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

Microbial *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

Brain development and behavioral responses are influenced by gut microbiota. Perturbations of the microbiota-gut-brain axis have been identified in autism spectrum disorders (ASD), suggesting that the microbiota could be involved in abnormal social and stereotyped behaviors in ASD patients. Notably, changes in microbiota composition and fecal, serum or urine levels of microbial metabolites are associated with ASD. Yet, a causal relationship between abnormal microbiota composition, altered microbial metabolite production, and ASD remains to be demonstrated. We hypothesized that *p*-Cresol (also known as 4-Cresol), a microbial metabolite that was described as more abundant in ASD patients, contributes to ASD core behavioral symptoms. Here we show that mice exposed to *p*-Cresol for 4 weeks in drinking water presented social behavior deficits, stereotypies, and perseverative behaviors, but no changes in anxiety, locomotion, or cognition. Abnormal social behavior induced by *p*-Cresol was associated with decreased activity of central dopamine neurons involved in the social reward circuit. Further, *p*-Cresol modified the relative abundance of specific bacterial taxa which correlated with social behavior. In addition, social behavior deficits were transferrable from *p*-Cresol-treated mice to control mice by fecal matter transplantation. In contrast, the microbiota from control mice restored both social interactions and dopamine neurons excitability when transplanted to *p*-Cresol-treated mice. Altogether, our results suggest that microbial metabolites *p*-Cresol could be involved in the development of core autistic behaviors via a gut microbiota-dependent mechanism. Further, this study paves the way for therapeutic interventions targeting the production of *p*-Cresol by gut bacteria to treat patients with ASD.

INTRODUCTION

Autism spectrum disorders (ASD) are frequent (1:100) neurodevelopmental pathologies characterized by social interaction and communication deficits, perseverative/stereotyped behaviors and restricted interests, as well as abnormal sensory processing[1]. Also, ASD often co-occur 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 abundances of several bacterial taxa[6-8]. Dysbiosis in ASD patients is 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 exhibited social behavior deficits, 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 can induce 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 microbial metabolites linked to ASD, 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 urinary 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 *Clostridioides 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 exposure 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[31-34] 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[35]. 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[36], 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[37]. 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[38].

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 in the Y-maze spontaneous alternation task (Figure 1N). Finally, *p*-Cresol-treated mice were clearly separated from control mice along the PC1 axis in a PCA analysis of scores recorded in the dyadic social interaction and stereotypies tests (Figure 1O).

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).

We then investigated whether *p*-Cresol-induced behaviors were abolished when treatment was discontinued (Supplementary Figure 3A). 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 (Supplementary Figure 3B-O). 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 (Supplementary Figure 3P).

Altogether, these results suggest that *p*-Cresol selectively induces ASD core symptoms which persist in the absence of *p*-Cresol, 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'[39]. Altered VTA connectivity and impaired VTA dopamine neurons activity were demonstrated in ASD patients[40] and in ASD models[19, 20, 41-44] 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 of *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.

***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 2A-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 3A). Compared to control mice transplanted with a normal microbiota, mice transplanted with 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 3B-F, Supplementary Figure 5A, B). In contrast, the number of followings was not impacted (Figure 3G). Social behavior deficits were accompanied by stereotypies with increased number of head shakes (Figure 3H), while circling episodes, grooming and digging time were not impacted (Figure 3I, 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 3J). In conclusion, the microbiota from *p*-Cresol-treated mice transfers social behavior deficits and to some extent stereotypies 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 4A). Before FMT, *p*-Cresol-treated mice displayed social behavior deficits and stereotypies (Figure 4B-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 4B, 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 4D, F). In the three-chamber test, the sociability index was also normalized (Figure 4H, 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 normalization of contact mean duration (Figure 4I). 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 4J, 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 4L). 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 4M, 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[45]. 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 rodents, none of them selectively induced ASD core symptoms. 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, 4-EPS 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 ASD core behaviors by inducing social behavior deficits and repetitive/perseverative behaviors, while not impacting behaviors related to frequent ASD comorbidities (stress, anxiety, hyperactivity, cognition).

