**Gut microbial metabolite *p*-Cresol promotes autistic-like behaviors in mice through remodeling of the gut microbiota**

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

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**ABSTRACT**

**Background:** Perturbations of the microbiota-gut-brain axis have been identified in autism spectrum disorders (ASD), suggesting that the microbiota could be involved in the development or maintenance of abnormal social and stereotyped behaviors in ASD patients. Yet, the underlying mediators and mechanisms remain unclear. We hypothesized that microbial metabolites produced by the gut microbiota contribute to behavioral deficits in ASD. We focused on *p*-Cresol, a microbial metabolite previously described as abnormally elevated in ASD patients.

**Methods:** Wild-type mice were chronically treated with *p*-Cresol in drinking water to mimic intestinal exposure. We combined behavioral phenotyping, electrophysiology, microbiota 16S sequencing and fecal microbiota transplantations to decipher the consequences of *p*-Cresol exposure.

**Results:** We showed that *p*-Cresol selectively induced behavioral alterations reminiscent of ASD core symptoms: social behavior deficits, stereotypies and perseverative behaviors, but no changes in anxiety, locomotion or cognition. We further showed that *p*-Cresol decreases the activity of dopamine neurons in the ventral tegmental area (VTA), a key brain region for social reward processing. In addition, we reveal that *p*-Cresol remodels the intestinal microbiome, impacting specific bacterial taxa associated with social behavior deficits and stereotypies. We further demonstrated that social behavior deficits are transferred to control mice after transplantation of microbiota from *p*-Cresol-treated mice. Finally, both social interactions and VTA dopamine neurons activity were normalized in *p*-Cresol treated mice after transplant of microbiota from control mice.

**Conclusions**: Our study suggests that the microbial metabolite *p*-Cresol could be involved in the development of autistic behaviors through remodeling of the gut microbiota.

## INTRODUCTION

Autism spectrum disorders (ASD) form a class of frequent (1:68) and severe neurodevelopmental pathologies diagnosed during early childhood (1, 2). ASD is characterized by lifelong social interaction and communication deficits and patterns of perseverative and stereotyped behaviors, associated with restricted interests (2). ASD often co-occurs with anxiety, hyperactivity and intellectual disability (2). ASD is also often associated with gastrointestinal (GI) dysfunction and increased intestinal permeability (3). Children with ASD and concurrent GI symptoms exhibit more pronounced emotional/behavioral problems and restrictive/repetitive behaviors compared to ASD peers without GI symptoms (4, 5). ASD patients exhibit gut microbiota dysbiosis, with reduced bacterial β-diversity and changed abundances of specific bacterial taxa (6). In addition, fecal microbiota transplantation (FMT) from healthy individual to ASD patients alleviated both GI symptoms and ASD core symptoms (7, 8). ASD is also associated with altered urinary, plasmatic or fecal levels of microbial metabolites exclusively produced by the microbiota: short-chain fatty acids (SCFA), indoles and aromatic metabolites derived from tyrosine (9-14). These observations in ASD patients can be recapitulated in environmental, idiopathic and genetic models of ASD, which exhibit a dysbiotic microbiota (15-18) and altered levels of microbial metabolites (15, 19). Furthermore, manipulations of the gut microbiota by FMT or probiotic treatment alleviate behavioral alterations in ASD models (15-17). Finally, mice born from mothers transplanted with feces from ASD patients, but not from healthy individuals, exhibit social behavior deficits and abnormal patterns of microbial metabolites (19).

While the link between disrupted patterns of microbial metabolites and behavioral impairments remains mostly unknown, chronic treatment of rodents with microbial metabolites upregulated in ASD patients or ASD mouse models induced behavioral changes. Notably, treatment with the SCFA propionate provokes a wide range of behavioral changes in rats, and notably social interaction deficits (20). Also, the tyrosine derivative 4-ethylphenylsulfate induced anxiety in mice (15). Finally, indoles negatively impact the socioemotional behavior of rats (21). Altogether, these data suggest that intestinal dysbiosis could contribute to ASD core and associated behaviors and that this might be mediated, at least in part, by microbial metabolites.

