The microbial metabolite *p*-Cresol induces autistic-like behaviors in mice by remodeling of the gut microbiota

p-Cresol induces autistic-like behaviors in mice

Bermudez-Martin, P.[1, 2], Becker, J. A. J.[3,#], Fernandez, S. P.[1, 2], Costa-Campos, R.[1, 2], Barbosa, S.[1, 2], Martinez-Gili, L.[4], Myridakis, A.[4], Dumas, M.-E.[4], Bruneau, A.[5], Cherbuy, C.[5], Langella, P.[5], Chabry, J.[1, 2], Barik, J. [1, 2], Le Merrer, J.[3,#], Glaichenhaus, N. [1, 2], Davidovic, L.[1, 2*]

[1] Centre National de la Recherche Scientifique, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France

[2] Université Côte d'Azur, Nice, France

[3] Physiologie de la Reproduction et des Comportements, INRAE UMR-0085, CNRS UMR-7247, Inserm, Université François Rabelais, IFCE, 37380, Nouzilly, France

[4] Division of Computational and Systems Medicine, Department of Surgery and Cancer, Imperial College London, Exhibition Road, South Kensington, London SW7 2AZ, United Kingdom

[5] Institut Micalis, INRAE, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

Current address: UMR-1253 iBrain, Inserm, Université de Tours, CNRS, Faculté des Sciences et Techniques, Parc de Grandmont, F 37200 Tours, France

Corresponding author:

*Mailing address: Institut de Pharmacologie Moléculaire et Cellulaire UMR7275, 660 route des Lucioles 06560 Valbonne, France; E-mail: davidovic@ipmc.cnrs.fr; Phone: +33(0)493 957 763

Keywords: microbiota, autism, behavior, reward system, metabolite, *p*-Cresol, 4-Cresol

ABSTRACT

Background: Changes in microbiota composition and microbial metabolites levels are associated with autism spectrum disorders (ASD), suggesting that the microbiota-gut-brain axis could be involved in the development of social behavior deficits and stereotyped behaviors in ASD patients. Yet, a causal relationship between abnormal microbiota composition, altered microbial metabolite production and ASD behaviors remains to be demonstrated. Here, we hypothesized that *p*-Cresol (also known as 4-Cresol), a microbial metabolite described as overabundant in ASD patients, contributes to ASD core behavioral symptoms.

Methods: We treated for C57BL/6J male mice with *p*-Cresol in drinking water for 4 weeks, starting 4.5 weeks after birth. We characterized these animals for behavior, dopamine neurons electrophysiology and microbiota composition using 16S sequencing. We then used fecal matter transplantation (FMT) to further investigate the role of microbiota composition in *p*-Cresol-induced changes in behavior.

Results: We showed that *p*-Cresol did induce social behavior deficits, stereotypies and perseverative behaviors, but not changes in anxiety, locomotion or cognition. We further demonstrated that *p*-Cresol decreased the activity of dopamine neurons in the ventral tegmental area (VTA) and modified the relative abundance of specific bacterial taxa which correlated with behavioral changes. We also showed that social behavior deficits could be transferred from *p*-Cresol-treated mice to control mice by fecal matter transplantation (FMT). In contrast, the microbiota from control mice restored both social interactions and VTA dopamine neurons excitability when transferred to *p*-Cresol-treated mice.

Conclusions: The microbial metabolite *p*-Cresol induces ASD core behavioral symptoms in mice via a gut microbiota-dependent mechanism.

INTRODUCTION

Autism spectrum disorders (ASD) are frequent (1:68) and severe neurodevelopmental pathologies diagnosed during early childhood (1). ASD are characterized by social interaction and communication deficits, patterns of perseverative/stereotyped behaviors and restricted interests, as well as abnormal sensory processing (1). Also, ASD often co-occurs with anxiety, hyperactivity and intellectual disability (1). ASD is also associated with gastrointestinal (GI) dysfunction and increased intestinal permeability (2). Children with ASD and concurrent GI symptoms exhibit more pronounced social impairments, sensory over-responsivity and anxiety compared to ASD peers without GI symptoms (3-5). In addition, ASD patients exhibit gut microbiota dysbiosis characterized by reduced bacterial β-diversity and changes in the relative abundance of specific bacterial taxa and notably increased abundance of the *Clostridioides* genus and decreased abundance of the *Bifidobacterium* genus (6-8).

Dysbiosis in ASD patients is often associated with altered urinary, plasmatic or fecal levels of microbial metabolites such as short-chain fatty acids (SCFA), indoles and tyrosine-derived metabolites (9-16). In a pilot study, fecal microbiota transplantation (FMT) from healthy individuals to ASD patients durably alleviated both GI symptoms and ASD core symptoms (17). Dysbiosis (18-22) and altered levels of microbial metabolites (18, 21, 22) have also been observed in rodent models of ASD : the maternal immune activation (MIA), the diet-induced obesity (DIO) and the valproate environmental models, the BTBR T+tf/J idiopathic model, and the *Shank3b*-KO genetic model. Furthermore, changes in microbiota composition induced by FMT or probiotic treatment alleviated behavioral alterations in several of these ASD models (18-20). Finally, mice born from mothers transplanted with feces from ASD patients, but not from neurotypical individuals, exhibited social behavior deficits, microbiota dysbiosis and abnormal patterns of microbial metabolites (23).

The link between altered levels of microbial metabolites and behavioral impairments in ASD remains mostly unknown. However, several microbial metabolites whose levels are altered in ASD patients or ASD mouse models induced behavioral changes when administered to

rodents. Treatment with the SCFA propionate induced social interaction deficits, stereotypies, cognitive deficits and anxiety in rats (14). The tyrosine derivative 4-ethylphenylsulfate (4-EPS) induced anxiety in mice (18). Finally, indoles impacted socioemotional behavior in rats (24). Altogether, these data suggested that dysbiosis could contribute to ASD core and associated symptoms via the production of microbial metabolites.

Among the different microbial metabolites which are more abundant in ASD patients compared to sex- and age-matched neurotypical controls, the small aromatic metabolite *p*-Cresol (*para*-Cresol, 4-Cresol, 4-methylphenol) is one of those for which the results are the more consistent across studies. Urinary (11, 12) and fecal (15, 16) levels of *p*-Cresol were found to be increased ASD patients in four independent studies. Also, *p*-Cresol levels correlated with the severity of ASD behavioral alterations (11, 12). *p*-Cresol is the product of tyrosine degradation by the intestinal microbiota and mainly by *Clostridium difficile* and other species from the *Clostridioides* genus (25, 26), which is more abundant in ASD patients (6-8).

Based on these findings, we hypothesized that increased *p*-Cresol levels contribute to the induction or maintenance of ASD core symptoms. To test this hypothesis, we investigated in mice the impact of *p*-Cresol on behavior, dopamine neurons electrophysiology and microbiota composition.

METHODS AND MATERIALS

Extended methods and materials are available in Supplementary Information.

Ethics. Animal housing and experimentation were conducted in facilities certified by local authorities (Direction Départementale de Protection des Populations des Alpes-Maritimes, accreditation #EC-06-152-5) and performed according to policies on the care and use of laboratory animals of European Communities Council Directive (2010/63EU) and under the agreement from the French Ministry of Research.

Animals treatment. Weaned C57BL/6J mice (21 to 28 day-old) were ordered to Charles Rivers (France). This developmental stage was chosen to avoid confounding effects of maternal exposure to *p*-Cresol, while still being in the time window for late neurodevelopment during which the microbiota-gut-brain axis can intervene (27). Since the sex ratio for ASD is biased towards 3 males diagnosed for 1 female suggesting a higher susceptibility of males (28), only males were considered in this study. Mice were randomly assigned to experimental groups. After 6 days of acclimation to the animal facility, mice were treated for at least 4 weeks with *p*-Cresol (Sigma-Aldrich) dispensed in drinking water at a concentration of 0.25g/l for a target dose of 50 mg/Kg/24 h. This dose per day corresponded to 7 times less the reported LD50 dose of 350 mg/Kg for acute oral administration of *p*-Cresol by gavage in mice, based on available toxicity data (29).

Quantification of *p*-Cresol in urine and serum. Urine (20 µL) and serum (100 µL) samples were spiked with 10 µL internal standard solution (myristic acid-d_{27} in isopropanol, 750 mg/mL), subjected to methanol extraction and analyzed using gas chromatography-mass spectrometry (GC-MS) (30).

Behavioral testing. Standard behavioral tests that have extensively been used to characterize genetic and environmental models of ASD (31-35) were implemented to assess: social behavior (three-chamber sociability test, dyadic social interactions), repetitive behaviors (motor stereotypies, marble burying test, Y-maze exploration), locomotor activity (actimetry), anxiety (open-field, novelty suppressed feeding) and cognition (novel object recognition test).

***Ex vivo* patch-clamp electrophysiological recordings**. Spontaneous excitatory post-synaptic currents (sEPSCs) or excitability were measured in 250 µm brain sections encompassing the ventral tegmented area (VTA) using visualized whole-cell voltage-clamp and current-clamp recordings, respectively, as described (36). Depolarizing (0-300 pA) or hyperpolarizing (0- 450 pA) 800 ms current steps were used to assess excitability and membrane properties of VTA dopamine neurons. sEPSCs were assessed in voltage-clamp mode at a voltage of -65mV in the presence of picrotoxin (50 µM) using the same internal solution. Offline analyzes were performed using Clampfit 10.2 (Axon Instruments, USA).

Fecal microbiota analysis by 16S rRNA gene sequencing. Genomic DNA was obtained from fecal samples using the QIAamp power fecal DNA kit (Qiagen). The V3-V4 hypervariable region of the 16S rRNA gene were amplified by PCR and sequenced (Illumina Miseq technology). High-quality filtered reads were assembled and processed using FROGS pipeline to obtain operational taxonomic units (OTU) and their respective taxonomic assignment (37), yielding 591 final clusters. Clusters were then affiliated to OTU by using a Silva123 16S reference database and the RDP (Ribosomal Database Project) classifier taxonomic assignment procedure. Richness and diversity indexes of bacterial community were computed using the Phyloseq package (v 1.19.1) in RStudio software (38). Statistical differences in the microbial communities between groups were evaluated using constrained analysis of principal coordinates and permutational multivariate ANOVA. To identify discriminating taxa and OTU associated with the control or *p*-Cresol class and estimate their effect size, we used the linear discriminant analysis (LDA) effect size (LEfSe) method (39).

Fecal microbiota transplantation (FMT). Mice were gavaged with omeprazole (50mg/Kg/24 h, Day 1-3) each morning. On Day 4, mice received 5 consecutive gavages of 200μL of Moviprep (Norgine) at 90 min intervals. On Day 5, recipient mice received 1 omeprazole gavage and 3 consecutive gavages of 200 μL of a fecal slurry at 2 h intervals. The fecal slurry consisted of pools of individual fecal pellets from donor mice homogenized in ice-cold ddH_2O (w:v=1:50). Behavioral phenotyping or electrophysiological recordings were performed three weeks post FMT.

Statistics. Two-group comparisons were performed using 2-tailed Mann-Whitney's U-test. Multiple group comparisons were performed using two-way ANOVA. *Post hoc* comparisons were performed using Šidák's correction for multiple comparison. For comparison analysis of frequency distributions, Kolmogorov-Smirnov's test was used. Statistical analysis was performed using GraphPad Prism version 6.00 for iOS (GraphPad Software, USA). Principal Component Analysis (PCA) of behavioral data was performed for each dataset consisting of scores from the dyadic social interaction test and scores from the direct monitoring of motor stereotypies. Correlations were performed using Spearman's ρ correlation coefficient rank test with Benjamini-Hochberg's multiple testing correction. Statistical significance was set at an adjusted *p*-value below 0.05. Only significant differences are displayed.

RESULTS

p-Cresol induces ASD core symptoms in mice

To mimic exposure to *p*-Cresol through the GI tract, we treated C57BL/6J male mice with *p*-Cresol in drinking water starting at 4.5 weeks of age (Supplementary Figure 1A). A 4-week treatment with *p*-Cresol did not induce changes in body weight, drink or food intake (Supplementary Figure 1B-D), suggesting that it did not have overt toxicity effects. Compared

to control animals, *p*-Cresol-treated mice exhibited a 4-fold increase in levels of *p*-Cresol in urine, but not in serum (Supplementary Figure 1E, F).

We then analyzed *p*-Cresol-treated mice for social interaction deficits and repetitive/perseverative behaviors (as proxies for ASD core symptoms) as well as anxiety, hyperactivity and cognitive deficits (as proxies of ASD comorbidities). In the 3-chamber test, *p*-Cresol-treated mice presented reduced sociability (Figure 1B) and no preference for the mouse interactor towards the toy mouse (Figure 1C) compared to control mice. Although the number of close contacts with the mouse interactor was higher than with the toy mouse (Supplementary Figure 2G), their mean duration was reduced (Figure 1D). During dyadic social interactions, *p*-Cresol-treated mice displayed a decrease in time spent in social contact compared to control mice (Figure 1E). The time, number and mean duration of both nose and paw contacts as well as the number of followings were also reduced (Figure 1F-J, Supplementary Figure 2H, I). This indicates that *p*-Cresol treatment reduces social interactions and deteriorates the quality of social contacts.

As for repetitive/perseverative behaviors, *p*-Cresol-treated mice displayed more frequent head shakes and circling events (Figure 1K, L), but similar numbers of rearing episodes and time spent in digging or self-grooming as compared to control mice (Supplementary Figure 2J-L). Thus, the occurrence of stereotyped behaviors was increased in *p*-Cresol-treated mice, as confirmed in the marble burying test (Figure 1M). Consistently, *p*-Cresol treatment increased the frequency of perseverative same arm returns during the Y-maze continuous spontaneous alternation task (Figure 1N). Finally, *p*-Cresol-treated mice were clearly separated from control mice along the PC1 axis (Figure 1O) in a PCA analysis of scores recorded in the dyadic social interaction and stereotypies tests.

As for other behaviors, *p*-Cresol-treated and control mice displayed similar nocturnal and diurnal locomotor activity as assessed in actimetry chambers, and travelled the same distance in the open-field (Supplementary Figure 2M-O). This indicated that *p*-Cresol did not induce hyperactivity. Also, the number of entries and time spent in the open-field center, as well as the latency to feed in the novelty-suppressed feeding test were not impacted (Supplementary

Figure 2P-R), suggesting that *p*-Cresol did not alter anxiety levels. Finally, *p*-Cresol-treated mice explored objects similarly to control mice and displayed a similar recognition index for the novel object in the novel object recognition task, indicating that their exploratory behavior and cognitive ability were preserved (Supplementary Figure 2S, T). Altogether, these results suggest that *p*-Cresol selectively induces ASD core symptoms, but does not impact the other behavioral dimensions investigated here.

_p_-Cresol impairs dopamine neurons excitability in the VTA and induces long-lasting autistic-like behaviors that persist after washout

We then sought to identify the neuronal circuits that were impacted by *p*-Cresol. We focused on dopamine neurons from the VTA that are part of a 'socially engaged reward circuit' (40). Altered VTA connectivity and impaired VTA dopamine neurons activity were demonstrated in ASD patients (41) and in ASD models (19, 20, 42-45) respectively. We used whole-cell patch-clamp to record VTA dopamine neurons in acute brain slices from control and *p*-Cresol-treated animals (Figure 1P-W). First, *p*-Cresol-treated animals displayed reduced excitability of VTA dopamine neurons, with a reduction in the number of evoked action potentials (Figure 1P-R). Second, both the amplitude and frequencies of miniature spontaneous excitatory postsynaptic currents (sEPSC) were reduced in VTA dopamine neurons in *p*-Cresol-treated animals compared to controls (Figure 1S-W). Therefore, *p*-Cresol treatment results in a decreased activity of dopamine neurons in the VTA.

