in brain activation within the dorsal motor nucleus of the vagus nerve (DMX) and the locus coeruleus (LC) regions in a dose-dependent manner. Further immune co-labelling experiments elucidated that the primary cells activated within the LC were tyrosine hydroxylase positive. To delve deeper into the mechanistic aspects, we conducted chemogenetic activation experiments on LC norepinephrine neurons with two doses of clozapine N-oxide (CNO). Low dose of CNO at 0.5 mg/kg induced no change in locomotion but anxiety-like behaviour, while high dose of CNO at 2 mg/kg resulted in locomotion impairment and anxiety-like behaviour. These findings support the neuroactive roles of indole in mediating gut-brain communication. It also highlights the LC as a novel hub in the gut-brain axis, encouraging further investigations.

1. Introduction

The gut-brain axis assumes a pivotal role in upholding homeostasis. The microbiome's significance in modulating gut-brain signaling has surfaced prominently, leading to the establishment of the concept of a microbiota-gut-brain axis, both in physiological and pathological conditions (Margolis et al., 2021). Refinements in the interactions among the gut, brain, and microbiome have been recognized in animal models of diverse psychiatric and neurological disorders, including conditions such as anxiety (Simpson et al., 2021), depression (Yang et al., 2020), Parkinson's disease (Hirayama and Ohno, 2021), and autism spectrum disorder (Nitschke et al., 2020; Li et al., 2023). The advancement of these findings, accompanied by clinical data, has contributed to the revised understanding of the pathophysiology underlying various brain disorders (Morais et al., 2021), previously thought to arise solely from brain-specific pathophysiological processes (Mayer et al., 2022). Deciphering the detailed mechanism of how gut microbial signals reach the brain sheds light on a new area of therapeutic targeting for these brain disorders.

Gut microbial signals reach the brain either by exerting localized effects on enteric cells, the autonomic nervous system, or through the circulatory system. Yet the biological mediators of these signal transductions remain largely unknown. Food-related metabolites constitute a large portion of known gut microbial signals; one example is the short-chain fatty acids (SCFAs), which come from the fermentation of food fiber by gut microbes (Dalile et al., 2019). Some signal mediators come

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from dietary amino acids metabolized by the gut microbiota. Tryptophan, an essential amino acid, is obtained through the diet. Common natural food sources of tryptophan include oats, bananas, milk, tuna fish, peanuts, and chocolate (Agus et al., 2018). Three neuroactive compounds, including peripheral 5-hydroxytryptamine (5-HT or serotonin) (Ridaura and Belkaid, 2015), kynurenine (Kennedy et al., 2017), and indole (Jaglin et al., 2018), could be produced by gut microbes from tryptophan. While microbes influence the modulation of the first two metabolites, the synthesis of indole relies solely on gut microbial metabolism (Jaglin et al., 2018).

The gut microbiota primarily metabolizes tryptophan into indole, predominantly facilitated by the enzymatic activity of tryptophanase. Subsequent metabolic pathways involve the transformation of indole through interactions with intestinal and hepatic xenobiotic metabolizing enzymes, resulting in the creation of a spectrum of oxidized and conjugated derivatives (Hubbard et al., 2015). Indole demonstrates its roles in bacterial physiology, including sporulation, biofilm formation, and antibiotic resistance. Indole has reported roles in host physiology as well, including metabolism and neuroactivity. For example, indole can prompt the production of glucagon-like peptide-1 (GLP-1), an incretin that stimulates insulin secretion by pancreatic β cells, by acting on enteroendocrine L cells (Chimerel et al., 2014). By utilizing gnotobiotic male rodents and comparing the two conditions: one associating with an indole-producing *E. coli* strain and the other with a non-indole-producing knockout mutant, we found that indole led to anxiety-like behaviour and a sense of helplessness in the F344 rat strain (Jaglin et al., 2018), and amplified emotional behavioural impairments arising from exposure to unpredictable chronic mild stress in the C3H/HeN mouse strain (Mir et al., 2020). Our prior work also demonstrated that intra-cecal administration of indole within the hindgut of male rats impaired locomotion and activated the dorsal vagal complex (Jaglin et al., 2018).

In this study, we aim at identifying the relevant neural nuclei involved in this process, and to ascertain whether the effects of indole on emotional behaviours are consistent across strains, specifically in mice. Our results showed that oral gavage of indole led to locomotor impairment and anxiety-like behaviour in two strains of mice. Locus coeruleus (LC) norepinephrine neurons were strongly activated by indole treatment, and chemogenetic activation of LC norepinephrine neurons resembled the anxiety-like behaviour observed in the mice. Our data support the role of indole as a biological mediator of gut-brain signaling and highlight the need for further investigation into the gut microbiota-indole-LC pathway in psychological disorders.

2. Materials and methods

2.1. Animals

Adult (6–8 weeks) C3H/HeN and C57BL/6J mice (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were used. TH-Cre mice were generated by crossing TH-Cre (Jax No. 008601) with CD-1 mice (ICR, Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) (Savitt et al., 2005). All husbandry and experimental procedures in this study were approved by the Animal Care and Use Committees (IACUC) at the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences (CAS), with the IACUC number of SIAT-IACUC-200720-NS-LL-A0746–02. Mice were group-housed at 22–25 °C on a circadian cycle of 12-h light and 12-h dark with ad-libitum access to food (Beijing Keaoxieli Feed Company, Beijing, China) and water in specific pathogen-free (SPF) animal rooms.

2.2. Indole treatments

As mentioned earlier, indole is produced by gut bacteria. We therefore administered indole to mice by gavage, to simulate microbial overproduction of indole in the gut. In our previous study on rats (Jaglin et al., 2018), we had introduced indole into the cecum. Nevertheless, as Carpenedo et al. (1998) showed that gavage with indole in rats led to the presence of neuroactive oxidized derivatives in brain (Carpenedo et al., 1998), we decided to use this method of administration, because it is less invasive for the animals. The doses we used, i.e. 200 and 400 mg/kg body weight (BW), are in the same order of magnitude as those used by Carpenedo et al. (1998) (Carpenedo et al., 1998) and as the one we used in our previous study on rats (Jaglin et al., 2018).

The mice were acclimated to their environment for 10 days before the start of the experiment. Additionally, they underwent daily habituation to the gavage procedure, beginning 7 days prior to the experiment's commencement. This habituation process involved manually restraining the mouse for 30 s, during which we simulated the oral gavage using an empty flexible feeding probe (1.6 mm × 38 mm; Cat# FTP-20-38-50, Phymep, Paris, France). Subsequently, the mouse was gently placed on a cotton pad held by the experimenter, who then softly stroked the mouse's back for 3 min. Then on the treatment day, the tube was filled with either the indole solution or vehicle alone. This handling and habituation phase is crucial to minimize brain activations caused by the handling of the animals and the oral gavage treatment, as our study focuses on the neuronal circuits implicated in anxiety-like behaviours.

For the c-Fos activated brain nuclei mapping, on the day of the experiment, C3H/HeN mice were gavaged with either 0.15 mL of corn oil (n = 4; Sigma-Aldrich, Darmstadt, Germany), or indole (Sigma-Aldrich, Darmstadt, Germany) dissolved in corn oil at a dose of 200 mg/kg BW (n = 4) or 400 mg/kg BW (n = 4), freshly prepared. The oral gavages were done between 11:00 (to ensure that the mice's bowels were emptied) and 17:30. After 1.5 h, the mice were deeply anesthetized with a pentobarbital i.p. (intraperitoneal) injection and transcardially perfused with 4% paraformaldehyde (PFA; Boster Biological Technology, Pleasanton, CA, USA) in 1× PBS using a peristaltic pump (BT100-2 J et YZ1515X, Longer, Amersham, UK). Then the brains were collected and post-fixed in 4% PFA for another 48 h. Thereafter, post-fixed brains were stored in 30% sucrose in 1× PBS and kept at 4 °C until further sectioning and immunostaining. Similarly, procedure was carried out in C57BL/6J mice to study the activation of Locus coeruleus (LC) and its specific cell type, only there were two groups of mice: oil controls (n = 4) and indole of 200 mg/kg BW (n = 4).