Mice displaying social avoidance exhibited an increase in *p*-Cresol levels measured in gut tissue[46]. However, the 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[47]. Nonetheless, the intravenous administration of *p*-Cresol did not recapitulate chronic intestinal exposure and the broad observed effects may have resulted from acute activity of the compound. 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, indoles or 4-EPS impact the brain remains unknown. Some of these metabolites are ligands to the host's receptors: 3-indoxylsulfate and indole-3-propionate are ligands to the aryl-hydrocarbon receptor[48] and the xenobiotic sensor pregnane X receptor[49] respectively, while propionate binds to GPR41 and GPR43[50]. Yet, their signaling role has been mostly investigated in the context of metabolic, GI or autoimmune disorders[48-50], 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. In support of this, its circulating serum levels are not increased in *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 upon transplantation to control mice. We indeed show that a *p*-Cresol-rich environment reduces bacterial β-diversity, as described in ASD patients[6-8], and provides a selective growth advantage for several taxa, in line with a previous *in vitro* study[26]. The correlation between the abundances of 26 OTU and 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 *Muribaculaceae* and *Peptococcaceae* families are overabundant in *p*-Cresol-treated animals and their abundances correlate 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 2.5-fold increase range identified in ASD patients[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 FMT

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[51]. The VTA is a key subcortical node of the mesolimbic reward pathway[52] and ASD patients display defects in VTA connectivity[40]. Moreover, blocking VTA dopamine neurons that are part of a 'socially engaged reward circuit' is sufficient to diminish social interactions in rodents[39, 53]. Further, impaired dopamine neurons activity in the VTA has been previously described in models targeting the ASD genes *Shank3b*, *Nlgn3* and *Ube3a*[20, 41-43] and in the MIA and DIO environmental models of ASD[19, 44]. The disruption of the social reward pathway both in ASD patients and in our model also argues for its construct validity. Dopaminergic circuits are sensitive to changes in gut microbiota composition[54] and gut-innervating vagal sensory neurons control striatal dopamine release[55]. 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 dysbiosis results in impaired VTA dopamine neurons activity which eventually disrupts the social reward pathway and causes social behavior 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. 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[56]. 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), ANR and MRC (MR/M501797/1). JAJB and JLM thank Region Centre-Val de Loire (ARD2020 Biomedicaments GPCRAb) and the Labex MabImprove for financial support.

CONFLICT OF INTEREST

None to declare.

SUPPLEMENTARY INFORMATION

Supplementary information is available at MP's website

REFERENCES

1. Lai MC, Lombardo MV, Baron-Cohen S. Autism. *Lancet* 2014; **383**(9920)**:** 896-910.
2. Vuong HE, Hsiao EY. Emerging Roles for the Gut Microbiome in Autism Spectrum Disorder. *Biol Psychiatry* 2017; **81**(5)**:** 411-423.
3. Mazurek MO, Vasa RA, Kalb LG, Kanne SM, Rosenberg D, Keefer A *et al.* Anxiety, sensory over-responsivity, and gastrointestinal problems in children with autism spectrum disorders. *J Abnorm Child Psychol* 2013; **41**(1)**:** 165-176.
4. Nikolov RN, Bearss KE, Lettinga J, Erickson C, Rodowski M, Aman MG *et al.* Gastrointestinal symptoms in a sample of children with pervasive developmental disorders. *J Autism Dev Disord* 2009; **39**(3)**:** 405-413.
5. Gorrindo P, Williams KC, Lee EB, Walker LS, McGrew SG, Levitt P. Gastrointestinal dysfunction in autism: parental report, clinical evaluation, and associated factors. *Autism Res* 2012; **5**(2)**:** 101-108.
6. Xu M, Xu X, Li J, Li F. Association Between Gut Microbiota and Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. *Front Psychiatry* 2019; **10:** 473.
7. Iglesias-Vazquez L, Van Ginkel Riba G, Arija V, Canals J. Composition of Gut Microbiota in Children with Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. *Nutrients* 2020; **12**(3).
8. Liu F, Li J, Wu F, Zheng H, Peng Q, Zhou H. Altered composition and function of intestinal microbiota in autism spectrum disorders: a systematic review. *Transl Psychiatry* 2019; **9**(1)**:** 43.
9. Emond P, Mavel S, Aidoud N, Nadal-Desbarats L, Montigny F, Bonnet-Brilhault F *et al.* GC-MS-based urine metabolic profiling of autism spectrum disorders. *Analytical and bioanalytical chemistry* 2013; **405**(15)**:** 5291-5300.
10. Yap IK, Angley M, Veselkov KA, Holmes E, Lindon JC, Nicholson JK. Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls. *J Proteome Res* 2010; **9**(6)**:** 2996-3004.