We then investigated whether *p*-Cresol-induced behaviors were abolished when treatment was discontinued (Figure 2A). We observed that the effect of *p*-Cresol on social interactions and stereotyped/perseverative behaviors persisted and were of similar magnitude, even after a 4-week washout (Figure 2B-I, Supplementary Figure 3A-F). Control and *p*-Cresol-treated mice were clearly separated along the PC1 axis, and before and after washout along the PC2 axis in a PCA analysis of social behavior and stereotypies scores (Figure 2J).

_p_-Cresol induces microbiota dysbiosis

Previous studies suggested that *p*-Cresol could have bacteriostatic properties (26). We reasoned that irreversible changes in microbiota composition could explain the persistence of *p*-Cresol behavioral effects after a 4-week washout. We therefore analyzed the bacterial composition and community structure of the fecal microbiota from *p*-Cresol-treated and control mice using bacterial 16S ribosomal RNA sequencing. *Bacteroidia* and *Clostridia* were the dominant classes in both groups (Supplementary Figure 4A). Bacterial α–diversity indices did not reveal differences in richness or evenness between groups (Supplementary Figure 4B-E). However, the β–diversity index based on Jaccard's distances revealed changes in microbial taxonomic abundance profiles upon *p*-Cresol treatment (Supplementary Figure 4F). We then sought to identify the bacterial taxa and OTU that discriminated *p*-Cresol-treated and control mice. Among the 91 OTU identified, 70 were discriminant ($|Log_{10}LDA\ score|>2$; $p<0.05$) and the largest effect sizes ($|Log_{10}LDA\ score|>3$) were observed for OTU related to the *Bacteroidales* and *Clostridiales* orders (Supplementary Table 1). We identified 29 OTU associated with *p*-Cresol exposure, mostly from the *Lachnospiraceae* and *Muribaculaceae* families. We also identified 41 OTUs associated with the control group, mostly from the *Lachnospiraceae*, *Ruminococcaceae* and *Muribaculaceae* families.

We then analyzed the association between the relative abundance of the 70 discriminant bacterial OTU (Supplementary Table 1) and social behavior and stereotypies scores impacted by *p*-Cresol (Figure 1E-L). Correlation analyzes revealed moderate to strong associations ($0.38 < |\rho| < 0.74$) between bacterial abundances and behavior, in particular social behavior (Table 1, Supplementary Table 2). Among the OTU that were associated with *p*-Cresol treatment, the abundance of 4 OTU from the *Bacteroidales* and the *Clostridiales* orders correlated with social interaction deficits. Among the OTU that were associated with the control group, the abundance of 20 OTU from the *Clostridiales* order (*Ruminococaceae* and *Lachnospiraceae* families) correlated with higher sociability. The abundance of two OTU correlated with reduced numbers of head shakes. All these OTU were differentially impacted by *p*-Cresol treatment, either downregulated if they correlated positively with behavioral

outcome or upregulated if they were correlated with the severity of behavioral impairments (Figure 3A-C). This supports a tight link between microbiota dysbiosis induced by *p*-Cresol and behavioral impairments relative to core ASD symptoms.

The microbiota from *p*-Cresol-treated mice induces social behavior deficits when transplanted to normal recipients

Having shown that *p*-Cresol remodeled the intestinal microbiota, we investigated whether these changes were responsible for *p*-Cresol-induced behavioral alterations. To this aim, we transplanted the fecal microbiota from *p*-Cresol-treated mice to normal recipients (Figure 4A). Compared to control mice transplanted with the microbiota of untreated animals, mice transplanted with the microbiota of *p*-Cresol-treated mice spent less time in social contact, exhibited decreased time, number and mean duration of nose and paw contacts (Figure 4B-F, Supplementary Figure 5A, B). In contrast, the number of followings was not impacted (Figure 4G). Social behavior deficits were accompanied by stereotypies with increased number of head shakes (Figure 4H), while circling episodes, grooming and digging time were not impacted (Figure 4I, Supplementary Figure 5C, D). Mice transplanted with the microbiota from *p*-Cresol-treated and control mice were clearly separated along the PC1 axis using PCA analysis of social interaction and stereotypies scores (Figure 4J). In conclusion, the microbiota from *p*-Cresol-treated mice transfers social behavior deficits and to some extent stereotypic behavior when transplanted to recipient mice.

Transfer of a normal microbiota to *p*-Cresol-treated recipient mice restores social behavior deficits and VTA dopamine neurons excitability

Having shown that the microbiota from *p*-Cresol-treated mice induced social behavior deficits when transplanted into normal recipients, we investigated whether *p*-Cresol-induced behavioral alterations could be restored by a normal microbiota. We therefore transplanted a normal microbiota to either control mice or mice that had been treated for 4 weeks with *p*-Cresol (Figure 5A). Before FMT, *p*-Cresol-treated mice displayed social behavior deficits and

stereotypies (Figure 5B-K) consistent with our previous results (Figures 1, 2). Recolonization of *p*-Cresol-treated mice with a normal microbiota restored all parameters to control levels in the dyadic social interaction test (Figure 5B, C, E, G, Supplementary Figure 6I, J). The quality of social contacts was also restored as the mean duration of both nose and paw contacts were rescued (Figure 5D, F). In the three-chamber test, the sociability index was also normalized (Figure 5H, Supplementary Figure 6F), as well as the preference for the interactor mouse (Supplementary Figure 6G, H). The quality of social interactions was also rescued as assessed by the mean duration of contacts which was restored to normal (Figure 5I). In contrast, reversal of stereotyped behaviors was incomplete, since head shakes were only modestly reduced and circling episodes were still present after FMT in recipient mice that had been treated with *p*-Cresol (Figure 5J, K). Normalization of *p*-Cresol-induced behaviors by the microbiota of untreated mice was confirmed by PCA analysis which showed that control mice prior FMT and control and *p*-Cresol-treated mice transplanted with a normal microbiota clustered on the right side of the PC1 axis, while *p*-Cresol-treated mice prior FMT stood alone on the left side (Figure 5L). Importantly, besides normalizing *p*-Cresol-induced social behavior deficits, transplantation of a normal microbiota also restored the excitability of dopamine neurons in the VTA (Figure 5M, N).

DISCUSSION

p-Cresol induces ASD core behavioral deficits

ASD result from interactions between genes and environment. While 10-25% of ASD cases are explained by mutations in specific genetic *loci*, twin studies have revealed that genetic and environmental factors share equal influence on ASD risk (46). The identification of environmental factors, including microbiota-linked factors, contributing to ASD is therefore critical to better understand the etiology of these multi-factorial pathologies.

Here we show that *p*-Cresol-treated mice exhibit social behavior deficits and stereotypies, demonstrating a possible causal relationship between elevated *p*-Cresol levels and ASD core symptoms. Like *p*-Cresol, other microbial metabolites are possibly linked to ASD. While several of these metabolites were demonstrated to modify behavior when administered to mice, none of them selectively induced social behavior deficits and stereotypies. For example, the microbial SCFA propionate did not only induce social interaction deficits and stereotypies, but also anxiety and hyperlocomotion (14). Indoles increased social contact and anxiety but reduced locomotor activity in rats (24). Lastly, and although 4-EPS shares structural similarity with *p*-Cresol sulfated conjugate, this compound increased anxiety and startle reflex in mice but had no impact on social behavior or stereotypies (18). Therefore, in contrast to mice treated with other microbial metabolites, *p*-Cresol has selective effects on the core dimensions of behavior impacted in ASD: social behavior and stereotyped/perseverative behaviors, while other behavioral dimensions (stress, anxiety, hyperactivity, cognition) related to frequent ASD comorbidities, are not impacted.

Mice displaying social avoidance exhibit an increase in *p*-Cresol levels measured in gut tissue (47). However, whether there was a causal relationship between social behavior and *p*-Cresol levels was not investigated. Also, a recent study showed that a single acute intravenous injection of *p*-Cresol exacerbated anxiety and reduced social preference in the BTBR idiopathic model of ASD (48). Nonetheless, the intravenous administration of *p*-Cresol did not recapitulate chronic intestinal exposure to *p*-Cresol and the broad observed effects may have

resulted from acute activity of the compound injected 15 min prior to the behavioral tests. Finally, although several studies have described *p*-Cresol as overabundant in ASD patients (11, 12, 15, 16), how *p*-Cresol actually connects with ASD clinical symptoms remained largely overlooked. The specific induction of ASD core symptoms upon *p*-Cresol intestinal exposure supports the face validity of our environmental ASD model.

p-Cresol-induced dysbiosis modulates the social behavior of the host

How microbial metabolites linked to ASD such as propionate, 4-EPS or indoles impact the brain remains unknown. Some of these metabolites are ligands to the host's receptors: 3-indoxylsulfate binds to the aryl-hydrocarbon receptor (49), indole-3-propionate is a ligand for the xenobiotic sensor pregnane X receptor (50), while propionate binds to GPR41 and GPR43 (51). Yet, their signaling role has been mostly investigated in the context of metabolic, GI or autoimmune disorders (49-51), and not in the context of neuropsychiatric disorders. It appears unlikely that *p*-Cresol exerts *per se* a direct systemic or even central effect on a receptor in our experimental paradigm of GI exposure to *p*-Cresol. In support of this, its circulating levels are not increased in the serum of *p*-Cresol-treated mice, making it unlikely to reach bioactive levels in the brain. Also, *p*-Cresol effects on behavior persist even in its absence after a 4-week washout period. Rather, we propose that GI exposure to *p*-Cresol triggers ASD behaviors by inducing a selective microbiota dysbiosis, which is sufficient to fully induce social behavior deficits and partially stereotypies when transferred to control recipient mice. We indeed show that a *p*-Cresol-rich environment does not induce broad changes in the microbial community structure, but rather appears bacteriostatic for some taxa and provides a selective growth advantage for other taxa, in line with a previous *in vitro* study (26). The correlation between the abundances of 26 dysregulated OTU with social behavior argues for a clear link between certain bacterial taxa and social abilities. Notably, members of the *Blautia* genus are reduced both in ASD patients (8) and in *p*-Cresol-treated animals and their abundances correlate positively with social abilities in our model. Conversely, OTU from the *Muriculobaculaceae* and *Peptococcaceae* families are overabundant in *p*-Cresol-treated animals and their abundance

correlates negatively with social behavior abilities. We anticipate that the selective overgrowth of yet-to-be-identified bacterial species in a *p*-Cresol rich environment could contribute to social behavior deficits in our model. Finally, our model displays a 4-fold increase in *p*-Cresol urinary excretion, within the range of the 2.5-fold increase determined in young ASD patients as compared to neurotypical controls (11, 12). Elevated urinary *p*-Cresol (11, 12) and dysbiosis (6-8) are observed both in ASD patients and in our model, underpinning its construct validity. Furthermore, its predictive validity is supported by our findings that transplantation of a normal microbiota normalizes behavior in *p*-Cresol-treated mice, as already shown in a pilot study in ASD patients (17).

p-Cresol-induced dysbiosis dysregulates central dopamine pathways and the social reward system

Social interactions are pleasurable events for humans and animals, as shown by the activation of the reward circuit by social stimuli – activation that is blunted in ASD patients (52). The VTA is a key subcortical node of the mesolimbic reward pathway (53) and ASD patients display defects in VTA connectivity (41). Moreover, blocking VTA dopamine neurons that are part of a 'socially engaged reward circuit' is sufficient to diminish social interactions in rodents (40, 54). These data suggest that impaired dopamine neurons activity in the VTA could disrupt the social reward pathway and contribute to social behavior deficits in *p*-Cresol-treated animals, as previously described in genetic models targeting the ASD genes *Shank3b*, *Nlgn3* and *Ube3a* (20, 42-44) and the MIA and DIO environmental models of ASD (19, 45). The disruption of the social reward pathway both in ASD patients and in our model argue for its construct validity. Dopaminergic circuits are sensitive to changes in gut microbiota composition (55) and gut-innervating vagal sensory neurons control striatal dopamine release (56). Further, normalization of microbiota composition by FMT abolished social interaction deficits and restored VTA dopamine neurons excitability in *p*-Cresol-treated mice. Taken together, these results support a model in which *p*-Cresol-induced changes in microbiota composition results

in impaired VTA dopamine neurons activity which eventually disrupts the social reward pathway and causes social interaction deficits.

Conclusions and perspectives

We found that *p*-Cresol-treated mice exhibited social interaction deficits and stereotypies reminiscent of ASD core symptoms in humans. These behaviors were dependent on changes in microbiota composition and were associated with a reduced activity of dopamine neurons in the VTA. This environmental model of ASD exhibited face, construct and predictive validity. The current report paves the way for further investigation of the role of *p*-Cresol in ASD and more broadly of the role played by microbial metabolites and the microbiota on the control of host behavior. Because *p*-Cresol levels are elevated in ASD patients, our study suggests that increased *p*-Cresol levels could contribute to ASD core symptoms in humans. Further, the ability of a normal microbiota to normalize social interaction deficits when transplanted into *p*-Cresol-treated mice supports the rationale for the ongoing clinical trials aiming at ascertaining the beneficial impact of FMT in ASD, as highlighted in a pilot study (17). Finally, prebiotic treatment with oligofructose-enriched inulin or probiotic treatment with *Lactobacillus casei* decreased *p*-Cresol urinary excretion in healthy volunteers (57). Future clinical studies could evaluate the benefits of such interventions to reduce the microbial production of *p*-Cresol and alleviate the core symptoms of ASD.

ACKNOWLEDGEMENTS

We thank Lucien Relmy for expert technical assistance with animal care and Thomas Lorivel for help with behavioral tests set-up. We are grateful to Dominique Gauguier (Centre de Recherche des Cordeliers, Paris) for helpful discussions regarding the procedure for fecal microbiota transplantations. LD and MED are grateful to the European Community 7th Framework Program under Coordinated Action NEURON-ERANET (grant agreement 291840) and MRC (MR/M501797/1). JAJB and JLM thank Region Centre-Val de Loire (ARD2020 Biomedicaments GPCRAb) and the Labex MabImprove for financial support.

DISCLOSURES

None to declare.

REFERENCES

1. Lai MC, Lombardo MV, Baron-Cohen S (2014): Autism. *Lancet*. 383:896-910.

2. Vuong HE, Hsiao EY (2017): Emerging Roles for the Gut Microbiome in Autism Spectrum Disorder. *Biol Psychiatry*. 81:411-423.

3. Mazurek MO, Vasa RA, Kalb LG, Kanne SM, Rosenberg D, Keefer A, et al. (2013): Anxiety, sensory over-responsivity, and gastrointestinal problems in children with autism spectrum disorders. *J Abnorm Child Psychol*. 41:165-176.

4. Nikolov RN, Bearss KE, Lettinga J, Erickson C, Rodowski M, Aman MG, et al. (2009): Gastrointestinal symptoms in a sample of children with pervasive developmental disorders. *J Autism Dev Disord*. 39:405-413.

5. Gorrindo P, Williams KC, Lee EB, Walker LS, McGrew SG, Levitt P (2012): Gastrointestinal dysfunction in autism: parental report, clinical evaluation, and associated factors. *Autism Res*. 5:101-108.

6. Xu M, Xu X, Li J, Li F (2019): Association Between Gut Microbiota and Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. *Front Psychiatry*. 10:473.

7. Iglesias-Vazquez L, Van Ginkel Riba G, Arija V, Canals J (2020): Composition of Gut Microbiota in Children with Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. *Nutrients*. 12.