The synthesis of the small aromatic metabolite *para*-Cresol (*p*-Cresol, 4-Cresol, 4-methylphenol) depends on tyrosine degradation by the intestinal microbiota, performed notably by *Clostridioides difficile* and other species from *Clostridioides* (22, 23). Recent meta-analyses revealed consistent overabundance of the *Clostridioides* genus and *Clostridioides difficile* species in the intestinal microbiota of ASD patients (6, 24-28). Furthermore, in ASD patients, urinary (11, 12, 29) or fecal (26, 30) levels of *p*-Cresol are significantly elevated. Importantly, *p*-Cresol levels correlate with the severity of ASD stereotyped behaviors and chronic

constipation (11, 12, 29). This led us to hypothesize that exposure to *p*-Cresol could contribute to the development and/or maintenance of ASD core symptoms. Here we investigated the effects of chronic treatment of C57BL/6J wild-type mice with *p*-Cresol on behavior, neurotransmission and microbiota.

## 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**. Since the sex ratio for ASD is biased towards 3 males diagnosed for 1 female, only male C57BL/6J animals were considered in this study (1, 31). Four-week-old mice (Charles Rivers) were randomly assigned to experimental groups and treated for 4 weeks with *p*-Cresol (Sigma-Aldrich) dispensed in drinking water at a concentration of 2.3 mM for a target dose of 50mg/Kg/24 h.

**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) (32).

**Behavioral testing.** A battery of behavioral tests was used to assess the behavioral dimensions impacted in ASD which has extensively been used to characterize genetic and environmental models of ASD (33-37): social behavior (three-chamber sociability test, dyadic social interactions), repetitive behaviors (motor stereotypies, marble burying test, Y-maze exploration), activity (actimetry), anxiety (openfield, 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 VTA using visualized whole-cell voltage-clamp and current-clamp recordings, respectively, as described (38). Depolarizing (0-300 pA) or hyperpolarizing (0- 450 pA) 800 ms current steps were used to assess excitability and membrane properties of VTA dopaminergic 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 OTUs and their respective taxonomic assignment (39), 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 (40). 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 (41).

**Fecal microbiota transplantation (FMT)**

Mice were gavaged with omeprazole (50mg/Kg/24 h, Day 1-4) each morning. On Day 4, mice received 5 consecutive gavages of 200μL of Moviprep (Norgine) at 90 min intervals. On Day 5, individual fecal pellets from donor mice were pooled, weighted and homogenized in ice-cold $ddH_2O$ (w:v=1:50). Recipient mice received 3 consecutive gavages of 200 μL of a 70μm-filtered slurry at 2 h intervals. Three weeks post FMT, mice were subjected to behavioral tests or electrophysiological recordings.

**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

### Chronic exposure to *p*-Cresol induces ASD core symptoms in mice

To mimic exposure to *p*-Cresol through the GI tract, we opted for a chronic 4-week exposure of male mice to *p*-Cresol in drinking water. This did not impair basic physiological parameters as the growth curve, drink or food intake of treated animals were not impacted (Supplementary Figure 1A-D). There was no elevation of *p*-Cresol in the serum post treatment (Supplementary Figure 1E). However, *p*-Cresol treatment yielded a 4-fold increase in *p*-Cresol urinary excretion (Supplementary Figure 1F).

Control mice and mice exposed chronically to *p*-Cresol were then subjected to a battery of behavioral tests to assess behaviors related to ASD core symptoms (social behavior, repetitive/perseverative behaviors) and comorbidities (anxiety, hyperactivity, cognitive deficits). 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) as compared to control mice. While their number of close contacts with the interactor was higher than with the toy mouse (Supplementary Figure 2G), the mean duration of these contacts was strongly reduced (Figure 1D). During dyadic social interactions, *p*-Cresol-treated mice displayed a decrease in time spent in social contact, as well as time, number and mean duration of both nose and paw contacts and number of followings, as compared to control mice (Figure 1E-J, Supplementary Figure 2H, I). This was indicative of reduced social interactions and deterioration of the quality of social contacts in *p*-Cresol-treated mice.