11. Altieri L, Neri C, Sacco R, Curatolo P, Benvenuto A, Muratori F *et al.* Urinary p-cresol is elevated in small children with severe autism spectrum disorder. *Biomarkers* 2011; **16**(3)**:** 252-260.
12. Gabriele S, Sacco R, Cerullo S, Neri C, Urbani A, Tripi G *et al.* Urinary p-cresol is elevated in young French children with autism spectrum disorder: a replication study. *Biomarkers* 2014; **19**(6)**:** 463-470.
13. Dieme B, Mavel S, Blasco H, Tripi G, Bonnet-Brilhault F, Malvy J *et al.* Metabolomics Study of Urine in Autism Spectrum Disorders Using a Multiplatform Analytical Methodology. *J Proteome Res* 2015; **14**(12)**:** 5273-5282.
14. Macfabe DF. Short-chain fatty acid fermentation products of the gut microbiome: implications in autism spectrum disorders. *Microbial ecology in health and disease* 2012; **23**.
15. De Angelis M, Piccolo M, Vannini L, Siragusa S, De Giacomo A, Serrazzanetti DI *et al.* Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. *PLoS One* 2013; **8**(10)**:** e76993.
16. Kang DW, Ilhan ZE, Isern NG, Hoyt DW, Howsmon DP, Shaffer M *et al.* Differences in fecal microbial metabolites and microbiota of children with autism spectrum disorders. *Anaerobe* 2017; **49:** 121-131.
17. Kang DW, Adams JB, Coleman DM, Pollard EL, Maldonado J, McDonough-Means S *et al.* Long-term benefit of Microbiota Transfer Therapy on autism symptoms and gut microbiota. *Sci Rep* 2019; **9**(1)**:** 5821.
18. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T *et al.* Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 2013; **155**(7)**:** 1451-1463.
19. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M. Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell* 2016; **165**(7)**:** 1762-1775.

20. Sgritta M, Dooling SW, Buffington SA, Momin EN, Francis MB, Britton RA *et al.* Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. *Neuron* 2019; **101**(2)**:** 246-259 e246.
21. Golubeva AV, Joyce SA, Moloney G, Burokas A, Sherwin E, Arboleya S *et al.* Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism. *EBioMedicine* 2017; **24:** 166-178.
22. de Theije CG, Wopereis H, Ramadan M, van Eijndthoven T, Lambert J, Knol J *et al.* Altered gut microbiota and activity in a murine model of autism spectrum disorders. *Brain Behav Immun* 2014; **37:** 197-206.
23. Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM *et al.* Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. *Cell* 2019; **177**(6)**:** 1600-1618 e1617.
24. Jaglin M, Rhimi M, Philippe C, Pons N, Bruneau A, Goustard B *et al.* Indole, a Signaling Molecule Produced by the Gut Microbiota, Negatively Impacts Emotional Behaviors in Rats. *Front Neurosci* 2018; **12:** 216.
25. Saito Y, Sato T, Nomoto K, Tsuji H. Identification of phenol- and p-cresol-producing intestinal bacteria by using media supplemented with tyrosine and its metabolites. *FEMS Microbiol Ecol* 2018; **94**(9).
26. Passmore IJ, Letertre MPM, Preston MD, Bianconi I, Harrison MA, Nasher F *et al.* Para-cresol production by Clostridium difficile affects microbial diversity and membrane integrity of Gram-negative bacteria. *PLoS Pathog* 2018; **14**(9)**:** e1007191.
27. Borre YE, O'Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF. Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol Med* 2014; **20**(9)**:** 509-518.
28. Loomes R, Hull L, Mandy WPL. What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis. *J Am Acad Child Adolesc Psychiatry* 2017; **56**(6)**:** 466-474.