8. Liu F, Li J, Wu F, Zheng H, Peng Q, Zhou H (2019): Altered composition and function of intestinal microbiota in autism spectrum disorders: a systematic review. *Transl Psychiatry*. 9:43.

9. Emond P, Mavel S, Aidoud N, Nadal-Desbarats L, Montigny F, Bonnet-Brilhault F, et al. (2013): GC-MS-based urine metabolic profiling of autism spectrum disorders. *Analytical and bioanalytical chemistry*. 405:5291-5300.

10. Yap IK, Angley M, Veselkov KA, Holmes E, Lindon JC, Nicholson JK (2010): Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls. *J Proteome Res*. 9:2996-3004.

11. Altieri L, Neri C, Sacco R, Curatolo P, Benvenuto A, Muratori F, et al. (2011): Urinary p-Cresol is elevated in small children with severe autism spectrum disorder. *Biomarkers*. 16:252-260.

12. Gabriele S, Sacco R, Cerullo S, Neri C, Urbani A, Tripi G, et al. (2014): Urinary p-Cresol is elevated in young French children with autism spectrum disorder: a replication study. *Biomarkers*. 19:463-470.

13. Dieme B, Mavel S, Blasco H, Tripi G, Bonnet-Brilhault F, Malvy J, et al. (2015): Metabolomics Study of Urine in Autism Spectrum Disorders Using a Multiplatform Analytical Methodology. *J Proteome Res*. 14:5273-5282.

14. Macfabe DF (2012): Short-chain fatty acid fermentation products of the gut microbiome: implications in autism spectrum disorders. *Microbial ecology in health and disease*. 23.

15. De Angelis M, Piccolo M, Vannini L, Siragusa S, De Giacomo A, Serrazzanetti DI, et al. (2013): Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. *PLoS One*. 8:e76993.

16. Kang DW, Ilhan ZE, Isern NG, Hoyt DW, Howsmon DP, Shaffer M, et al. (2017): Differences in fecal microbial metabolites and microbiota of children with autism spectrum disorders. *Anaerobe*. 49:121-131.

17. Kang DW, Adams JB, Coleman DM, Pollard EL, Maldonado J, McDonough-Means S, et al. (2019): Long-term benefit of Microbiota Transfer Therapy on autism symptoms and gut microbiota. *Sci Rep*. 9:5821.

18. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, et al. (2013): Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell*. 155:1451-1463.

19. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M (2016): Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell*. 165:1762-1775.

20. Sgritta M, Dooling SW, Buffington SA, Momin EN, Francis MB, Britton RA, et al. (2019): Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. *Neuron*. 101:246-259 e246.

21. Golubeva AV, Joyce SA, Moloney G, Burokas A, Sherwin E, Arboleya S, et al. (2017): Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism. *EBioMedicine*. 24:166-178.

22. de Theije CG, Wopereis H, Ramadan M, van Eijndthoven T, Lambert J, Knol J, et al. (2014): Altered gut microbiota and activity in a murine model of autism spectrum disorders. *Brain Behav Immun*. 37:197-206.

23. Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM, et al. (2019): Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. *Cell*. 177:1600-1618 e1617.

24. Jaglin M, Rhimi M, Philippe C, Pons N, Bruneau A, Goustard B, et al. (2018): Indole, a Signaling Molecule Produced by the Gut Microbiota, Negatively Impacts Emotional Behaviors in Rats. *Front Neurosci*. 12:216.

25. Saito Y, Sato T, Nomoto K, Tsuji H (2018): Identification of phenol- and p-Cresol-producing intestinal bacteria by using media supplemented with tyrosine and its metabolites. *FEMS Microbiol Ecol*. 94.

26. Passmore IJ, Letertre MPM, Preston MD, Bianconi I, Harrison MA, Nasher F, et al. (2018): Para-Cresol production by Clostridium difficile affects microbial diversity and membrane integrity of Gram-negative bacteria. *PLoS Pathog*. 14:e1007191.

27. Borre YE, O'Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF (2014): Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol Med*. 20:509-518.

28. Loomes R, Hull L, Mandy WPL (2017): What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis. *J Am Acad Child Adolesc Psychiatry*. 56:466-474.

29. Lewis RJ, Sax NI (2004): Sax's Dangerous Properties of Industrial Materials. 11th Edition ed. Hoboken, NJ, USA: Hoboken, N.J.; J. Wiley & Sons, pp 1004.

30. Fiehn O (2016): Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling. *Curr Protoc Mol Biol*. 114:30 34 31-30 34 32.

31. Becker JA, Clesse D, Spiegelhalter C, Schwab Y, Le Merrer J, Kieffer BL (2014): Autistic-like syndrome in mu opioid receptor null mice is relieved by facilitated mGluR4 activity. *Neuropsychopharmacology*. 39:2049-2060.

32. Pujol CN, Pellissier LP, Clement C, Becker JAJ, Le Merrer J (2018): Back-translating behavioral intervention for autism spectrum disorders to mice with blunted reward restores social abilities. *Transl Psychiatry*. 8:197.

33. Leboucher A, Bermudez-Martin P, Mouska X, Amri EZ, Pisani DF, Davidovic L (2019): Fmr1-Deficiency Impacts Body Composition, Skeleton, and Bone Microstructure in a Mouse Model of Fragile X Syndrome. *Front Endocrinol (Lausanne)*. 10:678.

34. Silverman JL, Yang M, Lord C, Crawley JN (2010): Behavioural phenotyping assays for mouse models of autism. *Nature reviews Neuroscience*. 11:490-502.

35. Becker JAJ, Kieffer BL, Le Merrer J (2017): Differential behavioral and molecular alterations upon protracted abstinence from cocaine versus morphine, nicotine, THC and alcohol. *Addict Biol*. 22:1205-1217.

36. Fernandez SP, Broussot L, Marti F, Contesse T, Mouska X, Soiza-Reilly M, et al. (2018): Mesopontine cholinergic inputs to midbrain dopamine neurons drive stress-induced depressive-like behaviors. *Nat Commun*. 9:4449.

37. Escudie F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, et al. (2018): FROGS: Find, Rapidly, OTUs with Galaxy Solution. *Bioinformatics*. 34:1287-1294.

38. McMurdie PJ, Holmes S (2012): Phyloseq: a bioconductor package for handling and analysis of high-throughput phylogenetic sequence data. *Pac Symp Biocomput*.235-246.

39. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. (2011): Metagenomic biomarker discovery and explanation. *Genome Biol*. 12:R60.

40. Hung LW, Neuner S, Polepalli JS, Beier KT, Wright M, Walsh JJ, et al. (2017): Gating of social reward by oxytocin in the ventral tegmental area. *Science*. 357:1406-1411.

41. Supekar K, Kochalka J, Schaer M, Wakeman H, Qin S, Padmanabhan A, et al. (2018): Deficits in mesolimbic reward pathway underlie social interaction impairments in children with autism. *Brain*. 141:2795-2805.

42. Krishnan V, Stoppel DC, Nong Y, Johnson MA, Nadler MJ, Ozkaynak E, et al. (2017): Autism gene Ube3a and seizures impair sociability by repressing VTA Cbln1. *Nature*. 543:507-512.

43. Bariselli S, Hornberg H, Prevost-Solie C, Musardo S, Hatstatt-Burkle L, Scheiffele P, et al. (2018): Role of VTA dopamine neurons and neuroligin 3 in sociability traits related to nonfamiliar conspecific interaction. *Nat Commun*. 9:3173.

44. Bariselli S, Tzanoulinou S, Glangetas C, Prevost-Solie C, Pucci L, Viguie J, et al. (2016): SHANK3 controls maturation of social reward circuits in the VTA. *Nat Neurosci*. 19:926-934.

45. Lecca S, Luchicchi A, Scherma M, Fadda P, Muntoni AL, Pistis M (2019): Delta(9)-Tetrahydrocannabinol During Adolescence Attenuates Disruption of Dopamine Function Induced in Rats by Maternal Immune Activation. *Front Behav Neurosci*. 13:202.

46. Bourgeron T (2016): Current knowledge on the genetics of autism and propositions for future research. *C R Biol*. 339:300-307.

47. Gacias M, Gaspari S, Santos PM, Tamburini S, Andrade M, Zhang F, et al. (2016): Microbiota-driven transcriptional changes in prefrontal cortex override genetic differences in social behavior. *Elife*. 5.

48. Pascucci T, Colamartino M, Fiori E, Sacco R, Coviello A, Ventura R, et al. (2020): P-Cresol Alters Brain Dopamine Metabolism and Exacerbates Autism-Like Behaviors in the BTBR Mouse. *Brain Sci*. 10.

49. Kim CH (2018): Immune regulation by microbiome metabolites. *Immunology*. 154:220-229.

50. Venkatesh M, Mukherjee S, Wang H, Li H, Sun K, Benechet AP, et al. (2014): Symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor PXR and Toll-like receptor 4. *Immunity*. 41:296-310.

51. Bolognini D, Tobin AB, Milligan G, Moss CE (2016): The Pharmacology and Function of Receptors for Short-Chain Fatty Acids. *Mol Pharmacol*. 89:388-398.

52. Pellissier LP, Gandia J, Laboute T, Becker JAJ, Le Merrer J (2018): mu opioid receptor, social behaviour and autism spectrum disorder: reward matters. *Br J Pharmacol*. 175:2750-2769.

53. Ranaldi R (2014): Dopamine and reward seeking: the role of ventral tegmental area. *Rev Neurosci*. 25:621-630.

54. Gunaydin LA, Grosenick L, Finkelstein JC, Kauvar IV, Fenno LE, Adhikari A, et al. (2014): Natural neural projection dynamics underlying social behavior. *Cell*. 157:1535-1551.

55. Gonzalez-Arancibia C, Urrutia-Pinones J, Illanes-Gonzalez J, Martinez-Pinto J, Sotomayor-Zarate R, Julio-Pieper M, et al. (2019): Do your gut microbes affect your brain dopamine? *Psychopharmacology (Berl)*. 236:1611-1622.

56. Han W, Tellez LA, Perkins MH, Perez IO, Qu T, Ferreira J, et al. (2018): A Neural Circuit for Gut-Induced Reward. *Cell*. 175:887-888.

57. De Preter V, Vanhoutte T, Huys G, Swings J, De Vuyst L, Rutgeerts P, et al. (2007): Effects of Lactobacillus casei Shirota, Bifidobacterium breve, and oligofructose-enriched inulin on colonic nitrogen-protein metabolism in healthy humans. *Am J Physiol Gastrointest Liver Physiol*. 292:G358-368.

FIGURES & TABLES

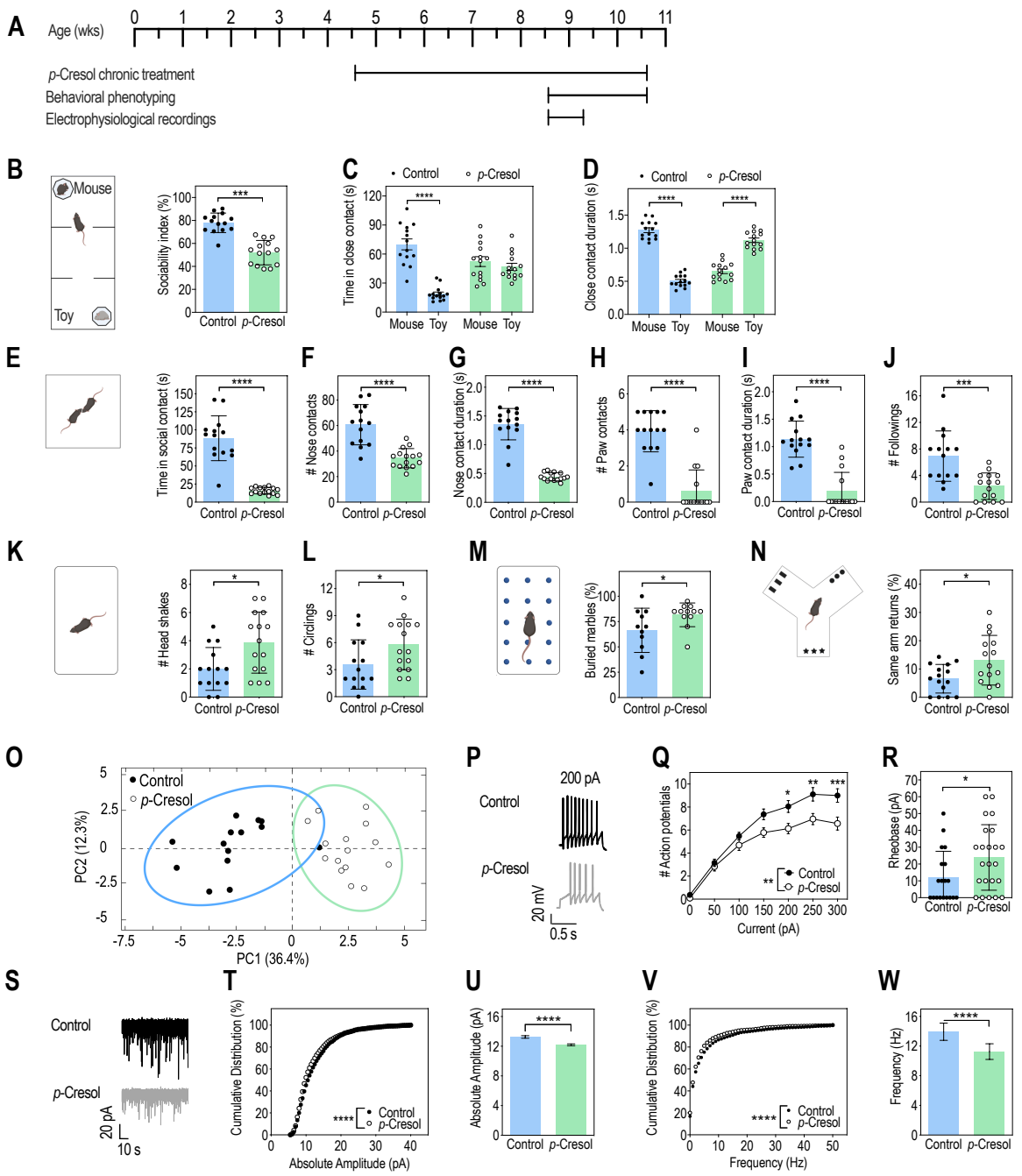


Figure 1. *p*-Cresol treatment induces autistic-like behaviors and alters VTA dopamine neurons excitability

(A) Timeline of the experiments

(B-D) Three-chamber test (n=14/group):

(B) Sociability index (ratio of the time spent in close contact with the mouse interactor normalized to the sum of the time in close contact with both mouse interactor and toy mouse); Mann-Whitney U-test: ***p<0.001.

(C) Time spent in close contact with the mouse interactor or the toy mouse; 2-way ANOVA: p(Treatment)=0.2601, p(Preference)=p<0.0001, p(Treatment x Mouse-Toy)=p<0.0001; Šidák's post hoc tests for treatment effect: ****p<0.0001.

(D) Mean duration of each close contact with the mouse interactor or the toy mouse; 2-way ANOVA: p(Treatment)=0.9416, p(Preference)=p<0.0001, p(Treatment x Mouse-Toy)=p<0.0001, Šidák's post hoc tests for treatment effect: ****p<0.0001.

(E-J) Dyadic social interaction test (n=14 Control, n=15 *p*-Cresol): (E) total time spent, (F) number of events and (G) mean duration of nose contacts, (H) number of events and (I) mean duration of paw contacts, (J) number of following events; Mann-Whitney U-test: ****p<0.0001, ***p<0001.