For the behavioural study using C3H/HeN mice, on the day of the experiment, mice were gavaged with either 0.15 mL of corn oil (n = 8) or 0.15 mL of indole dissolved in corn oil at a dose of 200 mg/kg BW (n = 9). The oral gavages were administered starting at 11:00. The open field test (OFT) was carried out 1.5 h after the gavage and lasted 20 min. The mice were placed in the test room at least 1 h before the test for habituation. Similarly, C57BL/6J mice were gavaged with either 0.15 mL of corn oil (n = 18) or 0.15 mL of indole at the dose of 200 mg/kg (n = 17) and followed by OFT and a randomly selected subset of mice were further tested in an elevated plus maze (EPM, n = 9 for corn oil controls, n = 8 for 200 mg/kg indole).

2.3. Histological study

A histological study was conducted to map c-Fos expression in the brain using immunofluorescence staining. Antibody staining was performed on single-well floating tissue sections. Sections were incubated successively with primary (4 °C, 12 h) and secondary antibodies (room temperature, 2 h). First, brain samples were embedded by OCT (optimal cutting temperature compound, Sakura, USA) and then sectioned on a cryostat (CM1950, Leica Biosystems, Nußloch, Germany) at 30 μm thickness. The sections were floated 3 times to wash out OCT, and blocked with Normal Goat Serum (NGS, Jackson Immunoresearch and Cat# 005–000-121), or Normal Donkey Serum (NDS, Jackson Immunoresearch and Cat#017–000-121) for 2 h, then incubated with primary antibody of c-Fos (rabbit anti-c-Fos, #2250, Cell Signaling Technology, 1:500) for 12 h in a 4 °C shaker. For LC characterization in C57BL/6J mice, chicken anti-Tyrosine Hydroxylase (#ab76442, Abcam, 1:500)

was also used in the primary antibody incubation. To reveal the co-localization with different fluorescent colours, suitable secondary antibodies were chosen. Sections were incubated with secondary antibodies for 2 h in a room temperature shaker. For marking the cell nuclei, the slices were incubated for 10 min with 4, 6-diamidino-2-phenylindol (DAPI, Invitrogen, Carlsbad, USA, 1:5000). All incubations were protected from light. The images were captured with Olympus VS120 virtual microscopy slide scanning system. For the c-Fos activated brain nuclei mapping using C3H/HeN and C57BL/6J mice, the names of the images were made anonymous allowing blind analysis. Brain sections of each animal were sorted according to the atlas of mouse brain and prescreened for brain nuclei with strong c-Fos positive (c-Fos$^+$) signals. The number of c-Fos$^+$ cells of the following brain nuclei were manually counted blindly with ImageJ software (https://imagej.nih.gov/): anterior cingulate cortex (ACC), lateral septum (LS), bed nucleus of the stria terminalis (BNST), hippocampus (HP), edinger-westphal nucleus (EW), basolateral amygdala nucleus (BLA), central amygdala nucleus (CEA), superior colliculus (SC), paraventricular nucleus of the thalamus (PV), dorsal raphe nucleus (DRN), periaqueductal gray (PAG), locus coeruleus (LC), dorsal motor nucleus of the vagus nerve (DMX), nucleus tractus solitarius (NTS) after digital sections and corresponding atlas watermarks were stacked accurately (Franklin and Paxinos, 2008). Several sections were randomly selected and analyzed in a given brain zone. The mean number of c-Fos$^+$ cells of all analyzed sections for each brain zone of one mouse was treated as individual data, and each n represents one mouse.

2.4. Behavioural tests

Open field test (OFT) was used for measuring locomotor activity and anxiety-like behaviour of the mice (Kraeuter et al., 2019). An open field arena (50 cm × 50 cm × 50 cm) made of white PVC was used. The open field was slightly illuminated from the top (about 50 lx). For analyses, we defined the center of the arena as a smaller concentric square inside, covering 25% of the area of the arena floor (i.e., 25 cm × 25 cm). The open field arena was cleaned with a 20% ethanol solution between mice. Animals were allowed to freely explore the open field for 20 min as the test section, and then the videos were divided to 2 sections of 10-min episodes for analysis. The number of entries to the center, time in the center, and total distance travelled in the open field (total activity) were recorded and then analyzed by Any-maze® software (version 4.96, Stoelting, IL, USA). For the OFT after chemogenetic activation of tyrosine hydroxylase (TH) positve neurons in LC, the test section was 10-min. The Elevated Plus Maze (EPM) was conducted as previously described (Li et al., 2018). Mice were placed on a four-arm plus maze consisting of two open arms and two closed arms (made of white PVC, each with dimensions of 30 cm length and 5 cm width), elevated 50 cm above the ground, for a 10-min test session. The EPM was cleaned with a 20% ethanol solution between mice. The videos were recorded, and the distance travelled, mean speed, entries into the open arms, time spent in the open arms and speed in open arms were analyzed by Any-maze® software (version 4.96, Stoelting, IL, USA).

2.5. Stereotaxic surgery

Stereotaxic surgeries and virus injections were performed as described previously (Li et al., 2018). Briefly, animals were anesthetized with sodium pentobarbital and placed in a stereotaxic apparatus (RWD, Shenzhen, China). The mice were ensured to be under deep anesthesia throughout the entire process. The skull above the LC nucleus was thinned with a dental drill and carefully removed. Injections were made using a micro-syringe pump (UMP3/Micro4, USA) with a 10 mL syringe connected to a 33-Ga needle (Neuros, Hamilton, Reno, USA). Either AAV2/9-hEF1a-DIO-mCherry-WPRE-pA (Taitool, Shanghai, China) for the mCherry control group or AAV2/9-hEF1a-DIO-hM3D(Gq)-mCherry-ER2-WPRE-pA (Taitool, Shanghai, China) for the hM3Dq group was injected into the LC, respectively. The syringe was not removed until 10 min after the end of infusion to allow for the diffusion of the virus. The LC injection coordinates were AP: −5.3 mm, ML: – 0.8 mm, and DV: – 4.0 mm. Behavioural experiment after Clozapine N-oxide (CNO, C0832, Sigma-Aldrich, Darmstadt, Germany) i.p. injection was carried out at least 6–8 weeks after virus injection. For chemogenetic activation at a low dose of CNO (0.5 mg/kg BW) or a high dose of CNO (2 mg/kg BW), CNO was i.p. injected 1.5 h before the 10-min OFT. The animals were then sacrificed, and brains were collected to access virus expression and c-Fos activation using the above immunostaining method.

2.6. Statistics

For the c-Fos expression analyses with corn oil, indole 200 mg/kg BW, and indole 400 mg/kg BW, one-way ANOVA with Tukey's multiple comparisons test was used. In addition, to study the dose response of selected key brain regions towards indole treatment, a linear regression model was used. To compare the differences between two groups in other experiments, Student's *t*-test with Welch's correction was used. Data were presented as mean ± SEM and the level of significance was set at $P < 0.05$. Statistical analysis was performed with the GraphPad Prism software (version 7.00, La Jolla, CA, USA).

3. Results

3.1. Locus coeruleus (LC) is activated by indole treatment with a dose response curve in C3H/HeN mice

By mapping the c-Fos protein expression throughout the brains of C3H/HeN mice, we examined the effect of indole gavage on various key brain regions (Fig. 1A). We found that indole influences several brain regions, most importantly activating the locus coeruleus (LC) (Fig. 1B, C), a brain region implicated with anxiety (Li et al., 2018). One-way ANOVA with Tukey's test revealed that the mice orally treated with 400 mg/kg indole exhibited a greater number of c-Fos$^+$ cells in the LC than those treated with corn oil ($P = 0.03$, Fig. 1B, C). This pattern led us to investigate whether the effect of indole on LC's activation would be dose dependent. Indeed, we found an increasingly apparent progression of c-Fos immunofluorescence signals, with the control showing the least signal and the 400 mg/kg indole group exhibiting the brightest (Fig. 2A), as shown in the dose-response curve ($P = 0.009$; Fig. 2B). Although there were no significantly different numbers of c-Fos$^+$ cells in other brain regions that we examined across groups (Fig. 1B, C), we found a trend that the dorsal motor nucleus of the vagus nerve (DMX) was activated in the mice treated with 400 mg/kg indole ($P = 0.071$, Fig. 1B). We also found a significant dose-response curve in the DMX ($P = 0.024$; Fig. 2C). Overall, the brain c-Fos mapping shows that indole not only activates the LC and the DMX, but the extent of such activation also depends on the concentration of indole.