29. Lewis RJ, Sax NI. Sax's Dangerous Properties of Industrial Materials. 11th Edition edn. Hoboken, N.J.; J. Wiley & Sons: Hoboken, NJ, USA, 2004, p 1004.
30. Fiehn O. Metabolomics by Gas Chromatography-Mass Spectrometry: Combined Targeted and Untargeted Profiling. *Curr Protoc Mol Biol* 2016; **114:** 30 34 31-30 34 32.
31. Becker JA, Clesse D, Spiegelhalter C, Schwab Y, Le Merrer J, Kieffer BL. Autistic-like syndrome in mu opioid receptor null mice is relieved by facilitated mGluR4 activity. *Neuropsychopharmacology* 2014; **39**(9)**:** 2049-2060.
32. Pujol CN, Pellissier LP, Clement C, Becker JAJ, Le Merrer J. Back-translating behavioral intervention for autism spectrum disorders to mice with blunted reward restores social abilities. *Transl Psychiatry* 2018; **8**(1)**:** 197.
33. Leboucher A, Bermudez-Martin P, Mouska X, Amri EZ, Pisani DF, Davidovic L. Fmr1-Deficiency Impacts Body Composition, Skeleton, and Bone Microstructure in a Mouse Model of Fragile X Syndrome. *Front Endocrinol (Lausanne)* 2019; **10:** 678.
34. Silverman JL, Yang M, Lord C, Crawley JN. Behavioural phenotyping assays for mouse models of autism. *Nature reviews Neuroscience* 2010; **11**(7)**:** 490-502.
35. Fernandez SP, Broussot L, Marti F, Contesse T, Mouska X, Soiza-Reilly M *et al.* Mesopontine cholinergic inputs to midbrain dopamine neurons drive stress-induced depressive-like behaviors. *Nat Commun* 2018; **9**(1)**:** 4449.
36. Escudie F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K *et al.* FROGS: Find, Rapidly, OTUs with Galaxy Solution. *Bioinformatics* 2018; **34**(8)**:** 1287-1294.
37. McMurdie PJ, Holmes S. Phyloseq: a bioconductor package for handling and analysis of high-throughput phylogenetic sequence data. *Pac Symp Biocomput* 2012**:** 235-246.
38. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol* 2011; **12**(6)**:** R60.
39. Hung LW, Neuner S, Polepalli JS, Beier KT, Wright M, Walsh JJ *et al.* Gating of social reward by oxytocin in the ventral tegmental area. *Science* 2017; **357**(6358)**:** 1406-1411.