(K-L) Motor stereotypies (n=14 Control, n=15 *p*-Cresol): (K) number of head shakes and (L) circling events; Mann-Whitney U-test: *p<0.05.

(M) Marble burying test (n=11 Control, n=12 *p*-Cresol): percentage of buried marbles; Mann-Whitney U-test: *p<0.05.

(N) Y-maze spontaneous alternations (n=15/group): % of same arm returns; n=15/group; Mann-Whitney U-test: *p<0.05.

(O) PCA plot of behavioral scores recorded in the dyadic social interaction and direct monitoring of motor stereotypies (Figure 1 E-L and Supplementary Figure 2H-L); ellipses of the 95% confidence intervals are indicated for each group (n=14 Control, n=15 *p*-Cresol); PC, principal component.

(P-W) Electrophysiological recordings of dopamine neurons activity in the VTA:

(P) Representative traces of dopamine neuron activity in patch-clamp experiments after a 200pA current injection

(Q) Number of action potentials evoked by current injection in current-clamp experiments; n=5 animals/group, 19 cells recorded for Control, 23 cells recorded for *p*-Cresol; 2-way ANOVA: p(Treatment)=0.0066, p(Current)<0.0001, p(Treatment x Current)=0.0006; Šidák's post hoc tests for treatment effect, *p<0.05,**p<0.01, ***p<0.001.

(R) Rheobase, minimal electric current required to evoke an action potential; n=5 animals/group, 19 cells recorded for Control, n=23 cells recorded for *p*-Cresol; Mann-Whitney U-test: *p<0.05,

(S) Representative traces of spontaneous miniature excitatory postsynaptic currents (sEPSC)

(T) Cumulative frequencies of sEPSC absolute amplitude; n=5 animals/group, >2500 events/group; Kolmogorov-Smirnov test: ****p<0.0001.

(U) Mean absolute amplitude of sEPSC; Mann-Whitney U-test: ****p<0.0001.

(V) Cumulative frequencies of sEPSC frequency; n=5 animals/group, >2500 events/group; Kolmogorov-Smirnov test: ****p<0.0001.

(W) Mean frequency of sEPSC; Mann-Whitney U-test: ****p<0.0001.

(B-N, R) Data are shown as means ± SD.

(Q, U, W) Data are shown as means ± SEM.

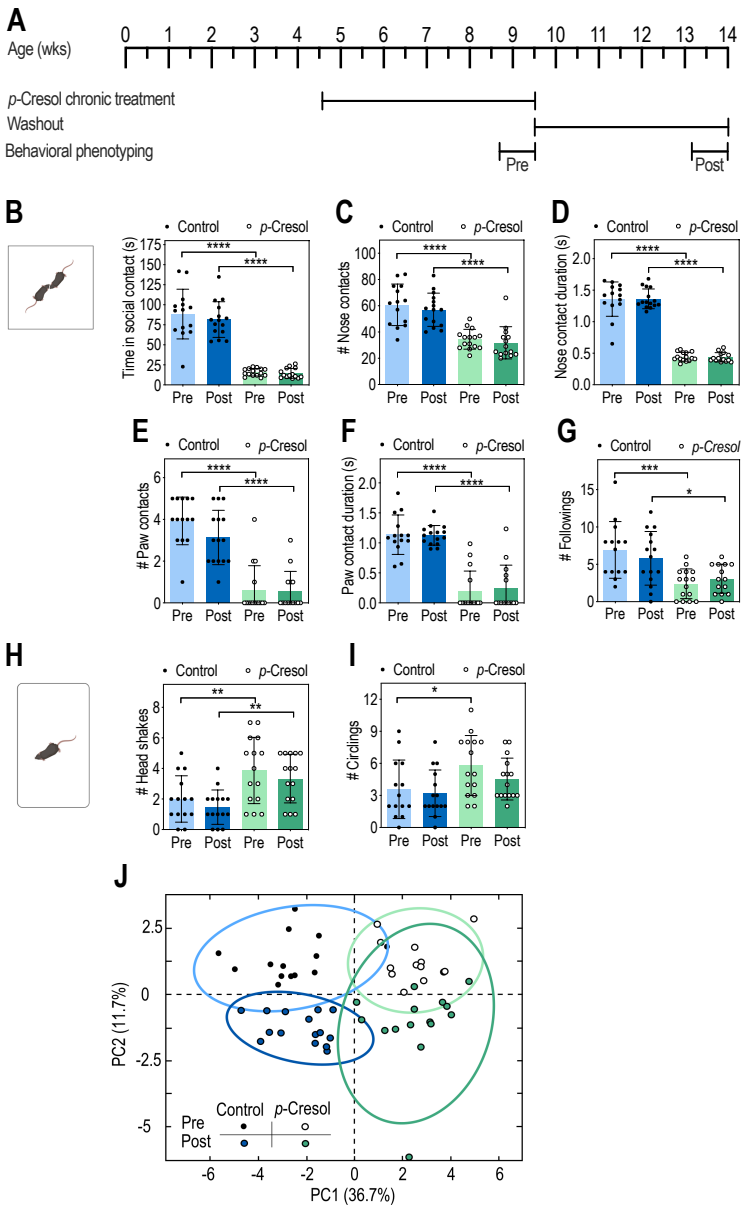


Figure 2. Autistic-like behaviors induced by *p*-Cresol persist after a 4-week washout period

(A) Timeline of the experiment

(B-G) Dyadic social interaction test (n=14 Control, n=15 *p*-Cresol):

(B) Total time spent in social contact; 2-way ANOVA: p(Treatment)<0.0001, p(Washout)=0.4194, p(Treatment x Washout)=0.5883; Šidák's post hoc tests for treatment effect: ****p<0.0001.

(C) Number of nose contacts; 2-way ANOVA: p(Treatment)<0.0001, p(Washout)=0.3237, p(Treatment x Washout)=0.8660; Šidák's post hoc tests for treatment effect: ****p<0.0001.

(D) Mean duration of each nose contact; 2-way ANOVA: p(Treatment)<0.0001, p(Washout)=0.9807, p(Treatment x Washout)=0.8922; Šidák's post hoc tests for treatment effect: ****p<0.0001.

(E) Number of paw contacts; 2-way ANOVA: p(Treatment)<0.0001, p(Washout)=0.1793, p(Treatment x Washout)=0.2109; Šidák's post hoc tests for treatment effect: ****p<0.0001.

(F) Mean duration of each paw contact; 2-way ANOVA: p(Treatment)<0.0001, p(Washout)=0.7906, p(Treatment x Washout)=0.6926; Šidák's post hoc tests for treatment effect: ****p<0.0001.

(G) Number of followings, 2-way ANOVA: p(Treatment)<0.0001, p(Washout)=0.7697, p(Treatment x Washout)=0.2517; Šidák's post hoc tests for treatment effect: ***p<0.001, *p<0.05.

(H,I) Motor stereotypies (n=14 Control; n=15 *p*-Cresol):

(H) Number of head shakes; 2-way ANOVA: p(Treatment)<0.0001, p(Washout)=0.2185, p(Treatment x Washout)>0.9999; Šidák's post hoc tests for treatment effect: **p<0.01.

(I) Number of circling events; 2-way ANOVA: p(Treatment)=0.0051, p(Washout)=0.2605, p(Treatment x Washout)=0.5796; Šidák's post hoc tests for treatment effect: *p<0.05 for Control, p>0.05 for *p*-Cresol.

(J) PCA plots of behavioral scores recorded in the dyadic social interaction and direct monitoring of motor stereotypies (Figure 2B-I, Supplementary Figure 3A-E); ellipses of the 95% confidence intervals are indicated for each group (n=14 Control; n=15 *p*-Cresol).

(B-I) Data are presented as dot-plots featuring means ± SD.

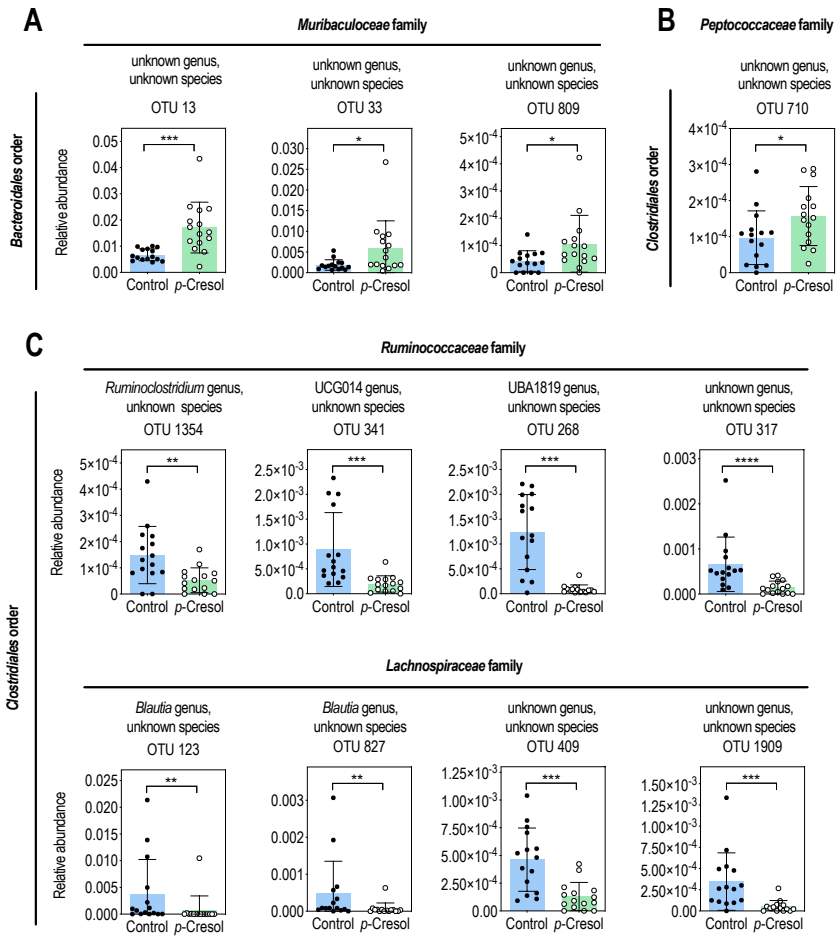


Figure 3. The abundances of microbial OTU correlating with social behavior are impacted by *p*-Cresol

(A, B) OTU associated with the *p*-Cresol class in LEfSE analysis and negatively correlated with social behavior abilities (n=15/group): abundances for members of the *Muribaculoceae* (A) and *Peptococcaceae* family (B) from the *Bacteroidales* and *Clostridiales* orders respectively; Mann-Whitney U-test: *p<0.05, **p<0.01, ***p<0.001.

(C) OTU associated with the Control class in LEfSE analysis and positively correlated with social behavior abilities (n=15/group): abundances for members of the *Ruminococcaceae* and *Lachnospiraceae* families from the *Clostridiales* order; Mann-Whitney U-test: **p<0.01, ***p<0.001, ****p<0.0001.

Data are presented as dot-plots featuring means ± SD.

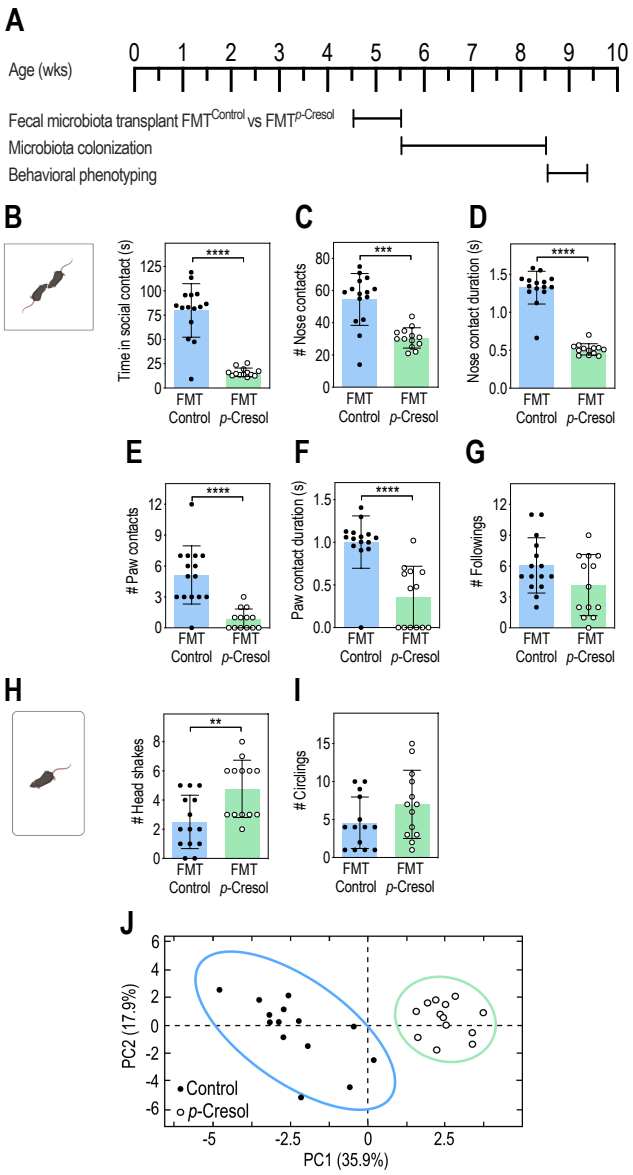


Figure 4. Microbiota remodeling following *p*-Cresol treatment induces social behavior deficits

(A) Timeline of the fecal microbiota transplant (FMT) experiment of fecal slurry from control or *p*-Cresol-treated donor animals to recipient wildtype mice.

(B-G) Dyadic social interaction test (n=15 Control, n=13 *p*-Cresol):

(B) Total time spent in social contact; Mann-Whitney U-test: ****p<0.0001.

(C) Number of nose contacts; Mann-Whitney U-test: ***p<0.001.

(D) Mean duration of nose contact; Mann-Whitney U-test: ****p<0.0001.

(E) Number of paw contacts; Mann-Whitney U-test: ****p<0.0001.

(F) Mean duration of each paw contact; Mann-Whitney U-test: ****p<0.0001.

(G) Number of followings; Mann-Whitney U-test: p=0.1563.

(H, I) Motor stereotypies: (n=14 Control, n=13 *p*-Cresol):

(H) Number of head shakes; Mann-Whitney U-test: **$p<0.001$.

(I) Number of circling events; Mann-Whitney U-test: $p=0.1762$.

(J) PCA plots of behavioral scores recorded in the dyadic social interaction and direct monitoring of motor stereotypies (Figure 4B-I, Supplementary Figure 5A-D); ellipses of the 95% confidence intervals are indicated for each group (n=15 Control, n=13 *p*-Cresol).

(B-I) Data are presented as dot-plots featuring means ± SD.