3.2. Indole impairs locomotion and induces anxiety-like behaviour in C3H/HeN mice

Knowing that indole activates LC, a brain region that has previously been shown to relate with locomotor arousal and anxiety (Li et al., 2018; Carter et al., 2010; McCall et al., 2015), we further examined whether indole would induce anxiety-like behaviour or affect other behaviours in C3H/HeN mice by subjecting the mice to the open field test (OFT) (Fig. 3A). Given the observed dose-dependent effects of 200 mg/kg and 400 mg/kg indole treatments on LC neural activation, in conjunction with previous reports (Jaglin et al., 2018), we opted to proceed with the 200 mg/kg dose of indole for further functional studies. Indeed, we found that during the first and second 10 min of the test, mice treated with 200 mg/kg indole travelled significantly less distance than the oil control group (Ps < 0.05; Fig. 3B-E). This indicates that indole could impair locomotion abilities. Although Student's *t*-test revealed no

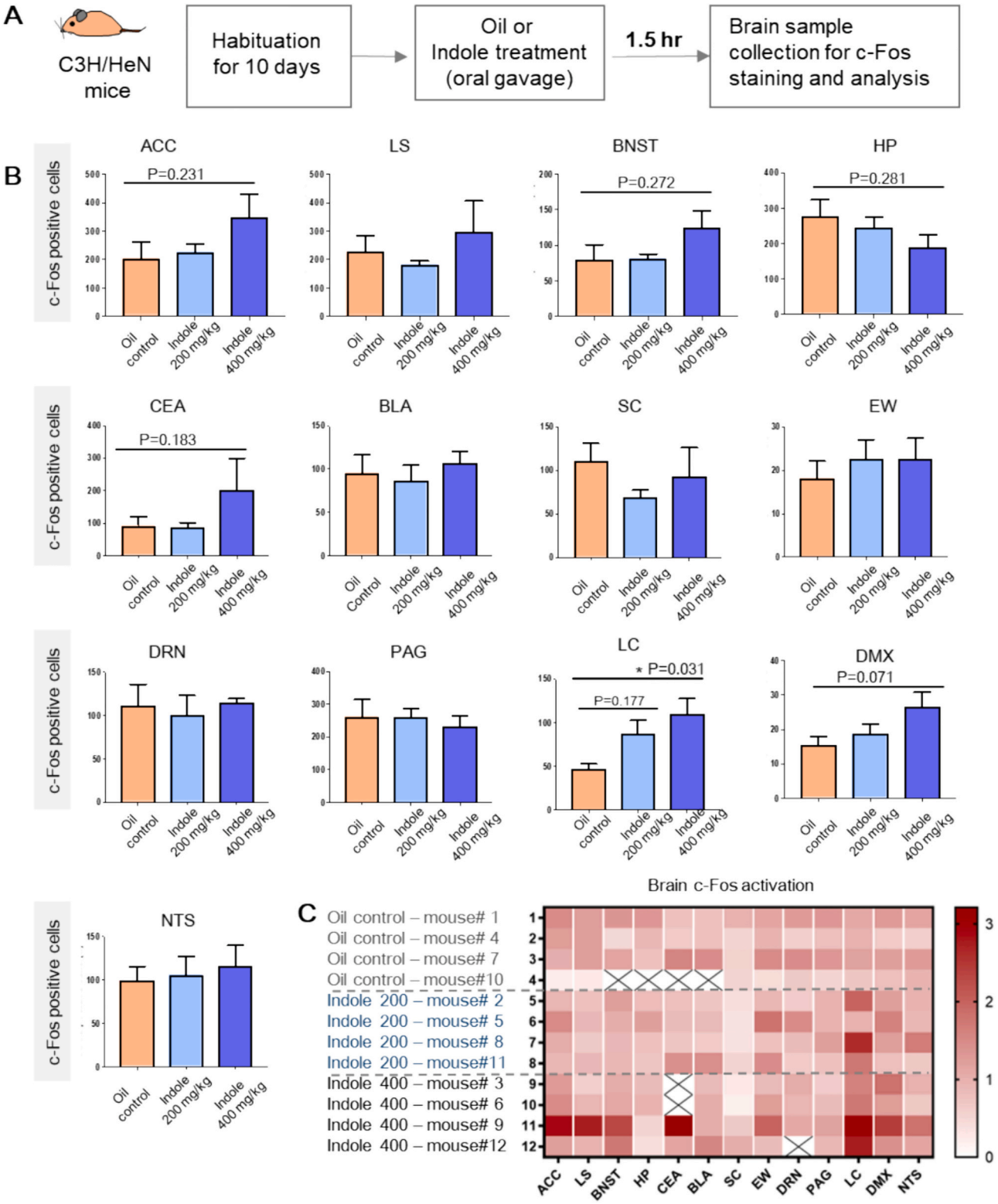

Fig. 1. Key brain areas activated by indole gavage in C3H/HeN mice.
(A) Schematic diagram of the gavage protocol and timing of brain sample collection for C3H/HeN mice. (B) Counting of c-Fos^{+} cells per brain area; Data are expressed as the mean ± SEM, One-way ANOVA with Tukey's test, * $P < 0.05$; $n = 4$ mice per group. (C) Heatmap representing of c-Fos activation for each mouse; anterior cingulate cortex (ACC), lateral septum (LS), bed nucleus of the stria terminalis (BNST), hippocampus (HP), central amygdala nucleus (CEA), basolateral amygdala nucleus (BLA), superior colliculus (SC), edinger-westphal nucleus (EW), dorsal raphe nucleus (DRN), periaqueductal gray (PAG), locus coeruleus (LC), dorsal motor nucleus of the vagus nerve (DMX), nucleus tractus solitarius (NTS); the white blocks with cross in the middle indicated lost brain sections during processing.