40. Supekar K, Kochalka J, Schaer M, Wakeman H, Qin S, Padmanabhan A *et al.* Deficits in mesolimbic reward pathway underlie social interaction impairments in children with autism. *Brain* 2018; **141**(9)**:** 2795-2805.
41. Krishnan V, Stoppel DC, Nong Y, Johnson MA, Nadler MJ, Ozkaynak E *et al.* Autism gene Ube3a and seizures impair sociability by repressing VTA Cbln1. *Nature* 2017; **543**(7646)**:** 507-512.
42. Bariselli S, Hornberg H, Prevost-Solie C, Musardo S, Hatstatt-Burkle L, Scheiffele P *et al.* Role of VTA dopamine neurons and neuroligin 3 in sociability traits related to nonfamiliar conspecific interaction. *Nat Commun* 2018; **9**(1)**:** 3173.
43. Bariselli S, Tzanoulinou S, Glangetas C, Prevost-Solie C, Pucci L, Viguie J *et al.* SHANK3 controls maturation of social reward circuits in the VTA. *Nat Neurosci* 2016; **19**(7)**:** 926-934.
44. Lecca S, Luchicchi A, Scherma M, Fadda P, Muntoni AL, Pistis M. Delta(9)-Tetrahydrocannabinol During Adolescence Attenuates Disruption of Dopamine Function Induced in Rats by Maternal Immune Activation. *Front Behav Neurosci* 2019; **13:** 202.
45. Bourgeron T. Current knowledge on the genetics of autism and propositions for future research. *C R Biol* 2016; **339**(7-8)**:** 300-307.
46. Gacias M, Gaspari S, Santos PM, Tamburini S, Andrade M, Zhang F *et al.* Microbiota-driven transcriptional changes in prefrontal cortex override genetic differences in social behavior. *Elife* 2016; **5**.
47. Pascucci T, Colamartino M, Fiori E, Sacco R, Coviello A, Ventura R *et al.* P-cresol Alters Brain Dopamine Metabolism and Exacerbates Autism-Like Behaviors in the BTBR Mouse. *Brain Sci* 2020; **10**(4).
48. Kim CH. Immune regulation by microbiome metabolites. *Immunology* 2018; **154**(2)**:** 220-229.
49. Venkatesh M, Mukherjee S, Wang H, Li H, Sun K, Benechet AP *et al.* Symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor PXR and Toll-like receptor 4. *Immunity* 2014; **41**(2)**:** 296-310.

50. Bolognini D, Tobin AB, Milligan G, Moss CE. The Pharmacology and Function of Receptors for Short-Chain Fatty Acids. *Mol Pharmacol* 2016; **89**(3)**:** 388-398.
51. Pellissier LP, Gandia J, Laboute T, Becker JAJ, Le Merrer J. mu opioid receptor, social behaviour and autism spectrum disorder: reward matters. *Br J Pharmacol* 2018; **175**(14)**:** 2750-2769.
52. Ranaldi R. Dopamine and reward seeking: the role of ventral tegmental area. *Rev Neurosci* 2014; **25**(5)**:** 621-630.
53. Gunaydin LA, Grosenick L, Finkelstein JC, Kauvar IV, Fenno LE, Adhikari A *et al.* Natural neural projection dynamics underlying social behavior. *Cell* 2014; **157**(7)**:** 1535-1551.
54. Gonzalez-Arancibia C, Urrutia-Pinones J, Illanes-Gonzalez J, Martinez-Pinto J, Sotomayor-Zarate R, Julio-Pieper M *et al.* Do your gut microbes affect your brain dopamine? *Psychopharmacology (Berl)* 2019; **236**(5)**:** 1611-1622.
55. Han W, Tellez LA, Perkins MH, Perez IO, Qu T, Ferreira J *et al.* A Neural Circuit for Gut-Induced Reward. *Cell* 2018; **175**(3)**:** 887-888.
56. De Preter V, Vanhoutte T, Huys G, Swings J, De Vuyst L, Rutgeerts P *et al.* 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* 2007; **292**(1)**:** G358-368.