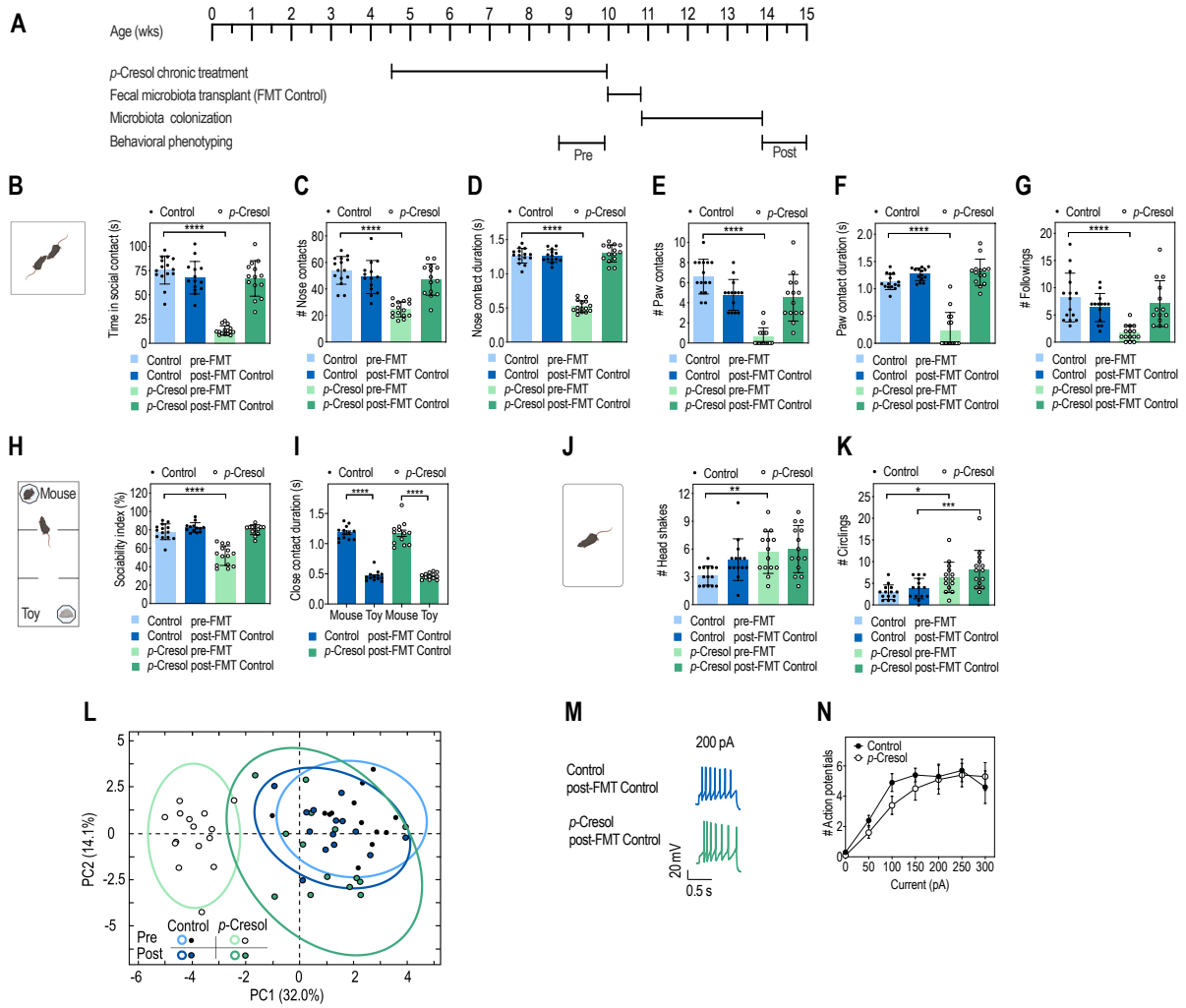


Figure 5. Transfer of microbiota from control mice to *p*-Cresol-treated mice restores social behavior deficits and VTA dopamine neurons excitability

(A) Timeline of the fecal microbiota transplant (FMT) experiment of fecal slurry from control donor mice to control or *p*-Cresol-treated recipient mice.

(B-G) Dyadic social interaction test (n=15/group pre-FMT, n=14/group post-FMT)

(B) Total time spent in social contact; 2-way ANOVA: p(Treatment)<0.0001, p(FMT Control)<0.0001, p(Treatment x FMT Control)<0.0001; Šidák's post-hoc tests for treatment effect: ****p<0.0001 for pre-FMT Control groups, p>0.05 for post-FMT Control groups

(C) Number of nose contacts; 2-way ANOVA: p(Treatment)<0.0001, p(FMT Control)= 0.0022, p(Treatment x FMT Control)<0.0001; Šidák's post-hoc tests for treatment effect: ****p<0.0001 for pre-FMT Control groups, p>0.05 for post-FMT Control groups

(D) Mean duration of each nose contact; 2-way ANOVA: p(Treatment)<0.0001, p(FMT Control)<0.0001, p(Treatment x FMT Control)<0.0001; Šidák's post-hoc tests for treatment effect: ****p<0.0001 for pre-FMT Control groups, p>0.05 for post-FMT Control groups

(E) Number of paw contacts; 2-way ANOVA: p(Treatment)<0.0001, p(FMT Control)=0.0221, p(Treatment x FMT Control)<0.0001; Šidák's post-hoc tests for treatment effect: ****p<0.0001 for pre-FMT Control groups, p>0.05 for post-FMT Control groups

(F) Mean duration of each paw contact; 2-way ANOVA: p(Treatment)<0.0001, p(FMT Control)<0.0001, p(Treatment x FMT Control)<0.0001; Šidák's post-hoc tests for treatment effect: ****p<0.0001 for pre-FMT Control groups, p>0.05 for post-FMT Control groups

(G) Number of followings; 2-way ANOVA: p(Treatment)=0.0020, p(FMT Control)=0.0516, p(Treatment x FMT Control)=0.0002; Šidák's post-hoc tests for treatment effect: ****p<0.0001 for pre-FMT Control groups, p>0.05 for post-FMT Control groups

(H, I) 3-chamber test (n=15/group pre-FMT, n=14/group post-FMT):

(H) Sociability index; 2-way ANOVA: p(Treatment)=0.0004, p(FMT Control)<0.0001, p(Treatment x FMT Control)=0.0032; Šidák's post-hoc tests for treatment effect: ****p<0.0001 for pre-FMT Control groups, p>0.05 for post-FMT Control groups

(I) Mean duration of each nose contact with the mouse interactor or toy mouse; 2-way ANOVA: p(FMT Control)=0.9067, p(Mouse-Toy)<0.0001, p(FMT Control x Mouse-Toy)=0.8713; Šidák's post-hoc tests for Mouse vs. Toy preference: ****p<0.0001.

(J, K) Motor stereotypies (n=15/group pre-FMT, n=15/group post-FMT).

(J) Number of head shakes; 2-way ANOVA: p(Treatment)=0.0021, p(FMT Control)=0.0715, p(Treatment x FMT Control)=0.2334; Šidák's post-hoc tests for treatment effect: **p<0.01 for pre-FMT Control groups, p>0.05 for post-FMT Control groups.

(K) Number of circling events; 2-way ANOVA: p(Treatment)<0.0001, p(FMT Control)=0.1133, p(Treatment x FMT Control)=0.5555; Šidák's post-hoc tests: *p<0.05 for pre-FMT Control groups, ***p<0.001 for post-FMT Control groups.

(L) PCA plots of behavioral scores recorded in the dyadic social interaction and direct monitoring of motor stereotypies (Figure 5B-G, J, K, Supplementary Figure 6I-L); ellipses of the 95% confidence intervals are indicated for each group (n=15/group pre-FMT, n=14/group post-FMT).

(M, N) Electrophysiological recordings of dopamine neurons activity in the VTA post-FMT Control:

(M) Representative traces of dopamine neurons activity in patch-clamp experiments performed post-FMT Control after a 200pA current injection

(N) Number of action potentials evoked by different current injection steps (n=3 animals/group, 10 cells recorded/group); 2-way ANOVA: p(Treatment)=0.5474, p(Current)<0.0001, p(Treatment x Current)=0.3640; Šidák's post hoc tests for treatment effect: p>0.05.

(B-K) Data are presented as dot-plots featuring means ± SD.

(N) Data are presented as means ± SD.

Table 1. The abundance of bacterial OTU discriminating *p*-Cresol-treated mice from control mice correlate with social behavior/stereotypies scores. Spearman's rank ρ correlation coefficients are annotated and color-coded: positive correlations (red), negative (blue). LEfSE score and associated p-value are also reported for each OTU. Only significant correlations are displayed (FDR-adjusted *p*-value < 0.05). *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 (n=30 animals: n=15 Control, n=15 *p*-Cresol). *See also Supplementary Table 1 for complete LefSE analysis and Supplementary Table 2 for complete correlation analysis.*

										ρ coefficients: OTU abundance-behavioral score							
	Taxonomy							***LEfSE metrics***		***Social interaction***						***Stereotypies***	
	Phylum	***Class***	***Order***	***Family***	***Genus***	***Species***	***OTU***	***Log_{10} LDA score***	***p-val.***	***Social contact time (s)***	***# Paw contacts***	***Paw contact duration (s)***	***# Nose Contacts***	***Nose contact duration (s)***	***# Followings***	***# Head shakes***	***# Circlings***
***p*-Cresol**	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Unknown*	*Unknown*	*13*	3.69	****	-0.57	-0.53	-0.55		-0.55			
					Unknown	*Unknown*	*33*	3.26	*	-0.62	-0.56	-0.52	-0.55	-0.57			
					Unknown	*Unknown*	*809*	2.80	*	-0.53		-0.508					
	Firmicutes	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*Unknown*	*Unknown*	*710*	2.42	*		-0.49	-0.38		-0.41			
Control	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Unknown*	*Unknown*	*838*	-2.60	**					0.58			
	Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiales vadinBB60*	*Unknown*	*Unknown*	*45*	-2.68	*	0.53							
				Lachnospiraceae	*Blautia*	*Unknown*	*827*	-2.41	**			0.61				-0.57	
						Unknown	*123*	-3.15	**			0.58		0.55			
					Lachnospiraceae NK4A136	*Unknown*	*497*	-2.22	*				0.53				
						Unknown	*28*	-3.84	**	0.53				0.52			
					Lachnospiraceae UCG006	*Unknown*	*635*	-2.18	**					0.54			
					Marvinbryantia	*Unknown*	*1039*	-2.48	*	0.57				0.55			
					Unknown	*Unknown*	*409*	-2.41	***	0.63	0.65	0.64	0.64	0.55	0.62		
					Unknown	*Unknown*	*1909*	-2.44	**	0.58	0.52	0.59		0.60			
					Unknown	*Unknown*	*311*	-2.50	*		0.53		0.59				
					Unknown	*Unknown*	*375*	-2.51	**	0.51				0.51	0.51		
					Unknown	*Unknown*	*364*	-2.68	***	0.57		0.60		0.65			
					Unknown	*Unknown*	*39*	-3.66	*	0.53				0.53			
				Ruminococcaceae	*Ruminiclostridium*	*Unknown*	*570*	-2.11	*	0.57		0.507		0.56			
						Unknown	*1354*	-2.42	**	0.58	0.55	0.57		0.61			
					Ruminococcaceae UCG014	*Unknown*	*332*	-2.40	**	0.56							
						Unknown	*341*	-2.58	****	0.54	0.58	0.59		0.58			
					UBA1819	*Multiaffiliation*	*268*	-2.80	****	0.69	0.54	0.54	0.60	0.68			
					Unknown	*Unknown*	*452*	-2.40	*	0.51			0.55		0.55		
					Unknown	*Unknown*	*317*	-2.49	****	0.64	0.63	0.74		0.72		-0.62	
	Proteobacteria	*Gammaproteobacteria*	*Betaproteobacteriales*	*Burkholderiaceae*	*Parasutterella*	*Unknown*	*321*	-2.43	**	0.53				0.51			

SUPPLEMENTARY INFORMATION

The microbial metabolite *p*-Cresol induces autistic-like behaviors in mice by remodeling of the gut microbiota

Microbial *p*-Cresol induces autistic-like behaviors in mice

Bermudez-Martin, P., Becker, J. A., Fernandez, S. P., Costa-Campos, R., Barbosa, S., Martinez-Gili, L., Myridakis, A., Dumas, M.-E., Bruneau, A., Cherbuy, C., Langella, P., Chabry, J., Barik, J., Le Merrer, J., Glaichenhaus, N., Davidovic, L.

Supplementary information provided:

Supplementary Methods and Materials

Supplementary figures and supplementary figures legends (6)

Supplementary tables legends (3)

Supplementary Methods and Materials

Ethical considerations. Animal housing and experimentation were conducted in facilities certified by regional authorities (Direction Départementale de Protection des Populations des Alpes-Maritimes, accreditation #EC-06-152-5). The procedures involved in this study were approved by the local ethics committee for animal experimentation (Ciepal-Azur) and the Ministère de l'Enseignement Supérieur et de la Recherche, in agreement with the European Communities Council Directive (2010/63EU) for animal experiments.

Animals treatment. Weaned C57BL/6J mice (21 to 28 day-old) were ordered to Charles Rivers (France). This developmental stage was chosen to avoid confounding effects of maternal exposure to *p*-Cresol, while still being in the time window for late neurodevelopment during which the microbiota-gut-brain axis can intervene (1). Since the sex ratio for ASD is biased towards 3 males diagnosed for 1 female suggesting a higher susceptibility of males (2, 3), only were considered in this study. Animals were randomly assigned to experimental groups and housed in medium-size (up to 5 animals) open cages filled with wooden bedding, one plastic house and nesting material for enrichment, in a temperature-(22-24°C) and hygrometry- (70-80%) controlled room, under 12 hours light–dark cycle (8:00 a.m.- 8:00 p.m.). Mice had *ad libitum* access to water and standard chow (reference 4RF25, Mucedola). Mice were habituated for 6 days to our animal facility before starting treatment with *p*-Cresol (reference W233706-SAMPLE-K, Sigma-Aldrich) dispensed in sterile drinking water at a concentration of 0.25 g/L. Bottles were renewed twice weekly. This amounts in adult mice to a dose of 50mg/Kg/24 h based on a mean body mass of 25 g and a mean drinking water consumption of 5mL/24 h. This dose per day corresponded to 7 times less the reported LD50 dose of 350mg/Kg for acute oral administration of *p*-Cresol by gavage in mice, based on available toxicity data (4). Body weight, consumption of water and chow were monitored weekly.

Quantification of *p*-Cresol in urine and serum by gas chromatography coupled to mass spectrometry (GC-MS). Mice were individually placed in a clean empty plastic cage and urine was collected over 10 min and centrifuged (12 000 g, 5 min). The supernatant was snapped-frozen in liquid nitrogen and stored at -80°C. Blood was collected by cardiac puncture, transferred in Eppendorf tubes and allowed to clot on bench RT for 30 min. After centrifugation (12000g, 10 min, RT), serum was collected, snapped-frozen in liquid nitrogen and stored at -80°C. Prior to gas chromatography-mass spectrometry (GC-MS), urine (20 µL) and serum (100 µL) samples were prepared as follows: i) samples were spiked with 10 µL internal standard solution (myristic acid-d_{27} in isopropanol, 750 mg/mL), ii) 850 µL of ice cold methanol were added, followed by centrifugation for 20 min (4°C, 16000 g), iii) 750 µL of supernatants were transferred to silanized dark 2mL autosampler vials and evaporated to dryness in a rotational vacuum concentrator (45°C, 20 mbar, 2 h), iv) 50 µL of methoxyamine solution (2% in pyridine) were added and the samples were incubated overnight at R, and v) 100 µL of N-methyl-trimethylsilyl-trifluoroacetamide (MSTFA)+1%,trimethylchlorosilane (TMCS) solution were added, the samples were incubated at 60°C for 1 h and transferred to dark autosampler vials with 250 µL silanized inserts. The detailed instrumental conditions for GC-MS analysis were based on a previous study (5), with minor modifications to quantify *p*-Cresol. Briefly, the samples were analyzed in an Agilent 7890B-5977B Inert Plus GC-MS system. Two µL of each sample were injected in a split inlet (1:10 ratio). The chromatographic column was an Agilent ZORBAX DB5- MS (30 m X 250 µm X 0.25 µm + 10m, Duraguard). The temperature gradient was 37.5 min long and the mass analyzer was operated in full scan mode between 50-600 m/z. Blank and pooled quality control (QC) samples in several dilutions were included in the analysis to ensure that the measurements were reproducible. There was no external contaminations and the monitored analytes were responding linearly to the detector.