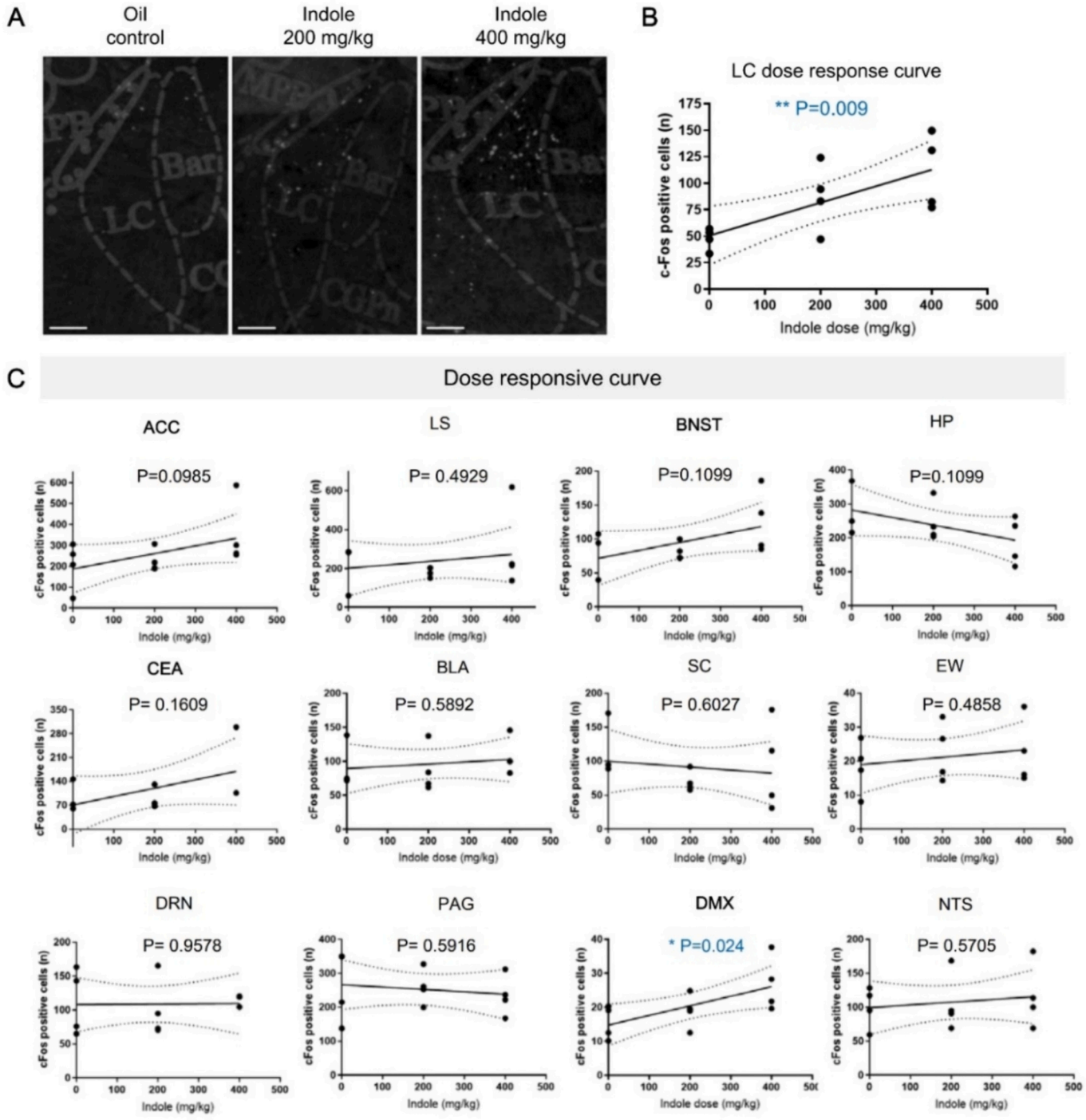

Fig. 2. Locus coeruleus (LC) and dorsal motor nucleus of the vagus nerve (DMX) were activated by indole treatment with a dose response curve in C3H/HeN mice. (A) Representative figures showed c-Fos immunofluorescence in LC of mice receiving indole 200 mg/kg or indole 400 mg/kg using corn oil as control; scale bars = 100 μm; $n = 4$ mice per group. (B) Linear regression analysis of the dose response curve of LC c-Fos$^+$ cell counting to corn oil, indole 200 mg/kg, and indole 400 mg/kg treatment; n = 4 mice per group, ** $P < 0.01$; data are expressed as individual values. (C) Linear regression analysis of the dose response curve of ACC, LS, BNST, HP, CEA, BLA, SC, EW, DRN, PAG, DMX and NTS c-Fos$^+$ cell counting to corn oil, indole 200 mg/kg, and indole 400 mg/kg treatment; anterior cingulate cortex (ACC), lateral septum (LS), bed nucleus of the stria terminalis (BNST), hippocampus (HP), central amygdala nucleus (CEA), basolateral amygdala nucleus (BLA), superior colliculus (SC), edinger-westphal nucleus (EW), dorsal raphe nucleus (DRN), periaqueductal gray (PAG), dorsal motor nucleus of the vagus nerve (DMX), nucleus tractus solitarius (NTS); n = 4 mice per group, * $P < 0.05$; data are expressed as individual values.

significant difference between the control and the indole group in the parameters of entries to the center and time in the center during the first 10 min in the open field (Ps > 0.05, Fig. 3B), C3H/HeN mice treated with indole entered less frequently to the center than the controls ($P = 0.0263$; Fig. 3D, E) during the second 10 min section. Furthermore, after normalizing with the parameter of locomotor activity (total distance), the indole group had a significantly lower value of "entries to the center/total distance" compared with control group (mean ± SEM: 0.89 ± 0.13 vs 0.40 ± 0.15; $P = 0.0260$; $n = 8$ mice for oil control group, $n = 9$ mice for indole; Student's *t*-test with Welch's correction). Overall, these results support the hypothesis that indole caused behavioural anomalies including locomotion impairment and anxiety-like behaviours.

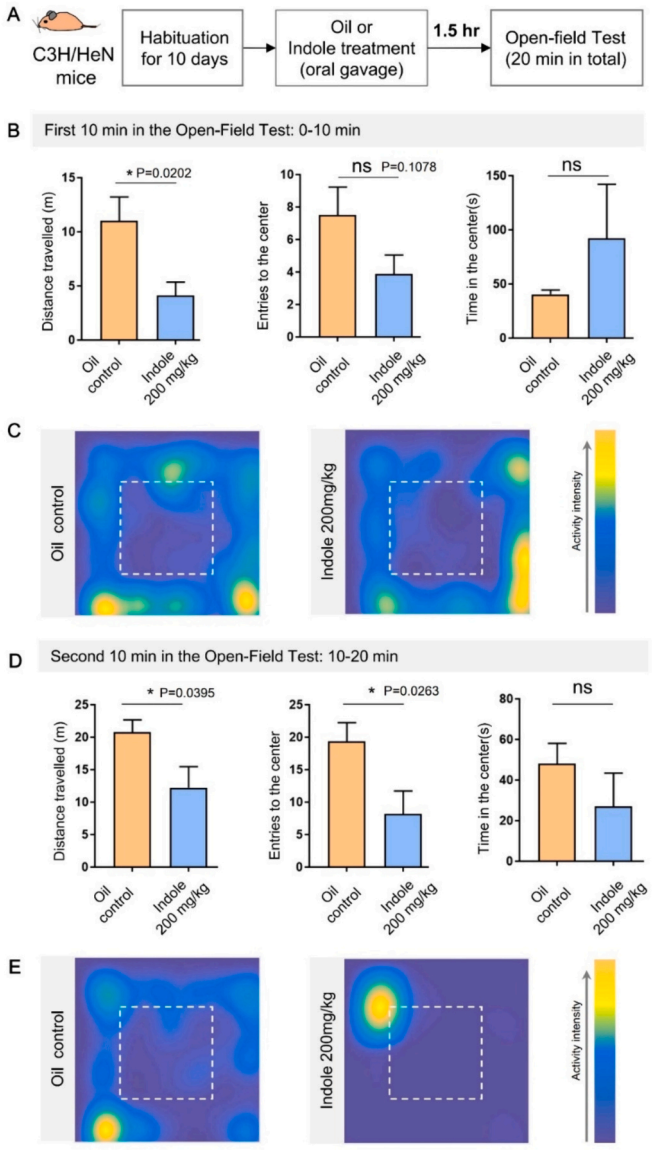

Fig. 3. Locomotion and anxiety-like behaviours in the open field test (OFT) after indole gavage treatment in C3H/HeN mice.
(A) Schematic diagram of gavage protocol and timing of behavioural tests for C3H/HeN mice. (B) Total distance travelled, entries to the center and time in the center during the first 10-min period in the OFT. (C) Representative heatmap showed the activity of C3H/HeN mice in the open field during the first 10-min period. (D) Total distance travelled, entries to the center and time in the center during the second 10-min period in the OFT. (E) Representative heatmap showed the activity of C3H/HeN mice in the open field during the second 10-min period. Data are expressed as mean ± SEM. ns, not significant; * $P <$ 0.05, Student's *t*-test with Welch's correction; $n = 8$ mice for oil control group, $n = 9$ mice for indole 200 mg/kg group.

3.3. Indole impairs locomotion and induces anxiety-like behaviour in C57BL/6 J mice with strong activation of locus coeruleus norepinephrine neurons

Different levels of baseline locomotion and anxiety-like behaviours have been reported among various inbred mouse strains (Trullas and Skolnick, 1993; Moloney et al., 2015; Milner and Crabbe, 2008). The C57BL/6J mouse strain is prominently featured as one of the most widely used inbred strains in diverse behavioural tests and serves as the genetic background for numerous transgenic mouse lines. In our study, aimed at checking whether the effect of indole is conserved across different mouse strains, we further employed C57BL/6J mice to investigate the impact of indole on animal behaviours in the OFT and assess its influence on brain activation (Fig. 4). During the first 10 min in the open field, there was no difference between C57BL/6J mice treated with 200 mg/kg indole and the controls in terms of the total distance travelled, the number of entries to the center, and the time in the center (Ps > 0.05; Fig. 4B, C). However, during the second 10 min of the OFT, the Student's *t*-test revealed that mice treated with indole travelled a significantly shorter distance than the mice treated with oil ($P = 0.0064$; Fig. 4D, E), indicating that indole may lead to difficulties in locomotion. In addition to locomotion anomalies, we also found that indole