FIGURES & TABLES

Figure 1

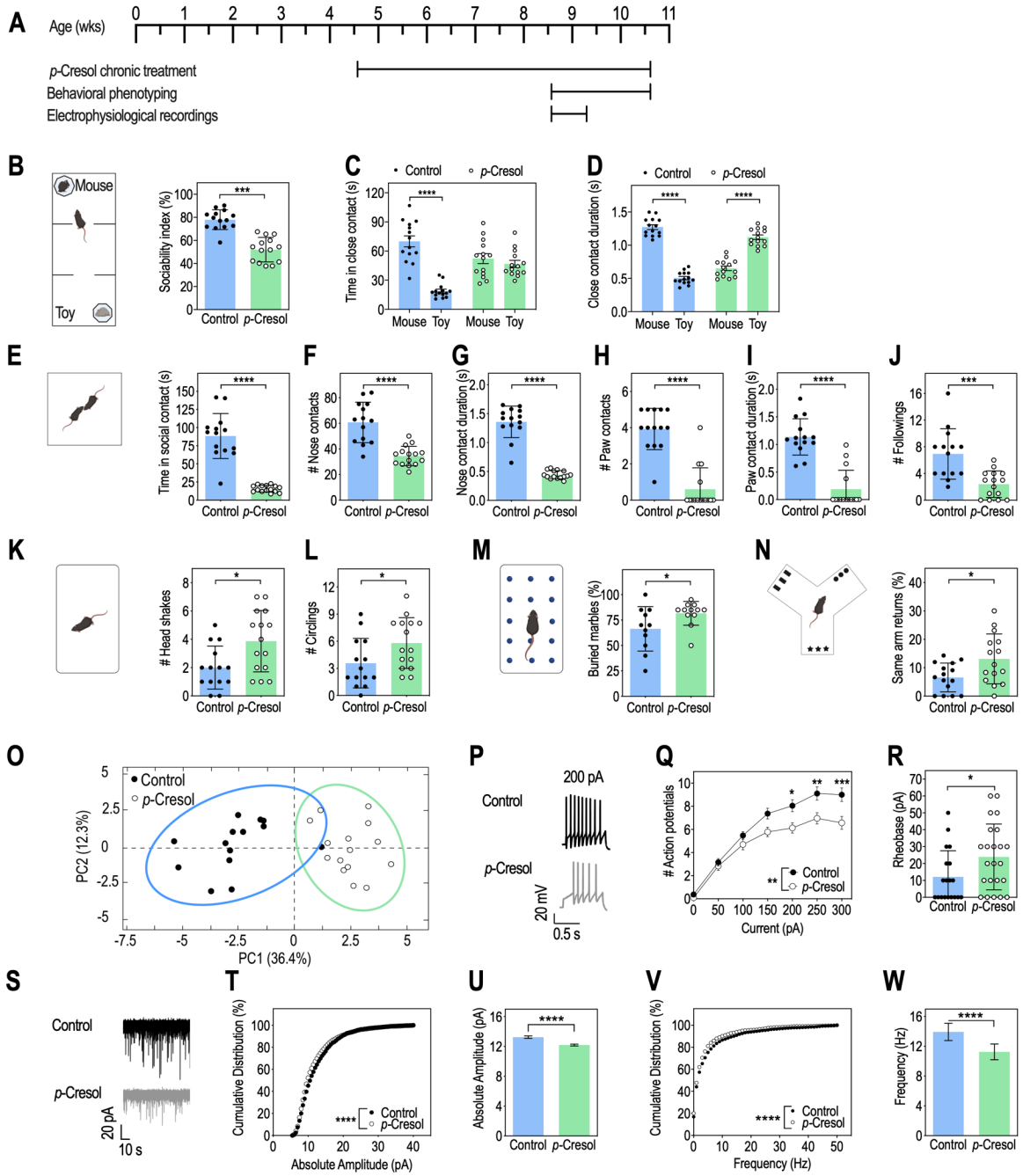

Figure 1. *p*-Cresol treatment induces autistic-like behaviors and alters VTA dopamine neurons excitability