Behavioral testing

Behavioral phenotyping started after a 4-week treatment with *p*-Cresol and lasted for 1-2 weeks during which treatment was continued. All behavioral experiments were performed during the day. The animals followed multiple behavioral tests respecting their resting hours, with a minimum of 2 days between tests. Standard behavioral tests that have extensively been used to characterize genetic and environmental models of ASD (6-10) were implemented to assess the behavioral dimensions impacted in ASD.

Social abilities

Three-chamber sociability test. The stimulus mouse was habituated to the cylindrical cages (20 min sessions, repeated 3 days before the testing day). The apparatus consisted in a rectangular non-transparent plexiglass box (60x30cm), divided in three compartments (object chamber, empty chamber and unfamiliar mouse chamber) connected by open doors (4 cm width) to allow the mouse to move freely between the different compartments. The unfamiliar mouse or the toy mouse were placed in wired cylindrical cages. The test consisted in a habituation phase to the empty apparatus only in the presence of empty cylindrical cages during 5 min and a sociability phase where the unfamiliar mouse or the toy mouse is introduced in the cylindrical cages. Time spent in the 2 chambers were video-tracked over 10 min and analyzed *a posteriori* during the habituation and sociability phase using the ANY-maze software. The number and the time spent in close contact with each of the empty cylindrical cage (during the habituation phase) as well as with the stimulus mouse or with the toy mouse contained in wired cylindrical cage (during the sociability phase) were manually scored by an experienced experimenter blind to the experimental group. The mean duration of close contacts was calculated from these data (7, 11-13). The relative position of stimulus unknown mouse (versus mouse toy) was counterbalanced between groups. There was no bias in chamber preference, empty cylindrical cages preference or interaction ratio during the habituation phase (Supplementary Figure 2A-E, Supplementary Figure 6A-E).

Dyadic social interactions. Direct social interactions were recorded in open-field arena with a low light intensity (15 Lux). After a 3 min. habituation period, the subject mouse was put in presence with un unfamiliar sex- and age-matched interactor and their interaction was recorded for 10 min. Manual scoring by an experienced experimenter blind to the experimental group was performed *a posteriori* by recording time spent in social contact events, number of nose and paw contacts, time spent in nose and paw contact, number and time spent in self-grooming, number of self-grooming events after social contact, number of rearing and grooming events, number of circling episodes and head shakes. The mean duration of nose and paw contacts was calculated from these data (7, 11-13).

Stereotypic/perseverative behaviors

Motor stereotypies. The subject mouse was placed in a clean standard home cage (21x11x17 cm) covered with a thick layer of fresh sawdust (4 cm) and recorded for 10 min with light intensity set at 40 Lux. Manual scoring by an experienced experimenter blind to the experimental group was performed *a posteriori* by computing the number of events of head shake, rearing, digging, grooming, circling episodes and total time spent digging and grooming (6).

Marble burying test. Marble burying was used as a measure of perseverative behavior (14). The subject mouse was placed in a clean standard home cage (21x11x17 cm) filled with 4 cm of fresh sawdust on which 20 glass marbles (diameter 1,5 cm) were disposed with light intensity set at 40 Lux. The number of marbles buried (more than half of its surface outside of sawdust) during a 30 min session was monitored by an experienced experimenter blind to the experimental group (6).

Y maze spontaneous alternation task. Spontaneous alternation behavior was used to assess behavioral flexibility and perseveration (15-17). The apparatus consisted in three arms made of plexiglass (40x9x16cm) containing different symbols to be differentiated. We placed the mice in the centre and allowed it to explore the arms for 5 min with a low light intensity of 15

Lux. We measured the willingness of mice to explore new environments and we scored different patterns as spontaneous alternation (SPA), alternate arm returns (AAR) and same arm return (SAR). We then calculated the percentage of SPA, AAR and SAR (6, 18).

Locomotor activity

Actimetry. The measurement of individual spontaneous activity was performed in actimetry chambers (Imetronic) consisting of cages equipped with infrared beams able to detect in real time horizontal and vertical movements (rearing events). Animals were individually placed in actimetry chambers under a 12-hr light/dark cycle, with free access to food and drinking water. To avoid biases in measurements due to stress possibly inducing hyperlocomotion in a novel environment, a habituation period (Day 0: 11:00 p.m. - Day 1: 8:00 a.m.) preceded the 24 h-recording of horizontal activity (Day 1: 8:00 a.m. – Day 2: 8:00 a.m.) (8).

Anxiety

Open field test. Mice were individually placed in the corner of a white and opaque quadratic arena (40x40cm) and allowed to explore for 10 min under low illumination (40 Lux). Total distance travelled, time spent in center and number center entries were analyzed by videotracking using the ANY-Maze software (8, 9).

Novelty suppressed feeding test. Mice were food-deprived for 16 h, with unlimited access to drinking water. A pellet of food was positioned in the centre of an open-field covered with 1 cm of sawdust with light intensity set at 60 Lux. The subject mouse was then introduced in a corner of the arena and the latency (s) for the first bite in the pellet recorded. Immediately after the test, the animal was placed in a clean cage with *ad libitum* access to food and water (6, 18).

Cognition

Novel object recognition test. The novel object recognition test was performed in a rectangular arena (20cmx40cm). The test consisted of three sessions under constant light intensity (15 Lux). During the habituation session, the mice explored freely the empty arena for 5 min. F or

the training session, two identical objects were placed on each side of the arena and the mouse allowed to explore for 10 min. For the test session, one of the objects was replaced by a novel object and the mouse allowed to explore the objects for 10 min. The time spent exploring the familiar and novel objects by the subject animal were manually scored *a posteriori* on videorecordings of the sessions. A recognition index was calculated as the percentage of the time spent exploring the novel object over the total time spent exploring both objects (19).

***Ex vivo* patch-clamp electrophysiological recordings**

Electrophysiological recordings were performed on sub-groups of control or *p*-Cresol-treated which had not been subjected to behavioral tests but nevertheless belonged to larger cohorts in which behavioral impairments in the *p*-Cresol group were observed. Mice were anesthetized (Ketamine (150 mg/kg)/ Xylazine (10 mg/kg), transcardially perfused with artificial cerebrospinal fluid (aCSF solution: NaCl 119 mM, KCl 2.5 mM, NaH_2PO_4 1.25 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 26 mM, glucose 11 mM). Brains were removed from skull and sliced sagittally in 250 µm sections using a HM650V vibratome (Microm, France). Sections encompassing VTA were placed un ice-cold cutting solution (KCl 2.5 mM, NaH_2PO_4 1.25 mM, $MgSO_4$ 10 mM, $CaCl_2$ 0.5 mM, glucose 11 mM, sucrose 234 mM, $NaHCO_3$ 26 mM) bubbled with 95% O_2/5% CO_2. Slices were then incubated in aCSF at 37°C for 1 h, and then kept at room temperature. For recordings, sections were transferred in a thermo-controlled (32-34°C) recording chamber superfused with aCSF (2.5 ml/min). Spontaneous excitatory post-synaptic currents (sEPSCs) or excitability were measured using visualized whole-cell voltage-clamp and current-clamp recordings, respectively, using an upright microscope (Olympus France). Putative dopamine neurons were identified as previously described(20). Current-clamp recordings were performed using a Multiclamp 700B device (Molecular Devices, Sunnyvale, CA). Signals were collected and stored using a Digidata 1440A converter and pCLAMP 10.2 software (Molecular Devices, CA). In all cases, access resistance was monitored by a step at -10 mV (0.1 Hz) and experiments were discarded if the access resistance increased more than 20%. Internal solution (K-D-gluconate 135 mM, NaCl 5 mM, $MgCl_2$ 2 mM, HEPES 10 mM,

EGTA 0.5 mM, MgATP 2 mM, NaGTP 0.4 mM). Depolarizing (0-300 pA) or hyperpolarizing (0-450 pA) 800 ms current steps were used to assess excitability and membrane properties of VTA dopamine neurons. sEPSCs were assessed in voltage-clamp mode at a voltage of -65mV in the presence of picrotoxin (50 µM) using the same internal solution. Off-line analyzes were performed using Clampfit 10.2 (Axon Instruments, USA).

Fecal microbiota analysis by 16S rRNA gene sequencing

Fecal DNA sample preparation. Mice were individually placed for 10 min. in a sterile empty plastic cage, and feces were collected in Eppendorf tubes in the morning (10-11 a.m.), snapped-frozen in liquid nitrogen and stored at -80°C until further use. Genomic DNA was obtained from fecal samples using the QIAamp power fecal DNA kit (Qiagen), and DNA quantity was determined using a TECAN Fluorometer (Qubit® dsDNA HS Assay Kit, Molecular Probes).

16S sequencing. The V3-V4 hypervariable region of the 16S rRNA gene was amplified by PCR using the following primers: a forward primer 5′-**CTT TCC CTA CAC GAC GCT CTT CCG ATC T**AC GGR AGG CAG CAG-3 (28-nt illumina adapter (in bold) followed the 14 nt broad range bacterial primer 343F) and a reverse primer 5′-**GGA GTT CAG ACG TGT GCT CTT CCG ATC T**TA CCA GGG TAT CTA ATC CT-3′ (28-nt illumina adapter (in bold) followed by the 19 nt broad range bacterial primer 784R). The PCR reaction used 10 ng of fecal DNA as template, 0.5 µM primers, 0.2 mM dNTP, and 0.5 U of the DNA-free Taq-polymerase (MolTaq 16S, Molzym, Bremen, Germany). The amplifications were carried out using the following profile: 1 cycle at 94°C for 60 s, followed by 30 cycles at 94°C for 60 s, 65°C for 60 s, 72°C for 60 s, and finishing with a step at 72°C for 10 min. The PCR reactions were sent to the @Bridge platform (INRAe, Jouy-en-Josas, france) for sequencing (Illumina Miseq technology). Single multiplexing was performed using home-made 6 bp index, which were added to R784 during a second PCR with 12 cycles using forward primer (AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC) and reverse primer (CAA GCA GAA GAC GGC ATA CGA GAT-index-GTG ACT GGA GTT CAG ACG TGT). The resulting PCR products were

purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer instructions. Run quality was checked internally using PhiX, and sequences assigned to the corresponding based on the 6 pb index.

Sequence analysis. High-quality filtered reads were assembled and processed using FROGS pipeline (Find Rapidly OTU with Galaxy Solution) to obtain OTUs and their respective taxonomic assignment thanks to Galaxy instance (https://migale.inra.fr/galaxy) (21). Paired-end sequences were merged using Flash software using at least a 10 bp overlap between the forward and reverse sequences. Then, merged sequence were pre-filtered on the following criteria (amplicons size, presence of the 2 primers sequences, ambiguous base), yielding < 4,106 reads. The following successive steps involved de-noising and clustering of the sequences into OTUs using SWARM, chimera removal using VSEARCh yielding 21,616 clusters (< 3,106 reads). Then, cluster abundances were filtered at 0.005%, yielding 591 final clusters. Clusters were then affiliated to OTU by using a Silva123 16S reference database and the RDP (Ribosomal Database Project) classifier taxonomic assignment procedure. Taxa abundance table obtained for the dataset shows the sum of OTU abundances in each sample.

Statistical analysis. Richness and diversity indexes of bacterial community, as well as clustering and ordinations, were computed using the Phyloseq package (v 1.19.1) in RStudio software (22). Within sample community composition (α-diversity) was assessed by observed diversity (i.e. sum of unique OTUs per sample), Chao1 and Shannon index, as well as evenness-based Simpson's richness index. Divergence in community composition between samples was quantitatively assessed by calculating β-diversity index using Jaccard's distances matrix. Statistical difference in the microbial communities between groups were evaluated using constrained analysis of principal coordinates (PCoA) and permutational multivariate ANOVA (PERMANOVA). To identify discriminating taxa and OTU associated with the Control or *p*-Cresol class and estimate their effect size, we used the linear discriminant analysis (LDA) effect size (LEfSe) method implemented in Galaxy (23).

Fecal matter transplant (FMT)

Since antibiotic treatment impacts the behavior of rodents (24), we opted for an FMT procedure which did not include an antibiotic treatment step to deplete endogenous microbiota. Instead, we used a laxative treatment that was previously shown to efficiently deplete endogenous microbiota and favor the engraftment of the transplanted microbiota (25).

For induction experiments (Figure 4), recipient C57BL/6J mice were received at 3 weeks of age and habituated for 5 days to our facility. From Day 1 to 3, mice were gavaged with omeprazole (Esomeprazole, Biogaran, 50mg/Kg/24 h) each morning to lower gastric acidity and favor survival of the inoculum. In the evening of Day 3, food was removed, but mice had *ad libitum* access to water. On Day 4, mice received 5 consecutive gavages of 200μL of Moviprep reconstituted as recommended by the manufacturer (Norgine SAS, Rueil-Malmaison, France) at 1 hr 30 min intervals. On Day 5, mice were gavaged with omeprazole. The FMT inoculum was prepared by pooling individual fecal pellets from n=15 control or n=15 *p*-Cresol treated and behaviorally phenotyped donor mice. Fecal pools were then weighted and homogenized in ice-cold ddH_2O (weight:volume=1:50). Fecal slurry was filtered over a 70 μm mesh to remove fibers and clogs. Recipient mice received 3 consecutive gavages of 200 μL of the filtered fecal slurry at 2 h intervals. After the last gavage, mice were returned to clean cages with *ad libitum* access to food and water. Body weight was monitored on Day 4, 5 and 7 to verify that the mice recovered from the FMT procedure. Three weeks post FMT, mice were subjected to behavioral tests two days apart in the following order: dyadic social interactions and motor stereotypies.

For rescue experiments (Figure 5), mice were treated with *p*-Cresol for 4 wks as described in the animal treatment section, subjected to behavioral tests two days apart in the following order: direct social interactions, motor stereotypies and three-chamber test. Control and *p*-Cresol-treated mice were then subjected to the FMT procedure described above. The FMT inoculum was prepared by pooling individual fecal pellets from n=15 control donor untreated mice of the same age and sex. Three weeks post FMT, mice were subjected to behavioral

tests two days apart in the following order: direct social interactions, motor stereotypies and three-chamber test. The *ex vivo* electrophysiological recordings were also performed three weeks post FMT on parallel sub-groups of mice which were not subjected to behavioral testing.

Statistics

Comparisons between two groups were performed using 2-tailed unpaired Mann-Whitney's non-parametric U-test. Multiple group comparisons were performed using two-way ANOVA with factors stated in legends and text. Prior to ANOVA, normality of data was assessed using Kolmogorov-Smirnov's test. *Post hoc* comparisons were performed using Šidák's correction for multiple comparison. Detailed ANOVA statistics for each panel are provided in Supplementary Table 3. For comparison analysis of frequency distributions, Kolmogorov-Smirnov's test was used. Principal Component Analysis (PCA) of behavioral data was performed for each dataset consisting of scores from the dyadic social interaction test and scores from the direct monitoring of motor stereotypies using the webtool Clustvis (26). Correlations between microbial OTU abundances and behavioral scores were performed using Spearman's ρ correlation coefficient rank test with Benjamini-Hochberg's multiple testing correction. Statistical significance was set according to a two-tailed p-value or adjusted (if relevant) p-value(p)<0.05. Only significant differences are displayed on the graphs.