We further monitored repetitive/perseverative behaviors. As compared to control mice, *p*-Cresol-treated mice display more frequent headshakes and circling events (Figure 1K, L), but similar numbers of rearing episodes and time spent in self-grooming or digging than control mice (Figure 1J-L). Thus, the occurrence of stereotyped behaviors was increased in *p*-Cresol treated mice, as confirmed in the marble burying test where *p*-Cresol exposed mice buried more marbles (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, PCA analysis of scores recorded in the dyadic social interaction and stereotypies tests clearly separated groups along the PC1 axis (Figure 1O).

In contrast, *p*-Cresol treatment did not induce hyperactivity, as control and *p*-Cresol-treated mice displayed similar nocturnal and diurnal locomotor activity over 24 h in actimetry chambers, as well as similar total distance travelled in the open-field (Supplementary Figure 2M-O). Also, the time spent in the openfield center and the latency to feed in the novelty-suppressed feeding test were not impacted (Supplementary Figure 2P-R), indicating that *p*-Cresol did not increase 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 exposure selectively induced ASD core symptoms, while not affecting the other dimensions of behavior investigated here.

**_p_-Cresol treatment impairs dopamine neurons excitability in the ventral tegmental area and induces long-lasting behavioral alterations that persist after washout**

We then sought to identify the neuronal circuits impacted by *p*-Cresol-treatment. We focused on dopamine (DA) neurons from the VTA that are part of a 'socially engaged reward circuit' (42). Altered VTA connectivity and impaired VTA DA neurotransmission were demonstrated in ASD patients (43) and in ASD models (16, 17, 44-47), respectively. We used whole-cell patch-clamp to record VTA DA 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 DA neurons, with a reduction in evoked action potentials (Figure 1P-R). Second, both the amplitude and frequencies of miniature spontaneous excitatory postsynaptic currents (sEPSC) were reduced in VTA DA neurons from *p*-Cresol treated animals as compared to controls (Figure 1S-W). This indicates that *p*-Cresol exposure induces changes in VTA DA neurotransmission.

We then looked at whether the effect of *p*-Cresol treatment on behavior could be reversed by interrupting the treatment (Figure 2A). We observed that *p*-Cresol effects on social interactions, stereotypies and perseverative behaviors persisted and were of similar magnitude, even after a of 4-week washout (Figure 2B-I, Supplementary Figure 3A-F). PCA analysis of behavioral scores showed a clear separation of *p*-Cresol and Control groups along the PC1 axis and between the behavior before and after the washout along the PC2 axis (Figure 2J).

**The dysbiotic microbiota induced by _p_-Cresol exposure transfers social behavior deficits to recipient mice**

We reasoned that the persistence of *p*-Cresol behavioral effects even after a 4-week washout period could be explained by irreversible microbiome changes even in the absence of exposure to the compound. We therefore analyzed the bacterial composition and community structure of the fecal microbiota from *p*-Cresol treated and control mice by targeted sequencing of bacterial 16S ribosomal RNA. *Bacteroidia* and *Clostridia* were the dominant classes in both groups (Supplementary Figure 4A). Bacterial $\alpha$–diversity indices and bacterial $\beta$-diversity computed based on weighted UniFrac distances (based on relative abundances of operational taxonomic units (OTU)) did not differ significantly between groups (Supplementary Figure 4B-E). However, the Jaccard distance (based on the presence/absence of species) revealed a significant difference in microbial composition between the groups (Supplementary Figure 4F).