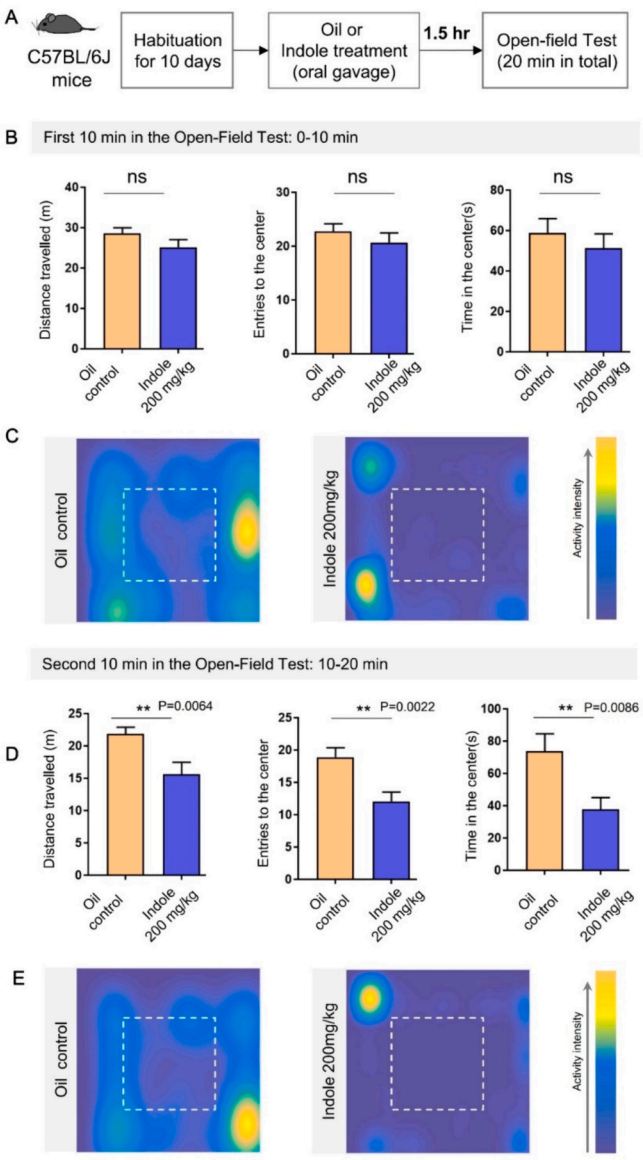

Fig. 4. Locomotion and anxiety-like behaviours in the open field test (OFT) after indole gavage treatment in C57BL/6J mice.
(A) Schematic diagram of gavage protocol and timing of behavioural tests for C57BL/6J mice. (B) Total distance travelled, entries to the center and time in the center during the first 10-min period in the OFT. (C) Representative heatmap showed the activity of C57BL/6J mice in the open field during the first 10-min period. (D) Total distance travelled, entries to the center and time in the center during the second 10-min period in the OFT. (E) Representative heatmap showed the activity of C57BL/6J mice during the second 10-min period. Data are expressed as mean ± SEM; ns, not significant; $^{**}P < 0.01$; Student's *t*-test with Welch's correction; $n = 18$ mice for oil control group, $n = 17$ mice for indole 200 mg/kg group.

influenced anxiety-like behaviours, such that the indole group entered the center less frequently than the control group ($P = 0.0022$; Fig. 4D, E) and spent less time in the center than the control group ($P = 0.0086$; Fig. 4D, E). The results of C57BL/6J mice corroborate the locomotion and anxiety-like behaviours issues seen in C3H/HeN mice. We further subjected the oil control group and the group treated with 200 mg/kg indole to the EPM test. In EPM test, the indole treated mice showed no significant difference in total distance travelled (mean ± SEM: 9.87 ± 0.74 vs 8.31 ± 0.47; $P = 0.2882$; $n = 9$ mice for oil control group, $n = 8$ mice for indole; Student's *t*-test with Welch's correction; data not plotted in the Figure). Yet, compared to the controls, indole treated mice showed a 33.10% drop in entries into the open arms, though not statistically significant (mean ± SEM: 12.33 ± 2.05 vs 8.25 ± 1.03; $P = 0.1017$; n = 9 mice for oil control group, n = 8 mice for indole; Student's *t*-test with Welch's correction; data not plotted in the Figure). Overall, the results point to a causal relationship between indole and behavioural abnormalities in C57BL/6J mice, including locomotion impairment and anxiety-like behaviours.

We next examined whether indole induced behavioural abnormalities in C57BL/6J mice were accompanied by activation of specific brain nuclei (Fig. 5). Fig. 5B shows the heatmap representing c-Fos activation for each mouse including anterior cingulate cortex (ACC), lateral septum (LS), bed nucleus of the stria terminalis (BNST), hippocampus (HP), central amygdala nucleus (CEA), basolateral amygdala nucleus (BLA), paraventricular nucleus of the thalamus (PV), edinger-westphal nucleus (EW), dorsal raphe nucleus (DRN), periaqueductal gray (PAG), LC, DMX, nucleus tractus solitarius (NTS), exhibiting similar pattern as observed in C3H/HeN mice (Fig. 1). Immunofluorescence staining of LC indeed showed increased c-Fos activation, with colocalization of TH^+ neurons (Fig. 5C). After counting the number of TH^+ and c-Fos^+ cells, we found that not only did the indole group expressed more c-Fos^+ cells in LC than the control group ($P = 0.0382$; Fig. 5D), but that more TH^+ neurons were activated in the indole group compared with the control ($P = 0.0356$; Fig. 5E). Among all the activated neurons in LC, the percentage of TH^+ neurons were 43.7% (Fig. 5F). Overall, our results show that the TH^+ neurons, and the LC-NE system, are likely to mediate the relationship between indole and anxiety-like behaviours.

3.4. *Chemogenetic activation of locus coeruleus norepinephrine neurons induces anxiety-like behaviour and locomotion impairment in a dose-sensitive manner*

Having found that indole treatment activated the LC-NE system and, simultaneously, influenced behaviours, we decided to investigate whether activation of the LC-TH^+ neurons alone would lead to anxiety-like behaviours and locomotion impairment. After injecting AAV-hM3Dq-mCherry virus or control virus AAV-mCherry in the LC of TH-Cre mice and i.p. injection of CNO (Fig. 6A), we found merged signals of virus expression (hM3Dq-mCherry) with c-Fos immunostaining in LC of the hM3Dq group, indicating successful virus infection and subsequent activation of TH^+ neurons in LC using hM3Dq-CNO system (Fig. 6B). The effect of LC-TH^+ neurons activation using hM3Dq-CNO was tested at two doses 0.5 mg/kg BW CNO and 2 mg/kg BW CNO (Fig. 6C-F). During the OFT after CNO injection, the hM3Dq-0.5 mg/kg CNO group and the controls travelled similar distance in the open field ($P = 0.4084$; Fig. 6D), while the hM3Dq-0.5 mg/kg CNO group spent less time in the center compared with control group ($P = 0.0485$; Fig. 6C, D; no significant difference in entries in the center parameter, $P = 0.8396$). In another experiment, with a higher dose of CNO (2 mg/kg BW), the hM3Dq-2 mg/kg CNO group showed strong decrease in total distance travelled compared with controls ($P = 0.0189$; Fig. 6E, F), though no significant change in time in the center between the control and hM3Dq-2 mg/kg CNO groups was observed ($P = 0.0810$), Entries to the center of the hM3Dq-2 mg/kg CNO were lower than those of the controls ($P = 0.0479$; Fig. 6E, F). Overall, we found that the activation of TH^+ neurons at low dose of CNO induced only anxiety-like behaviours while the