SUPPLEMENTARY REFERENCES

1.Borre YE, O'Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF (2014): Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol Med*. 20:509-518.

2.Loomes R, Hull L, Mandy WPL (2017): What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis. *J Am Acad Child Adolesc Psychiatry*. 56:466-474.

3.Waye MMY, Cheng HY (2017): Genetics and epigenetics of autism: A Review. *Psychiatry Clin Neurosci*.

4.Lewis RJ, Sax NI (2004): Sax's Dangerous Properties of Industrial Materials. 11th Edition ed. Hoboken, NJ, USA: Hoboken, N.J.; J. Wiley & Sons, pp 1004.

5.Fiehn O (2016): Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling. *Curr Protoc Mol Biol*. 114:30 34 31-30 34 32.

6.Becker JA, Clesse D, Spiegelhalter C, Schwab Y, Le Merrer J, Kieffer BL (2014): Autistic-like syndrome in mu opioid receptor null mice is relieved by facilitated mGluR4 activity. *Neuropsychopharmacology*. 39:2049-2060.

7.Pujol CN, Pellissier LP, Clement C, Becker JAJ, Le Merrer J (2018): Back-translating behavioral intervention for autism spectrum disorders to mice with blunted reward restores social abilities. *Transl Psychiatry*. 8:197.

8.Leboucher A, Bermudez-Martin P, Mouska X, Amri EZ, Pisani DF, Davidovic L (2019): Fmr1-Deficiency Impacts Body Composition, Skeleton, and Bone Microstructure in a Mouse Model of Fragile X Syndrome. *Front Endocrinol (Lausanne)*. 10:678.

9.Silverman JL, Yang M, Lord C, Crawley JN (2010): Behavioural phenotyping assays for mouse models of autism. *Nature reviews Neuroscience*. 11:490-502.

10.Becker JAJ, Kieffer BL, Le Merrer J (2017): Differential behavioral and molecular alterations upon protracted abstinence from cocaine versus morphine, nicotine, THC and alcohol. *Addict Biol*. 22:1205-1217.

11.Matsuo N, Tanda K, Nakanishi K, Yamasaki N, Toyama K, Takao K, et al. (2009): Comprehensive behavioral phenotyping of ryanodine receptor type 3 (RyR3) knockout mice: decreased social contact duration in two social interaction tests. *Front Behav Neurosci*. 3:3.

12.Katayama Y, Nishiyama M, Shoji H, Ohkawa Y, Kawamura A, Sato T, et al. (2016): CHD8 haploinsufficiency results in autistic-like phenotypes in mice. *Nature*. 537:675-679.

13.Spencer CM, Alekseyenko O, Serysheva E, Yuva-Paylor LA, Paylor R (2005): Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome. *Genes Brain Behav*. 4:420-430.

14.Thomas A, Burant A, Bui N, Graham D, Yuva-Paylor LA, Paylor R (2009): Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berl)*. 204:361-373.

15.Moustgaard A, Hau J, Lind NM (2008): Effects of dopamine D4 receptor antagonist on spontaneous alternation in rats. *Behav Brain Funct*. 4:49.

16.Le Marec N, Ethier K, Rompre PP, Godbout R (2002): Involvement of the medial prefrontal cortex in two alternation tasks using different environments. *Brain Cogn*. 48:432-436.

17.Delotterie D, Ruiz G, Brocard J, Schweitzer A, Roucard C, Roche Y, et al. (2010): Chronic administration of atypical antipsychotics improves behavioral and synaptic defects of STOP null mice. *Psychopharmacology (Berl)*. 208:131-141.

18.Meirsman AC, Le Merrer J, Pellissier LP, Diaz J, Clesse D, Kieffer BL, et al. (2016): Mice Lacking GPR88 Show Motor Deficit, Improved Spatial Learning, and Low Anxiety Reversed by Delta Opioid Antagonist. *Biol Psychiatry*. 79:917-927.

19.Fernandez SP, Muzerelle A, Scotto-Lomassese S, Barik J, Gruart A, Delgado-Garcia JM, et al. (2017): Constitutive and Acquired Serotonin Deficiency Alters Memory and Hippocampal Synaptic Plasticity. *Neuropsychopharmacology*. 42:512-523.

20.Fernandez SP, Broussot L, Marti F, Contesse T, Mouska X, Soiza-Reilly M, et al. (2018): Mesopontine cholinergic inputs to midbrain dopamine neurons drive stress-induced depressive-like behaviors. *Nat Commun*. 9:4449.

21.Escudie F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, et al. (2018): FROGS: Find, Rapidly, OTUs with Galaxy Solution. *Bioinformatics*. 34:1287-1294.

22.McMurdie PJ, Holmes S (2012): Phyloseq: a bioconductor package for handling and analysis of high-throughput phylogenetic sequence data. *Pac Symp Biocomput*.235-246.

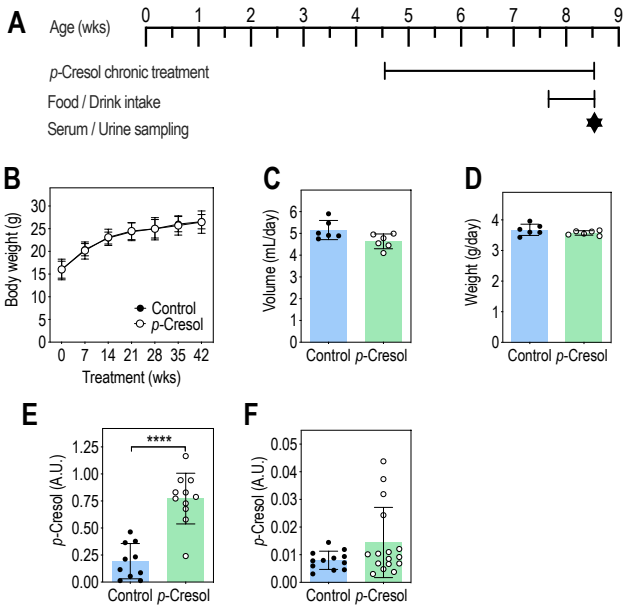
23.Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. (2011): Metagenomic biomarker discovery and explanation. *Genome Biol*. 12:R60.

24.Desbonnet L, Clarke G, Traplin A, O'Sullivan O, Crispie F, Moloney RD, et al. (2015): Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain Behav Immun*. 48:165-173.

25.Le Roy T, Debedat J, Marquet F, Da-Cunha C, Ichou F, Guerre-Millo M, et al. (2018): Comparative Evaluation of Microbiota Engraftment Following Fecal Microbiota Transfer in Mice Models: Age, Kinetic and Microbial Status Matter. *Front Microbiol*. 9:3289.

26.Metsalu T, Vilo J (2015): ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic acids research*. 43:W566-570.

Supplementary figures and supplemental figures legends (6)



Supplementary Figure 1. *p*-Cresol treatment does not impact general physiological parameters, increases urinary *p*-Cresol excretion but not its circulating levels

(A) Timeline of the experiment

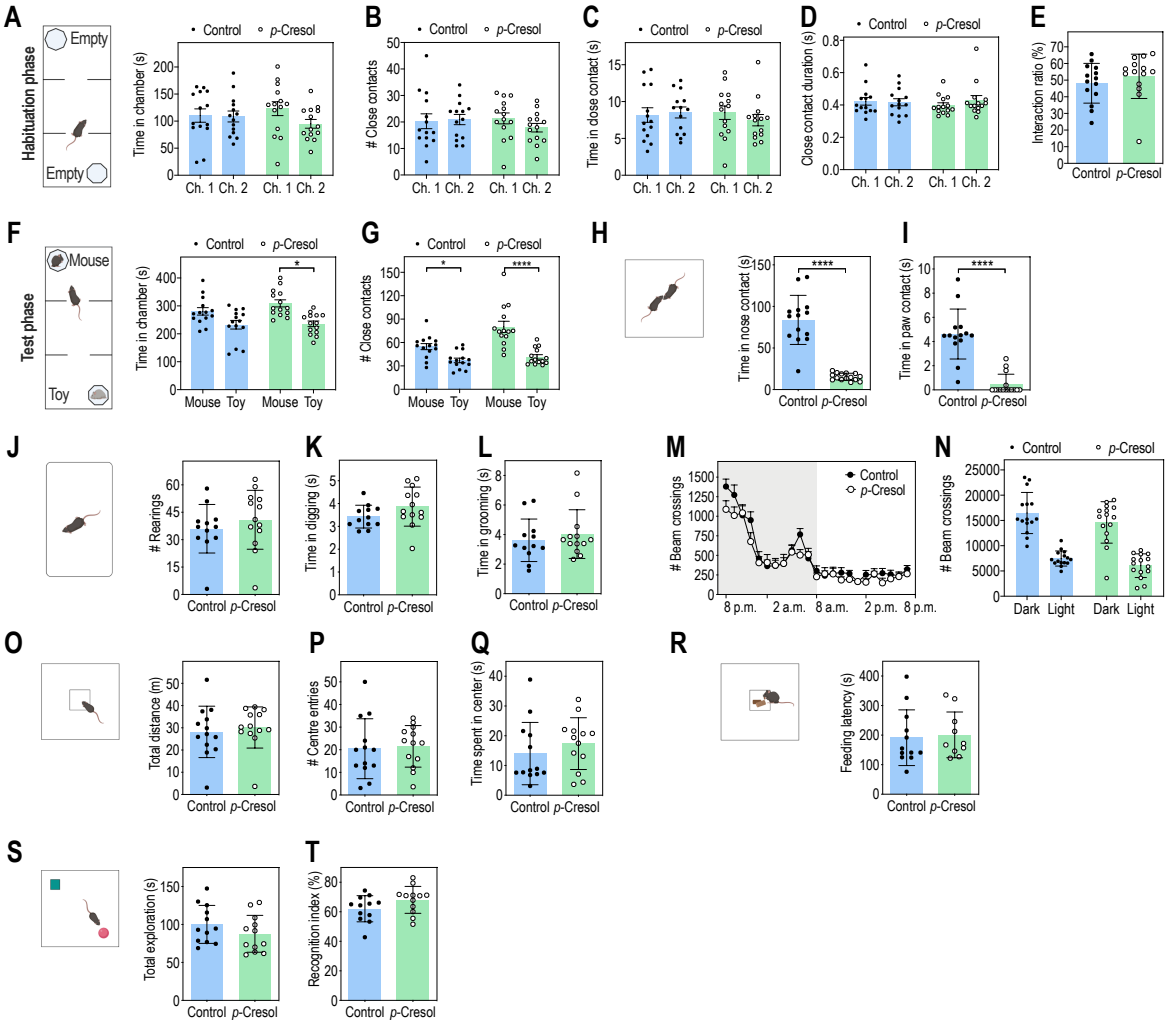
(B) Body weight follow-up (n=24/group); 2-way ANOVA: p(Treatment)=0.8884, p(Time)<0.0001, p(Treatment x Time)=0.9971; Šidák's post hoc tests for treatment effect: p>0.99.

(C) Drink intake (n=6/group, each point representing the mean consumption per animal in one cage of 5 animals); Mann-Whitney U-test: p=0.0931.

(D) Food intake (n=6/group, each point representing the mean consumption per animal in one cage of 5 animals); Mann-Whitney U-test: p=0.3939.

(E) Urinary levels of *p*-Cresol after 4 weeks treatment (n=10 Control, n=11 *p*-Cresol); Mann-Whitney U-test: ****, p<0.0001.

(F) Serum levels of *p*-Cresol after 4 weeks treatment (n=12 Control, n=16 *p*-Cresol); Mann-Whitney U-test: p=0.3238.



Supplementary Figure 2. *p*-Cresol treatment-induced behavioral alterations: additional parameters in the 3-chamber, dyadic social interactions and stereotypies tests and results from tests assessing locomotor activity, anxiety or cognition (relative to Figure 1)

(A-E) Additional parameters during the habituation phase in the 3-chamber test (n=14/group):

(A) Time spent in each chamber; 2-way ANOVA: p(Treatment)=0.9498, p(Chamber)=0.3018, p(Treatment x Chamber)=0.3599; Šidák's post hoc tests for chamber effect: p>0.05.

(B) Number of close contacts to the empty box in each chamber; 2-way ANOVA: p(Treatment)=0.6839, p(Chamber)=0.4390, p(Treatment x Chamber)=0.2648; Šidák's post hoc tests for chamber effect: p>0.05.

(C) Time close contacts to the empty box in each chamber; 2-way ANOVA: p(Treatment)=0.7265, p(Chamber)=0.6260, p(Treatment x Chamber)=0.3188; Šidák's post hoc tests for chamber effect: p>0.05.

(D) Mean duration of each close contacts to the empty box in each chamber; 2-way ANOVA: p(Treatment)=0.8330, p(Chamber)=0.4995, p(Treatment x Chamber)=0.3378; Šidák's post hoc tests for chamber effect: p>0.05.

(E) Interaction ratio; Mann-Whitney U-test: p=0.265.

(F, G) Additional parameters during the test phase in the 3-chamber test:

(F) Time spent in each chamber containing the mouse interactor or the toy mouse; 2-way ANOVA: p(Treatment)=0.0061, p(Mouse-Toy)=0.0019, p(Treatment x Mouse-Toy)=0.4797; Šidák's post hoc tests for Mouse vs. Toy preference effect: p>0.05 for Control group, * p<0.05 for *p*-Cresol group.

(G) Number of close contacts with the mouse interactor or the toy mouse; 2-way ANOVA: p(Treatment)=0.0035, p(Mouse-Toy)<0.0001, p(Treatment x Mouse-Toy)=0.0257; Šidák's post hoc tests for Mouse vs. Toy preference effect: *p<0.05, ****p<0.0001.

(H, I) Additional parameters in the dyadic social interaction test (n = 14 Control, n=15 *p*-Cresol):

(H) Total time spent in nose and (I) paw contact; Mann-Whitney U-test: ****p<0.0001.

(J-L) Additional parameters in the motor stereotypies test (n=11 Control, n=12 *p*-Cresol):

(J) Number of rearings; Mann-Whitney U-test: p=0.3400.

(K) Time spent in grooming; Mann-Whitney U-test: p=0.4613.

(L) Time spent in digging; Mann-Whitney U-test: p=0.1366.

(M, N) Locomotor activity in actimetry chambers (n=15/group):

(M) Horizontal activity follow-up over 24 h; 2-way ANOVA: p(Treatment)=0.1057, p(Time)<0.0001, p(Treatment x Phase)=0.3610; Šidák's post hoc tests for treatment effect: p>0.05.

(N) Cumulative activity in the dark or light phase; 2-way ANOVA: p(Treatment)=0.0909, p(Phase)<0.0001, p(Treatment x Phase)=0.7513; Šidák's post hoc tests for treatment effect: p>0.05.

(O-Q) Open-field test (n=13/group);

(O) Total distance travelled; Mann-Whitney U-test: p=0.3107.

(P) Number of centre entries; Mann-Whitney U-test: p=0.6047.

(Q) Time spent in the center of the area; Mann-Whitney U-test: p=0.3292.