(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

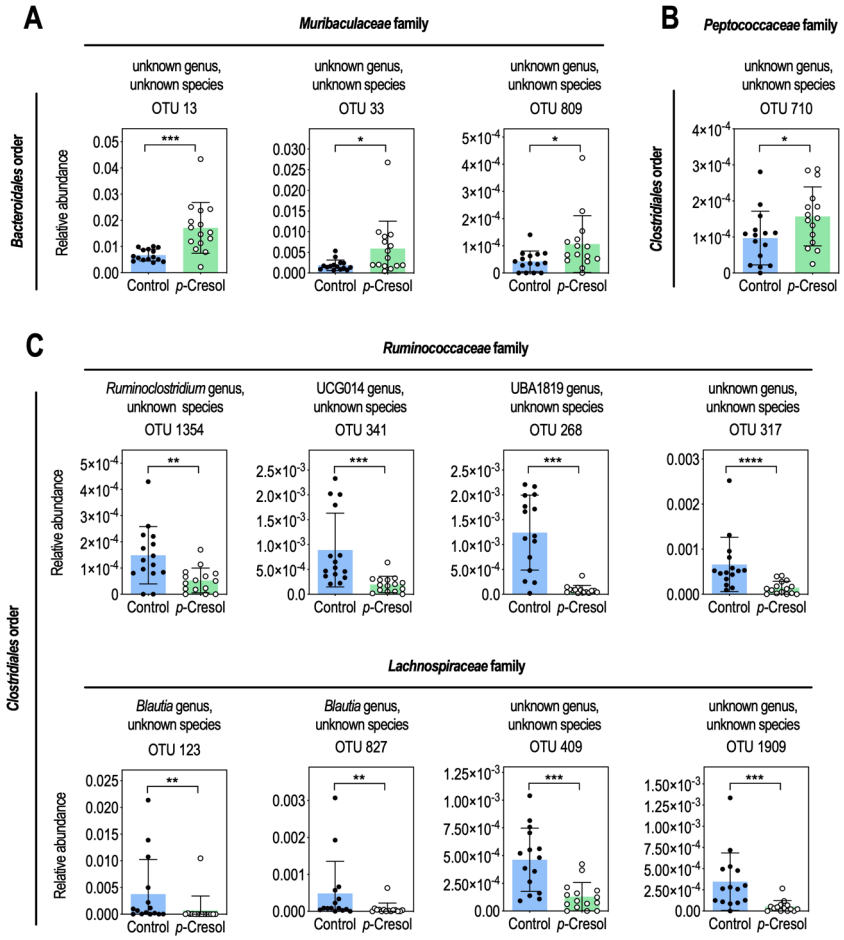

Figure 2. The abundances of microbial OTU correlating with social behavior are impacted by *p*-Cresol

(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 *Muribaculaceae* (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 3

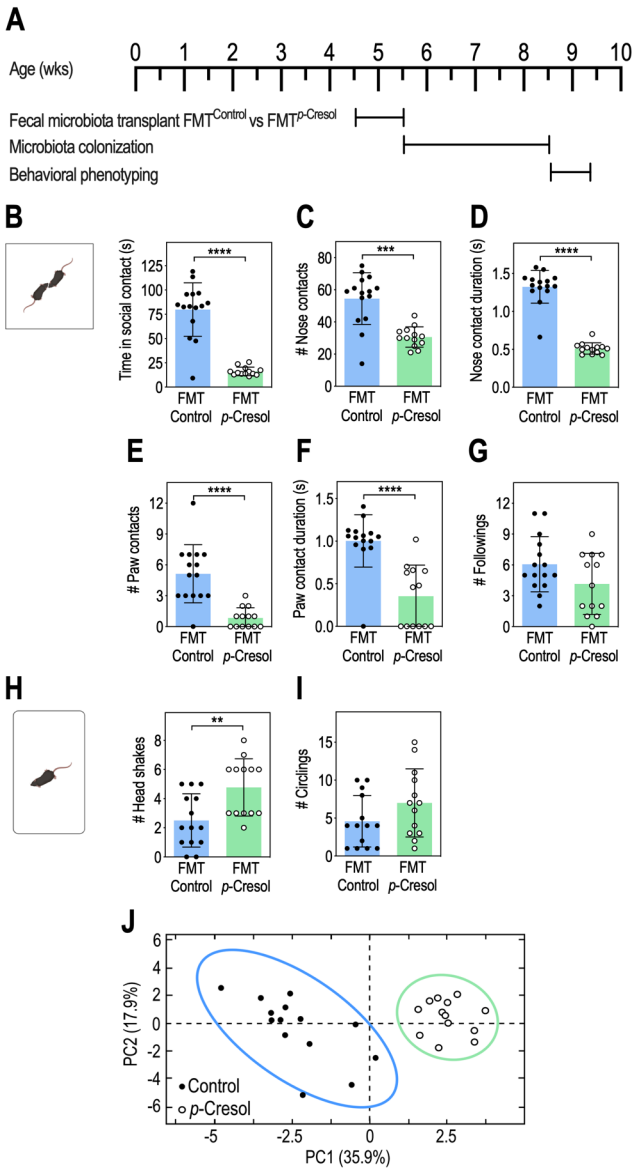

Figure 3. Microbiota remodeling following *p*-Cresol treatment induces social behavior deficits