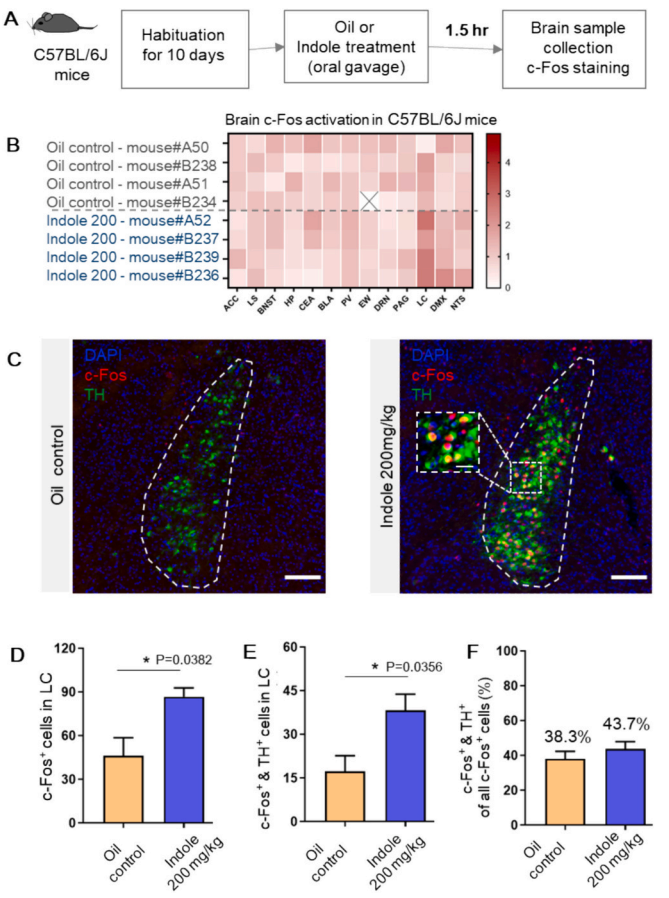

Fig. 5. Indole treatment induced strong activation of locus coeruleus (LC) norepinephrine neurons.
(A) Schematic diagram of the gavage protocol and timing of brain sample collection for C57BL/6J mice. (B) Heatmap representing of c-Fos activation for each mouse; anterior cingulate cortex (ACC), lateral septum (LS), bed nucleus of the stria terminalis (BNST), hippocampus (HP), central amygdala nucleus (CEA), basolateral amygdala nucleus (BLA), paraventricular nucleus of the thalamus (PV), edinger-westphal nucleus (EW), dorsal raphe nucleus (DRN), periaqueductal gray (PAG), locus coeruleus (LC), dorsal motor nucleus of the vagus nerve (DMX), nucleus tractus solitarius (NTS); the white blocks with cross in the middle indicated lost brain sections during processing; n = 4 mice per group. (C) Representative figures of the c-Fos activation in the LC of each mouse; blue, DAPI; red, c-Fos immunostaining; green, TH immunostaining; scale bars = 200 μm; insert bar = 50 μm. (D) The counting of c-Fos^+ cells in the LC of the indole group was higher than in the control group; (E) The activated c-Fos^+ and TH^+ cells in the LC of the indole group was higher than in the control group; (F) In the activated LC neurons, 43.7% was TH^+ cell in the indole group. For E-G, data are expressed as mean ± SEM; * $P < 0.05$; Student's *t*-test with Welch's correction; n = 4 mice per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activation of TH^+ neurons at high dose of CNO affected both locomotion and anxiety-like behaviours. Our data indicated that the LC-NE system mediated anxiety-like behaviours and locomotion anomalies induced by indole treatment, in a dose-sensitive way.

4. Discussion

To study the role of indole as a gut microbiota mediator in brain function, we used two strains of mice to show that administering indole via oral gavage consistently led to impaired locomotion and anxiety-like behaviour. Mapping of c-Fos protein expression in C3H/HeN mice subjected to two different indole doses provided clues about the brain

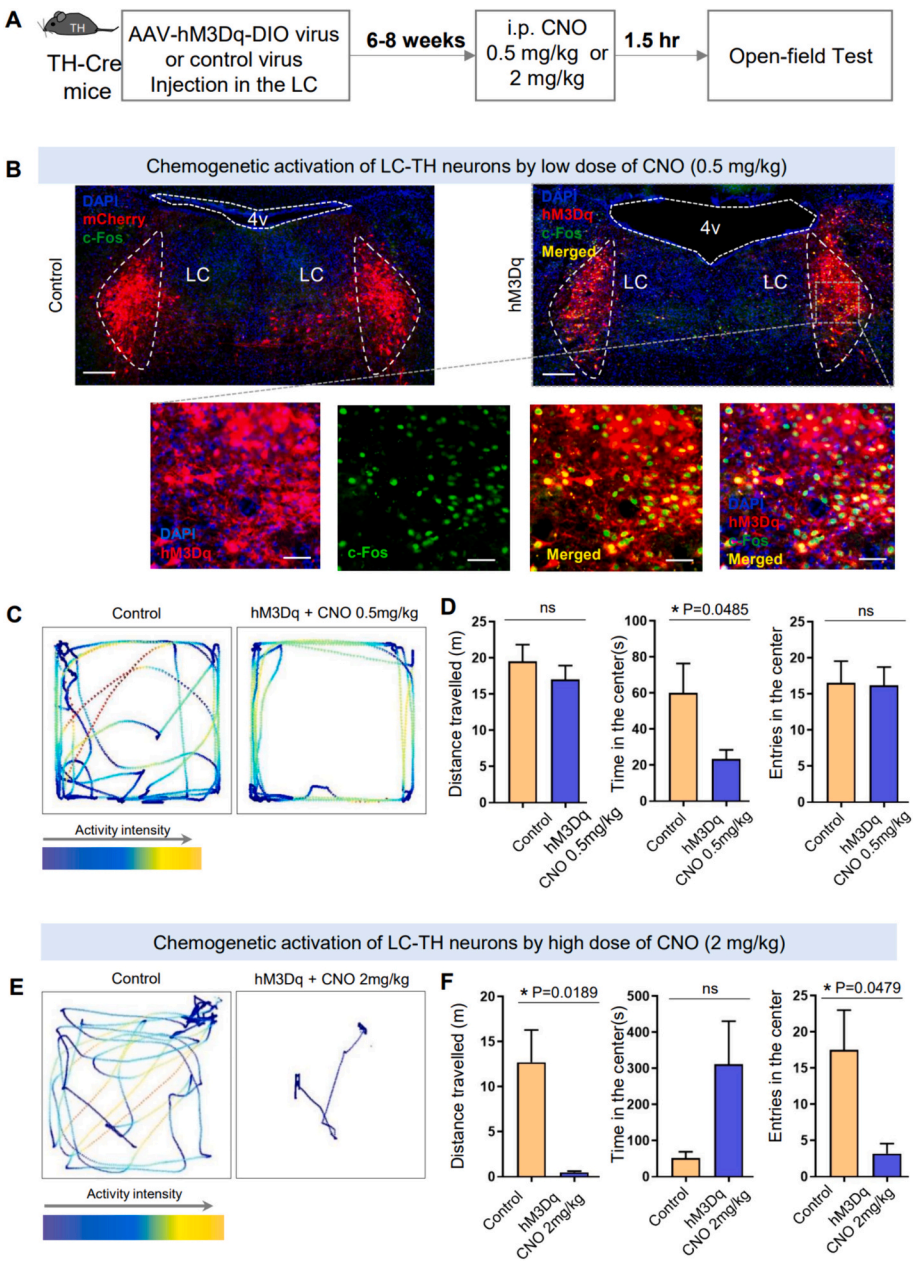

Fig. 6. Chemogenetic activation of LC TH-positive neurons resulted in anxiety-like behaviour and disturbed locomotion after low and high doses of CNO injection. (A) Schematic diagram of virus injection and chemogenetic activation of LC-TH$^+$ neurons using TH-Cre mice; LC, locus coeruleus; TH, tyrosine hydroxylase; HM3Dq – Human Muscarinic Receptor 3 Designed for DREADD Activation (q-type); DIO, Double-floxed Inverted Open reading frame; CNO, Clozapine-N-Oxide; i.p., intraperitoneal. (B) Representative figures showing the expression of both mCherry virus and hM3Dq-mCherry virus in the control and hM3Dq groups and the c-Fos activation in the hM3Dq group after CNO i.p. injection; blue, DAPI; red, mCherry or hM3Dq-mCherry; green, c-Fos, yellow, Merged, scale bars = 50 um. (C). Representative track plot of the mouse in the open field 1.5 h after 0.5 mg/kg BW CNO i.p. injection. (D). Quantitative analysis of the open field data of control and 0.5 mg/kg CNO-hM3Dq-LC activated groups showed no difference in the animals' locomotion but a significant decreased in the time in the center compared with control. Data are expressed as mean ± SEM; ns, not significant; * $P < 0.05$; Student's *t*-test with Welch's correction; $n = 13$ mice Control group, $n = 15$ mice for CNO 0.5 mg/kg-hM3Dq group. (E). Representative track plot of the mouse in the open field 1.5 h after 2 mg/kg BW CNO i.p. injection. (F). Quantitative analysis of the open field data revealed that 2 mg/kg CNO-hM3Dq-LC activated group showed strong decrease in locomotion induced together with a significant decrease in entries in the center compared with controls. Data are expressed as mean ± SEM; ns, not significant; * $P < 0.05$, Student's *t*-test with Welch's correction; $n = 6$ mice for Control group, n = 6 mice for CNO 2 mg/kg-hM3Dq group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nuclei potentially responsible of this indole-induced behavioural changes. This included anterior cingulate cortex (ACC), central amygdala nucleus (CEA), bed nucleus of the stria terminalis (BNST), dorsal motor nucleus of the vagus nerve (DMX) and locus coeruleus (LC), among which LC activation was significantly increased and showed a dose-responsive curve to indole treatments. Identification and chemogenetic activation of LC norepinephrine neurons proved the role of LC in mediating anxiety-like behaviours and locomotion of the mice (summarized in Fig. 7, created with BioRender.com).