We then sought to identify the bacterial taxa and OTU discriminating *p*-Cresol and control groups and their effect size. Among the 591 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 significantly associated with *p*-Cresol exposure, most of them belonging to the *Lachnospiraceae* (14 OTU) or the *Muribaculaceae* (8 OTU) families. We also identified 41 OTUs significantly associated with the control class, most of them also belonging to the *Lachnospiraceae* (19 OTU), *Ruminococcaceae* (9 OTU) or the *Muribaculaceae* (7 OTU) families.

We then looked for associations between the relative abundances of the 70 discriminant bacterial OTU identified by LefSe (Supplementary Table 2) and behavioral scores relative to social behavior and stereotypies significantly impacted by *p*-Cresol treatment (Figure 1E-N). Correlation analyzes revealed moderate to strong associations ($0.38 < |\rho| < 0.74$) between the gut microbiota composition and behavioral scores, in particular with social behavior (Table 1). The abundances of 4 OTU from the *Bacteroidales* and the *Clostridiales* orders associated with *p*-Cresol group correlated significantly with social interaction deficits. In contrast, a subset of 20 OTU from the *Clostridiales* order (*Ruminococaceae* and *Lachnospiraceae* families) were associated with the control group and higher sociability. Regarding stereotyped behavior, 2 OTU were associated the control group and with reduced headshakes. All these OTUs 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). These data support a tight link between microbiota dysregulations induced by *p*-Cresol exposure and behavioral dimensions relative to core ASD symptoms.

We then transplanted the microbiota from the groups analyzed by 16S sequencing to recipient control mice and scored social behavior and stereotypies in recipient mice (Figure 4A). FMT from *p*-Cresol-treated mice induced strong social interaction deficits, with reduction of the total time in social contact, as well as time in, number and average duration of nose and paw contacts (Figure 4B-F, Supplementary Figure 5A, B) while followings were not impacted (Figure 4G). The deficits were accompanied by increased head shakes (Figure 4H), while circling episodes, grooming and digging time were not impacted (Figure 4I, Supplementary Figure 5C, D). PCA analysis of the behavioral scores collected in these two tests indicated a clear separation between groups along the PC1 axis (Figure 4J). These data show that microbiota transplantation from mice exposed to *p*-Cresol is sufficient to transfer social behavior deficits and, to a lesser extent, stereotypies to wild-type recipient mice.

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

We then transplanted a normal control microbiota to recipient mice that had been exposed for 4 weeks to *p*-Cresol or water (Figure 5A). Before FMT, *p*-Cresol-treated mice displayed social behavior deficits and stereotypies, consistent with our initial data (Figure 1, 2). Recolonization of *p*-Cresol exposed mice with a normal control microbiota restored all parameters in dyadic social interaction test to control levels (Figure 5B, C, E, G, Supplementary Figure 6I, J). Importantly, the quality of social contacts was restored as both nose and paw contact durations were rescued (Figure 5D, F). In the 3-chamber test, FMT Control normalized the sociability index (Figure 5H, Supplementary Figure 6F) and preference for the interactor mouse, as shown by rescue to normal levels of the number, time and average duration of close contacts with the mouse interactor as compared to the toy mouse (Figure 5I, Supplementary Figure 6G-H). Reversal of stereotyped behaviors was incomplete, however, as head shakes were only modestly reduced and circling episodes were still present after FMT in recipient *p*-Cresol exposed mice (Figure 5J, K). Global normalization of *p*-Cresol behavioral scores after FMT was obvious from the PCA representation of behavioral scores which indicate clustering with control groups on the right side of the PC1 axis, while *p*-Cresol group before FMT stood alone on the left side (Figure 5L). Strikingly, social behavior restoration post-FMT Control in *p*-Cresol treated animals was accompanied by a normalization of evoked DA excitability in the VTA (Figure 5M, N).