(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 3B-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 4

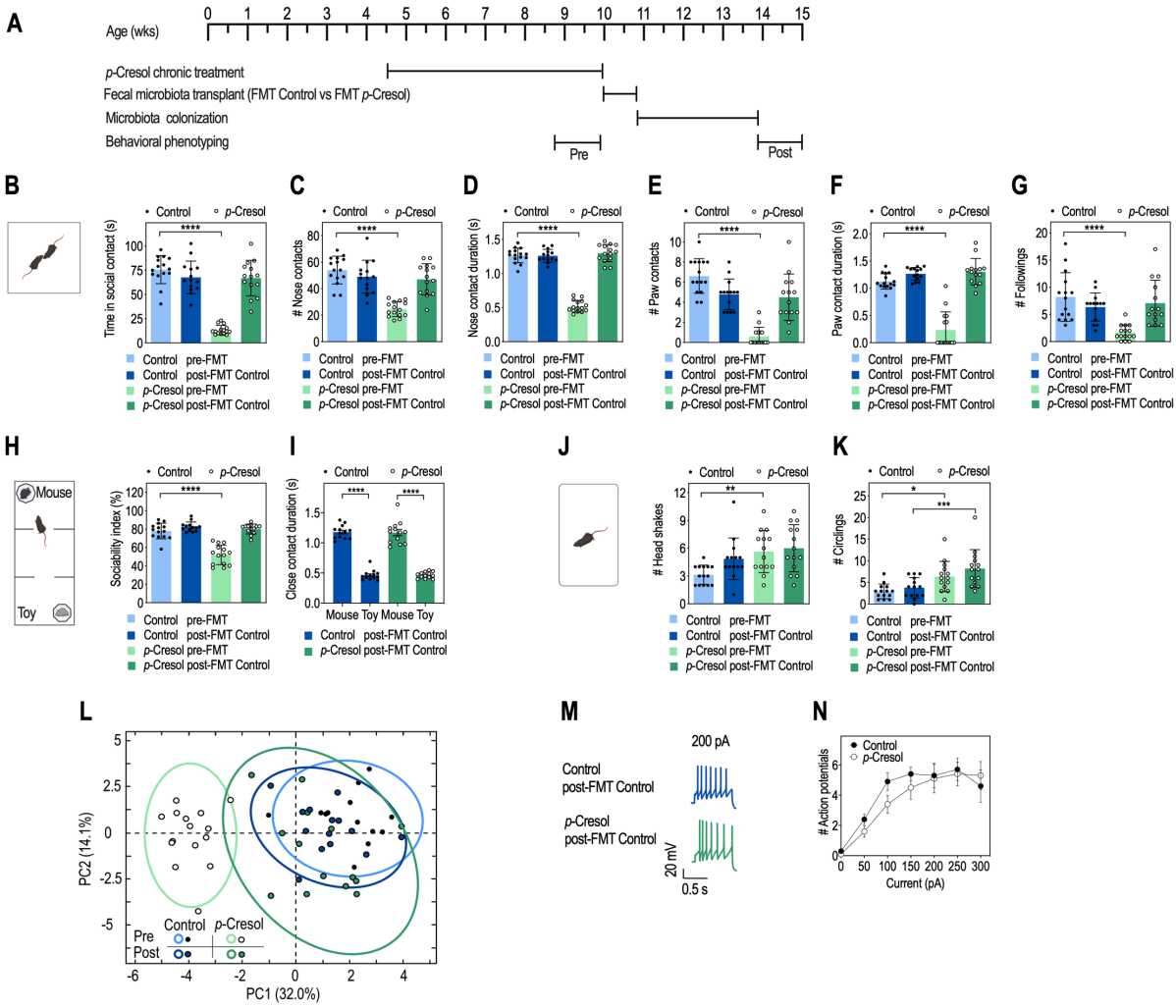

Figure 4. Transfer of microbiota from control mice to *p*-Cresol-treated mice restores social behavior deficits and VTA dopamine neurons excitability

(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 4B-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			