The roles of indole and its derivatives in modulating locomotion and emotional behaviours have been reported in rodent models. Research on indole derivatives, such as isatin, 3-indoxylsulfate, and oxindole, has shown implications in anxiety-like behaviours, reduced locomotor activity, spatial memory deficits, and other abnormalities (Jaglin et al.,

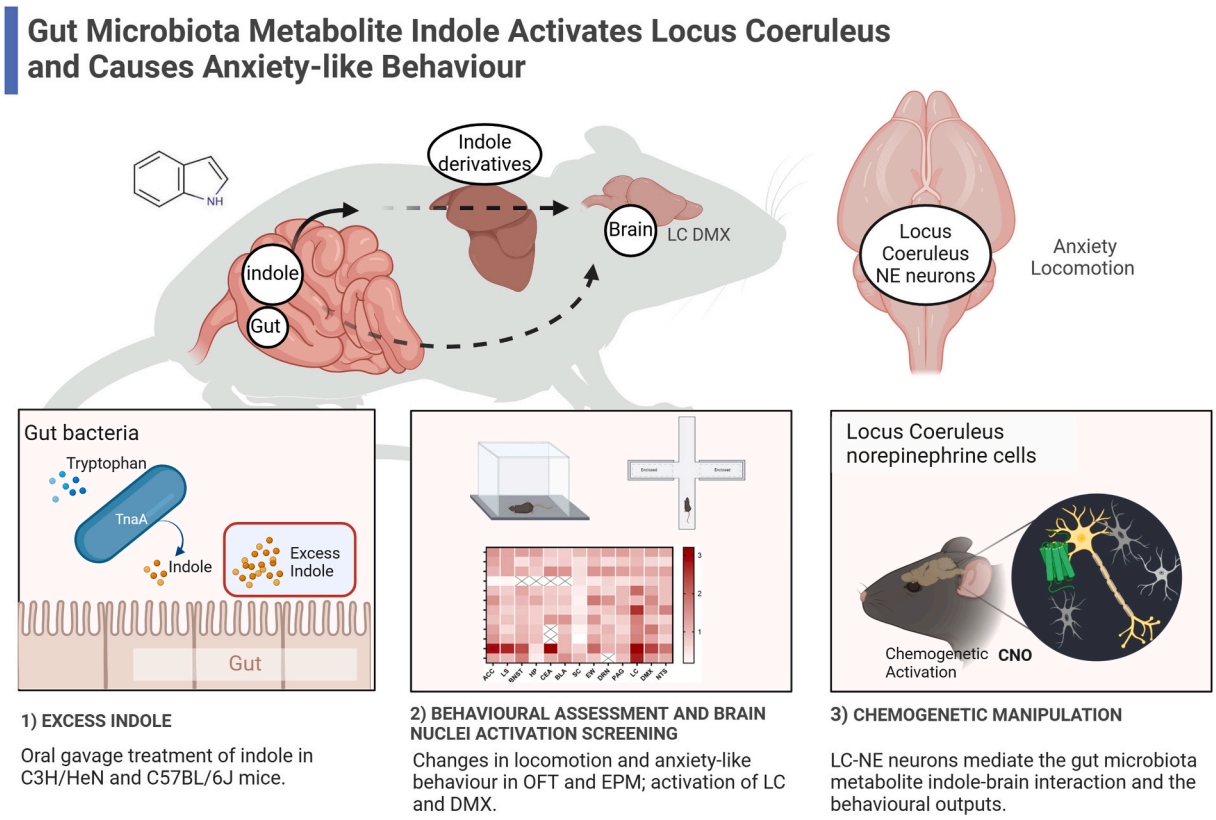

Fig. 7. Gut microbiota metabolite indole induces anxiety-like behaviour in mice mediated by brainstem locus coeruleus activation.
This summary diagram of our study illustrates the role of indole in activating locus coeruleus norepinephrine neurons and its contribution to behavioural changes. These findings provide support for indole's role as a neuroactive molecule within the gut-brain axis; LC, locus coeruleus; DMX, dorsal motor nucleus of the vagus nerve; CNO, Clozapine-N-Oxide.

2018; Carpenedo et al., 1998; McCall et al., 2015; Trullas and Skolnick, 1993; Brydges et al., 2021; Mannaioni et al., 1998; Karbowska et al., 2020a). The effect of indole on ACC activation and its possible involvement in spatial memory would be intriguing to investigate further in subsequent studies (Sutherland et al., 1988; Teixeira et al., 2006; Jin et al., 2020). Limited results on the function of indole on emotions were reported. Chronic production of indole in gnotobiotic rodents with indole-producing *E. coli* strain compared with the ones with a non-indole-producing knockout mutant *E. coli* strain induced anxiety-like and depression-like behaviours in F344 male rats and increased responses to a comprehensive emotion-related behavioural assessment in male C3H/HeN mice exposed to unpredictable chronic mild stress (Jaglin et al., 2018; Mir et al., 2020). Intra-cecal injection of indole at a dose of 500 mg/kg BW decreased locomotion in male F344 rats (Jaglin et al., 2018). In the current experiment, we found oral gavage of indole at a lower dose of 200 mg/kg BW in mice could already induce activation of brain nuclei, along with the 400 mg/kg dose. Thus, we tested the effect of 200 mg/kg indole oral gavage treatment on animal behaviours in the following experiments. Experiments by Carpenedo et al. (1998) showed that oral gavage with indole leads to increase of oxindole concentration in brain (Carpenedo et al., 1998). We then found that injecting indole in the cecum of conventional rats led to accumulation of oxindole and isatin in the rat brain (Jaglin et al., 2018). Furthermore, experiments we carried out in gnotobiotic rats (Jaglin et al., 2018) and mice (Mir et al., 2020) colonized with an *E. coli* strain, mimicking a chronic and moderate overproduction of indole, led to the presence of 3-indoxylsulfate in the systemic circulation. Taken together, these data show that indole, when present in the gut, whether it is given by gavage, introduced in the cecum, or produced by gut bacteria, is absorbed and further metabolized into oxidized derivatives that reach the systemic circulation and distant organs. One might suspect that such treatment could lead to kidney toxicity, as the main oxidized and conjugated metabolite of indole, 3-indoxylsulfate, is a uremic toxin. In fact, studies of 3-indoxylsulfate toxicity in the context of kidney disease usually involve dosing the animals in a chronic fashion (Karbowska et al., 2020a; Bobot et al., 2020). Furthermore, Karbowska et al. (2020) administered 200 mg/kg BW of 3-indoxylsulfate daily for 4 weeks to rats via drinking water without any renal disease being observed (Karbowska et al., 2020b). It was on these bases that we chose the doses of indole and the acute mode of administration used in the present study.