## DISCUSSION

### *p-Cresol exposure specifically induces ASD core behavioral deficits*

ASD result from interactions between genes and environment. While 10-25% of ASD cases can be explained by mutations in specific genetic *loci*, twin studies have revealed that genetics and environmental factors share equal influence on ASD risk (48). 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 chronic exposure of mice to the ASD-related gut microbial metabolite *p*-Cresol induces social behavior deficits and stereotypies, demonstrating a possible causal relationship between elevated *p*-Cresol levels and ASD core symptoms. Previous studies have shown that other microbial metabolites possibly linked to ASD, such as propionate, indole or 4-ethyl-phenylsulfate (a compound with structural similarity to the sulfated conjugate of *p*-Cresol) triggered different behavioral responses in wild-type rodents, however, with less specificity and relevance to ASD core symptoms. First, while the microbial SCFA propionate induced social interaction deficits and stereotypies, it also induced anxiety and hyperlocomotion, all of which could be reversed after washout (49). Second, while exposure to indole induced increased social contact, it also triggered hypolocomotion and anxiety in rats (21). Third, exposure to both 4-ethyl-phenylsulfate increased anxiety and startle reflex in mice, while it had no impact on social behavior or stereotypies (15). Our model of intestinal exposure to *p*-Cresol induces selective alterations in the core dimensions of behavior impacted in ASD: social behavior and stereotyped/perseverative behaviors, while other behavioral dimensions (stress, anxiety, hyperactivity, cognition) frequently altered in ASD patients (2), are not impacted. Of note, mice displaying social avoidance and despair-like behaviors exhibited an increase in the levels of *p*-Cresol measured in gut tissue (50), but the link between both was not investigated. Also, a recent study showed that a single acute intravenous injection of *p*-Cresol, broadly exacerbated anxiety and reduced social preference in the BTBR idiopathic model of ASD (51). However, the mode of administration of *p*-Cresol in the latter study does not recapitulate chronic intestinal exposure to *p*-Cresol through the microbiota and the observed effects may result from acute toxicity of the compound injected intravenously 15 min prior to the behavioral tests. Although several studies have described increased urinary (11, 12, 29) or fecal (26, 30) levels of *p*-Cresol in ASD patients as compared to neurotypical controls, 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 this environmental ASD model.

### *Dysbiosis induced by p-Cresol exposure modulates host's behavior*

The mechanisms mediating the effects of ASD-linked microbial metabolites such as propionate (14), 4-ethylphenyl sulfate (15) or indoles (52), on the brain remain unknown. Some of these metabolites are ligands to the host's receptors: 3-indoxylsulfate binds to the aryl-hydrocarbon receptor, indole-3-propionate is a ligand for the xenobiotic sensor pregnane X receptor, while propionate binds to GPR41 and GPR43, however, their signaling role has been mostly investigated in the context of metabolic, GI or autoimmune disorders (53-55), and not in the context of neuropsychiatric disorders and behavior. In the case of *p*-Cresol, it appears unlikely to exert *per se* a direct systemic or even central effect on a receptor given that its circulating levels are not elevated in the serum of mice exposed to *p*-Cresol (making it unlikely to reach bioactive levels in the brain) and its effects on behavior persist even in its absence after a 4-week washout period. Rather, we propose that *p*-Cresol triggers ASD behaviors by inducing a selective microbiota dysbiosis, which is sufficient to induce social behavior deficits and stereotypies when transferred to control recipient mice. We 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 (23). The fact that 26 dysregulated OTU selectively correlated with social and stereotyped behaviors argues for a clear link between certain OTU abundances and social abilities. Notably, members of the *Blautia* genus are reduced both in ASD patients (6, 25) and in *p*-Cresol-treated animals and their abundances correlate positively with social abilities in our model. Conversely, OTU 710 from the *Peptococcaceae* family is overabundant in *p*-Cresol treated animals and its abundance correlates negatively with social behavior abilities. Since one study suggests that *p*-Cresol production confers a selective growth advantage for *Clostridioides difficile* (also belonging to the *Peptococcaceae* family) (23), we anticipate that selective overgrowth of certain bacterial species could contribute to social behavior deficits in our model. Th Increased abundance of OTU 710 is in line with findings in ASD patients, in which increased abundances of *Peptococcaceae*, including *Clostridioides difficile* have been repeatedly observed (6, 24-28). 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). Given the parenthood between the biological correlates underlying the abnormal social behavior in our model and those underlying ASD behaviors in patients, with elevated urinary *p*-Cresol (11, 12) and gut microbiota dysbiosis (6, 28), our data demonstrate the construct validity of our model. Furthermore, predictive validity is supported by our findings that FMT of a normal microbiota is able to normalize behavior in mice exposed to *p*-Cresol, as already shown in a pilot study in ASD patients (7, 8).