(R) Novelty suppressed feeding (NSF) test (n=12 Control, n=10 *p*-Cresol): latency to first bite; Mann-Whitney U-test: p=0.5707

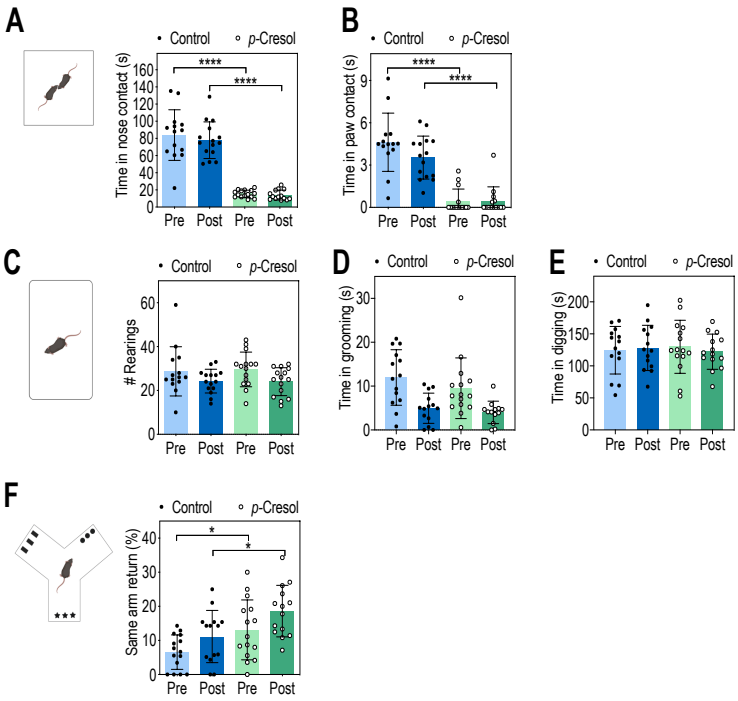
(S, T) Novel object recognition (NOR) (n=12/group):

(S) Total time spent exploring the objects during the test phase; Mann-Whitney U-test: p=0.1978.

(T) % of time spent exploring the old vs. the new object; Mann-Whitney U-test: p=0.1135.

(A-L, N-T) Data are presented as dot-plots featuring means ± SD.

(M) Data are shown as means ± SD.



Supplementary Figure 3. *p*-Cresol washout experiments: additional behavioral parameters for dyadic social interactions, stereotypies and Y-maze tests (relative to Figure 2)

(A, B) Dyadic social interaction test (n=14 Control, n=15 *p*-Cresol):

(A) Time spent in nose contact; 2-way ANOVA: p(Treatment)<0.0001, p(Washout)=0.4525, p(Treatment x Washout)=0.6450; Šidák's post hoc tests for treatment effect: ****p<0.0001.

(B) Time spent in paw contact; 2-way ANOVA: p(Treatment)<0.0001, p(Washout)=0.1638, p(Treatment x Washout)=0.1465; Šidák's post hoc tests for treatment effect: ****p<0.0001.

(C-E) Motor stereotypies (n=14 Control, n=15 *p*-Cresol):

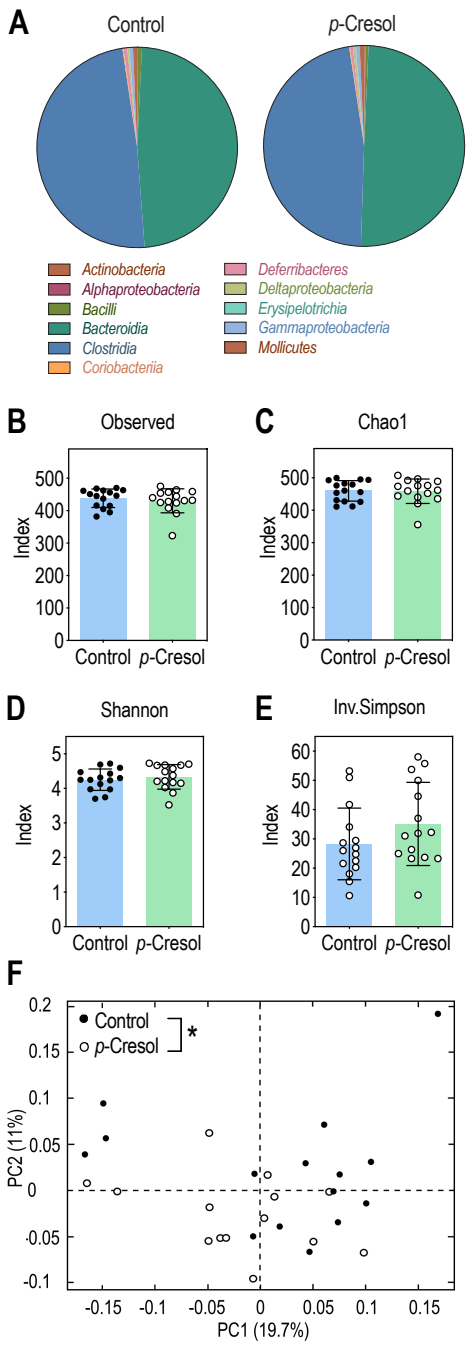
(C) Number of rearings; 2-way ANOVA: p(Treatment)=0.8820, p(Washout)=0.0188, p(Treatment x Washout)=0.7823; Šidák's post hoc tests for treatment effect: p>0.05.

(D) Time spent in grooming; 2-way ANOVA: p(Treatment)=0.2308, p(Washout)<0.0001, p(Treatment x Washout)=0.5929; Šidák's post hoc tests for treatment effect: p>0.05.

(E) Time spent in digging; 2-way ANOVA: p(Treatment)=0.9763, p(Washout) = 0.8304, p(Treatment x Washout)= 0.5579; Šidák's post hoc tests for treatment effect: p>0.05.

(F) Y-maze spontaneous alternations: percentage of same arm returns (n=15 Control, n=14 *p*-Cresol): 2-way ANOVA: p(Treatment) = 0.0007, p(Washout) = 0.0120, p(Interaction) = 0.8179; Šidák's post hoc tests for treatment effect: *p<0.05.

Data are presented as dot-plots featuring means ± SD.



Supplementary Figure 4. Microbiota analysis in *p*-Cresol-treated and control mice using 16S rRNA sequencing: α- and β-diversity indexes

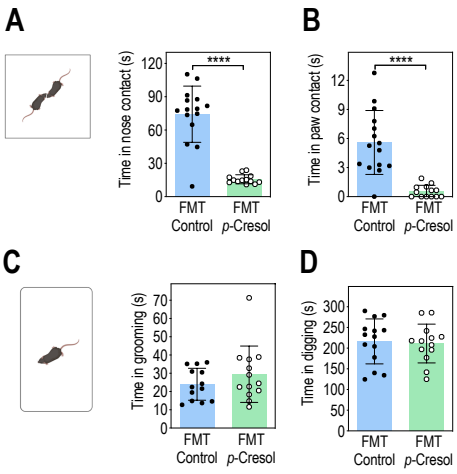
(A) Distribution of bacterial classes abundance (n=15 Control, n=15 *p*-Cresol).

(B-E) α-diversity indexes (n=15 Control, n=15 *p*-Cresol): Observed (B), Chao1 (C), Shannon (D) Inverse Simpson (E) diversity indexes; Mann-Whitney U-test: p>0.05.

(F) β-diversity index (n=15 Control, n=15 *p*-Cresol), PCoA of Jaccard's distances; PERMANOVA: *p<0.05.

(A) Data are presented as mean abundances for each group.

(B-E) Data are presented as dot-plots featuring means ± SD.



Supplementary Figure 5. FMT experiments from control or *p*-Cresol-treated mice to recipient wild-type mice: additional behavioral parameters for dyadic social interactions and stereotypies tests (relative to Figure 4)

(A, B) Dyadic social interaction test (n=15 animals/group):

(A) Total time spent in nose contact; Mann-Whitney U-test: ****p<0.0001

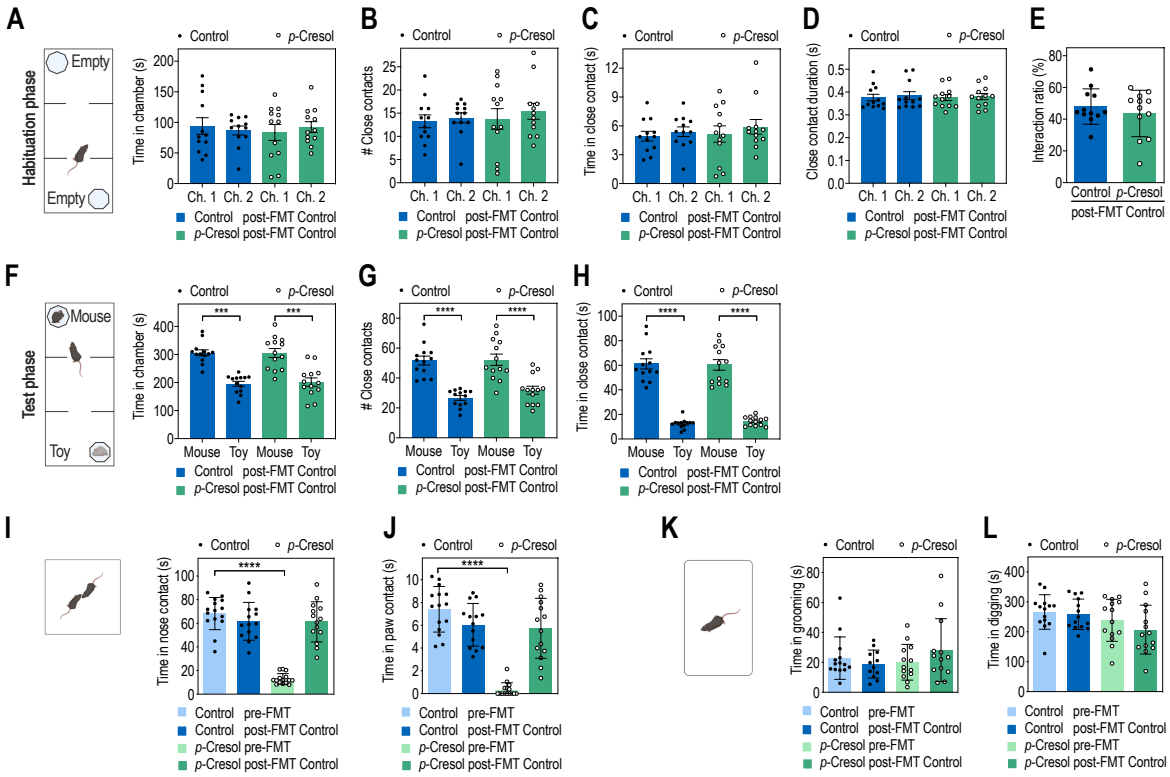
(B) Total time spent in paw contact; Mann-Whitney U-test: ****p<0.0001

(C, D) Motor stereotypies (n= 14 Control, n=13 *p*-Cresol):

(C) Time spent in grooming; Mann-Whitney U-test: p=0.3358.

(D) Time spent in digging; Mann-Whitney U-test: p=0.5502.

Data are presented as dot-plots featuring means ± SD.



Supplementary Figure 6. FMT experiments from control donors to control or *p*-Cresol treated recipient mice: additional behavioral parameters in the three-chamber, dyadic social interactions and stereotypies tests (relative to Figure 5)

(A-E) Additional parameters during the habituation phase in the 3-chamber test post-FMT control (n=13/group):

(A) Time spent in each chamber; 2-way ANOVA: p(Treatment)=0.8296, p(Chamber)=0.9514, p(Treatment x Chamber)=0.4749; Šidák's post hoc tests for chamber effect: p>0.05.

(B) Number of close contacts with the empty box in each chamber; 2-way ANOVA: p(Treatment)=0.6456, p(Chamber)=0.2947, p(Treatment x Chamber)=0.6878; Šidák's post hoc tests for chamber effect: p>0.05.

(C) Time in close contact with the empty box in each chamber; 2-way ANOVA: p(Treatment)=0.6728, p(Chamber)=0.1662, p(Treatment x Chamber)=0.7417; Šidák's post hoc tests for chamber effect: p>0.05.

(D) Mean duration of each close contact to the empty box in each chamber; 2-way ANOVA: p(Treatment)=0.9252, p(Chamber)=0.2163, p(Treatment x Chamber)=0.7061; Šidák's post hoc tests for chamber effect: p>0.05.

(E) Interaction ratio for Chamber 1 over Chamber 2; Mann-Whitney U-test: p=0.755.

(F-H) Additional parameters during the test phase in the 3-chamber test post-FMT (n=13/group)

(F) Time spent in each chamber containing the mouse interactor or the toy mouse; 2-way ANOVA: p(Treatment)=0.7588, p(Mouse-Toy) <0.0001, p(Treatment x Mouse-Toy)=0.8356; Šidák's post hoc tests for Mouse vs. Toy preference: ***p<0.001.

(G) Number of close contacts with the mouse interactor or the toy mouse; 2-way ANOVA: p(Treatment)=0.3399, p(Mouse-Toy)<0.0001, p(Treatment x Mouse-Toy)=0.4225; Šidák's post hoc tests for Mouse vs. Toy preference: ****p<0.0001.

(H) Time in close contact with the mouse interactor or the toy mouse; 2-way ANOVA: p(Treatment)=0.8480, p(Mouse-Toy)<0.0001, p(Treatment x Mouse-Toy)=0.6498; Šidák's post hoc tests for Mouse vs. Toy preference: ****p<0.0001.

(I, J) Additional parameters in the dyadic social interaction test pre- and post-FMT (n=15/group pre-FMT, n=14/group post-FMT):

(I) Time spent in nose contact; 2-way ANOVA: p(Treatment)<0.0001, p(FMT Control)<0.0001, p(Treatment x FMT Control) <0.0001; Šidák's post hoc tests for treatment effect: ****p<0.0001 for pre-FMT Control groups, p>0.05 post-FMT Control groups.

(J) Time spent in paw contact; 2-way ANOVA: p(Treatment)<0.0001, p(FMT Control)=0.0002, p(Treatment x FMT Control)<0.0001; Šidák's post hoc tests for treatment effect: ****p<0.0001 pre-FMT Control, groups p>0.05 post-FMT Control groups.

(K, L) Additional parameters in the motor stereotypies test pre- and post-FMT (n=14/group pre-FMT, n=14/group post-FMT):

(K) Time spent in grooming; 2-way ANOVA: p(Treatment)=0.7605, p(FMT Control)=0.2801, p(Treatment x FMT Control)=0.9682; Šidák's post hoc tests for treatment effect: p>0.05.

(L) Time spent in digging; 2-way ANOVA: p(Treatment)=0.0295, p(FMT Control)=0.274, p(Treatment x FMT Control)=0.5058; Šidák's post hoc tests for treatment effect: p>0.05.

Supplementary tables legends (3)

Supplementary Table 1. Association of bacterial taxa and OTU with Control or *p*-Cresol group (relative to Table 1)

Taxonomic information regarding the identified bacterial taxa, linear discriminant analysis score and corresponding *p*-values as determined by LefSE analysis of bacterial taxa abundances based on 16S rRNA sequencing are indicated. OTU, operational taxonomic unit (n=15 Control, n=15 *p*-Cresol).

Available as Excel file

Supplementary Table 2. Correlations between the abundance of discriminant microbial OTU identified by LEfSE analysis and social behavior/stereotypies scores impacted by *p*-Cresol treatment (relative to Table 1)

Table displays taxonomic information regarding the identified OTU, Spearman's rank ρ correlation coefficients between OTU abundances and behavioral scores significantly impacted by *p*-Cresol treatment in the dyadic social interactions and motor stereotypies tests (n=30 animals: n=15 Control, n=15 *p*-Cresol). The corresponding p-values and adjusted p-values are indicated, significant $p<0.05$ are shaded in grey.

Available as Excel file

Supplementary Table 3. Compilation of ANOVA statistics for behavioral data analyses and PERMANOVA statistics for Jaccard's distances analyses

Available as Excel file