In our study, decreased locomotion was found in the first and second 10-min time interval inside the open field for C3H/HeN mice, as well as in the second 10-min time interval for C57BL/6J mice. No change in the total distance travelled in the first 10-min time interval was found in the C57BL/6J mice, suggesting that C3H/HeN mice' locomotor activity was more sensitive to indole treatment. C3H/HeN mice are inherently blind (von Gall et al., 2000; Pfeffer et al., 2022), and, consequently, cannot be utilized in anxiety-like behaviour assays such as the light/dark box. The open field test (OFT), initially introduced by Hall in 1934, serves as a valuable tool for evaluating emotional behaviour in rodents. Within the context of behavioural assessments, an animal's behaviour in an open field, including changes in the number of lines crossed, is considered a key parameter indicative of the impact of anxiolytic compounds on exploratory behaviour. Anxiety-related behaviour evaluated in the OFT arises from the interplay of two contributing factors: solitary testing and agoraphobia (Crawley, 1985; Prut and Belzung, 2003). Now it is generally accepted that an increase in central movement or an extended duration spent in the central region of the apparatus, without significant alterations in overall locomotion, is interpreted as indicative of an anxiolytic-like response. Conversely, a reduction in these variables is associated with anxiogenic effects. Numerous studies have demonstrated the utility of OFT in screening for anxiolytic-like or anxiogenic

effects, yielding positive results for chlordiazepoxide and diazepam in eliciting anxiolytic-like responses (Crawley, 1981; Sanger and Zivkovic, 1988; Bruhwyler, 1990; Horvath et al., 1992; Angrini et al., 1998; Britton and Britton, 1981; Matsubara and Matsushita, 1982; Hard et al., 1985; Rex et al., 1996). Notably, initial investigations in this field indicated that chlordiazepoxide and diazepam significantly influenced locomotor activity in male C57 mice (Crawley, 1985; Christmas and Maxwell, 1970). Subsequent evaluations of diazepam in rodent models yielded conflicting results regarding its impact on locomotion, with studies reporting either decreased motor activity or the absence of a stimulant effect on locomotion (Prut and Belzung, 2003; Siemiatkowski et al., 2000; Boerngen-Lacerda and Souza-Formigoni, 2000), yet the anxiolytic-like effect of these drugs were not overruled by these locomotion parameter complications. Parameters indicative of anxiety-like behaviour in our study included decreased entries into the central area and a trend towards reduced time spent in the central region during OFT for C3H/HeN mice. Regarding anxiety-like behaviours in the OFT, C3H/HeN strains were reported to exhibit higher levels of anxiety-like behaviour and lower locomotion baselines in comparison to C57BL/6J mice (Moloney et al., 2015; Milner and Crabbe, 2008). Our data showed a similar baseline pattern. Collectively, our data suggest an increase in the anxiety state of the animals, though complicated by alterations in locomotor activity.

Our data provide strong support for the neuroactive roles of indole. In our quest to pinpoint the brain nuclei responsible for this gut microbiota signal, we conducted a comprehensive brain activation analysis in C3H/HeN mice. These mice were subjected to either oil gavage controls or gavage with indole at doses of 200 mg/kg or 400 mg/kg. Our statistical analysis of c-Fos$^+$ cell counts in brain nuclei, coupled with the generation of heatmaps depicting the activated brain states in comparison to control conditions, pointed towards the potential involvement of several brain regions, including ACC, BNST, CEA, LC, and DMX. However, the most compelling evidence strongly implicated the brainstem nucleus LC as a key player. To further validate our hypothesis regarding the LC's pivotal role, we performed linear regression analyses correlating c-Fos$^+$ cell counts with the doses of indole administered. The statistical results revealed a significant positive response in both LC activation and DMX activation to indole treatments. This finding aligns with our previous research in rats, which highlighted the dorsal vagal complex (DVC, DMX as a part of DVC) involvement and demonstrated increased c-Fos expression accompanied by abnormal eye-blinking following indole treatment (Jaglin et al., 2018).

Given the robust activation of LC in both C3H/HeN and C57BL/6J mice, LC emerged as an intriguing target for further investigation. To delve deeper into this, we conducted experiments to validate that a predominant portion of the indole-activated LC cells were tyrosine hydroxylase (TH) -positive. Subsequently, we employed TH-Cre mice in conjunction with AAV-DIO-hM3Dq chemogenetic activation tools to artificially activate LC-TH$^+$ neurons. Our results demonstrated that the activation of LC-TH$^+$ neurons led to a significant reduction in the time spent in the center during OFT when compared to control conditions. These findings underscore the pivotal role of LC-TH$^+$ neurons in mediating the effects of indole oral gavage treatment and shed light on the intricate interplay between gut microbiota-derived signals and the central nervous system. As the principal source of norepinephrine in the central nervous system, LC comprises a relatively small but densely populated group of cells, with approximately 1600 cells per LC in rodents (Berridge and Waterhouse, 2003; Schwarz and Luo, 2015). The LC-norepinephrine system provides widespread innervation throughout the entire neuroaxis, including the forebrain, brainstem, and cerebellum (Schwarz and Luo, 2015). The LC regulates a diverse array of functions, encompassing arousal, stress responses, mood, cognition, pain perception, and motor control, with significant roles complicated in various neurological and psychiatric disorders (Valentino and Van Bockstaele, 2008; Sara, 2009).

Carter et al. employed optogenetics to selectively manipulate LC-NE neurons activity in mice. Their work revealed that phasic stimulation (10-ms pulses at 10 Hz for 500 ms, delivered as five light pulses every 20 s for 1 h) led to a significant decrease in locomotor activity, while tonic stimulation (constant 10-ms light pulses at 3 Hz for 1 h) resulted in a significant increase in locomotor arousal, indicating the intricate and nuanced role of the LC in regulating locomotion (Carter et al., 2010). In our study, chemogenetic activation of the LC-NE neurons through a single low dose of CNO injection resulted in a slight decline in total distance travelled in OFT. McCall et al. and Li et al. demonstrated that both acute high-tonic LC activation (5 Hz, 10-ms pulse width, 473 nm for 30 min) or repetitions in LC activation (10 Hz, 10-ms pulse width, 473 nm for 3 min then off for 3 min for three times) induced anxiety-like behaviours in mice (Li et al., 2018; McCall et al., 2015). In our investigation, LC-NE neurons exhibited robust activation in response to indole treatment, surpassing all other brain regions examined. Notably, chemogenetic activation of LC-NE neurons with a single low dose of CNO already induced anxiety-like behaviour in the animals while higher dose of CNO-hM3Dq activation of LC-NE neurons changed both the locomotion and entries to the center in OFT, providing strong support for the role of the LC in mediating the abnormal behaviours induced by indole in mice.

Future studies will be necessary to understand the gut microbiota indole-to-LC interplay in anxiety-like behaviours. To construct a more comprehensive dose-response curve, it would be beneficial to investigate additional doses beyond 200 mg/kg indole and 400 mg/kg indole. Additionally, it is highly possible that more than one nucleus and pathway are involved in the indole-induced behavioural changes in the animals. In-depth examination of the role played by the DMX and its specific cell types, for example, using vagotomy in combination with optogenetic or chemogenetic manipulation of DMX neuron activities, as well as their interactions with the LC, will be important.

Furthermore, it is imperative to ascertain by which pathways indole activates the LC. Through its widespread projections, LC neuron activation can modulate behavioural outputs via NE release at its downstream target nuclei (Schwarz and Luo, 2015; Zhang et al., 2019a; Seo et al., 2021; Zhang et al., 2019b). In our study, locomotion and anxiety-like behaviours were affected by indole treatment. Considering the important roles LC plays in stress response, arousal and cognition (Sara, 2009; Poe et al., 2020), the activation of the LC by indole treatment may indicate additional neuroactive functions of indole and its oxidized derivatives, extending beyond their established effects on locomotion and anxiety regulation. Further research into how LC-NE neurons are activated by indole, whether directly or indirectly, and the specific LC-NE signaling pathways involved would be highly intriguing. By elucidating the pathways through which indole and its derivatives influence LC activity, the ultimate goal in the long run is to gain a more comprehensive understanding of the microbiota-gut-brain axis and its role in anxiety disorders. This understanding will pave the way for innovative treatments that leverage the microbiota, dietary interventions, and targeted pharmacological therapies related to LC-NE signaling or indole-oxidized derivatives, ultimately offering new hope for individuals suffering from anxiety.

Our study proved the neuroactive role of the gut microbiota metabolite indole in modulating anxiety-like behaviour and locomotion through the activation of central brain nuclei LC. Our finding highlights the LC as a novel hub in the gut-brain axis, offering promising avenues for future research investigations.

Author contributions

RS and LL designed and supervised research; MH, YQ, JS and SX performed the experiments with inputs from WL; NL, ME, MQ, CQ, LL, and RS analyzed and interpreted data; MH, YQ, LL and RS wrote the manuscript with inputs from all authors.

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CRediT authorship contribution statement

Hayatte-Dounia Mir: Methodology, Investigation. **Qingning Yang:** Writing – original draft, Methodology, Investigation, Formal analysis. **Elise Maximin:** Data curation. **Quentin Montardy:** Investigation, Formal analysis. **Shuqin Ji:** Investigation. **Qi Cheng:** Writing – original draft. **Xiaochun Shan:** Investigation. **Liping Wang:** Validation, Resources. **Laurent Naudon:** Investigation. **Sylvie Rabot:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization. **Lei Li:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

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Appendix A. Supplementary data

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