***Dysbiosis induced by p-Cresol exposure dysregulates central dopamine pathways and the social reward system***

We provide evidence for alterations in VTA DA neurons activity in *p*-Cresol treated mice. The VTA is a key subcortical node of the mesolimbic reward pathway (56). Social interactions are pleasurable events for humans and animals, as shown notably by the activation of the reward circuit by social stimuli – activation that is blunted in ASD patients (57), who also display altered VTA connectivity (43). Moreover, blocking the VTA DA neurons that are part of the 'socially engaged reward circuit' is sufficient to diminish social interactions (42, 58). These data suggest that impaired VTA DA neurotransmission in *p*-Cresol-treated animals disrupts the social reward pathway and this could be a possible neurobiological substratum for the social behavior deficits induced, as previously described in both genetic (17, 44-46) and environmental ASD models (16, 47). Abnormal VTA neurotransmission in our model therefore argues for its construct validity since disruption of the social reward pathway is observed in ASD (57). Furthermore, our findings that replacing the microbiota of *p*-Cresol-treated mice by a normal microbiota from control animals not only restored social behavior quantitatively and qualitatively to control levels, but also restored VTA DA neurotransmission, strongly argue that the gut microbiota conveys information to the VTA and can modulate its dopaminergic activity. This is also supported by several studies suggesting that dopaminergic circuits are sensitive to gut microbiota changes (59) and the recent discovery that gut-innervating vagal sensory neurons are major components of the central reward circuitry and control striatal dopamine release (60).

***Conclusions and perspectives***

Here, we propose a model for induction of ASD core symptoms in mice obtained by manipulation of the gut microbiota through *p*-Cresol exposure. We demonstrate the face, construct and predictive validity of this environmental ASD model. Future work will further ascertain its predictive validity using pharmacological interventions. Also, future functional metagenomics studies would enable to identify the specific bacterial species whose changes in abundance determine ASD-related social behavior deficits. 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 the host's behavior.

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**DISCLOSURES**

None to declare.

## REFERENCES


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

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

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

4.Prosperi M, Santocchi E, Balboni G, Narzisi A, Bozza M, Fulceri F, et al. (2017): Behavioral Phenotype of ASD Preschoolers with Gastrointestinal Symptoms or Food Selectivity. *J Autism Dev Disord*. 47:3574-3588.

5.Fulceri F, Morelli M, Santocchi E, Cena H, Del Bianco T, Narzisi A, et al. (2016): Gastrointestinal symptoms and behavioral problems in preschoolers with Autism Spectrum Disorder. *Dig Liver Dis*. 48:248-254.

6.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.

7.Kang DW, Adams JB, Gregory AC, Borody T, Chittick L, Fasano A, et al. (2017): Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. *Microbiome*. 5:10.

8.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.

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.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.

16.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.

17.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.

18.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.

19.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.

20.MacFabe DF (2015): Enteric short-chain fatty acids: microbial messengers of metabolism, mitochondria, and mind: implications in autism spectrum disorders. *Microb Ecol Health Dis*. 26:28177.

21.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.

22.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.

23.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.

24.Parracho HM, Bingham MO, Gibson GR, McCartney AL (2005): Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. *J Med Microbiol*. 54:987-991.

25.Luna RA, Oezguen N, Balderas M, Venkatachalam A, Runge JK, Versalovic J, et al. (2017): Distinct Microbiome-Neuroimmune Signatures Correlate With Functional Abdominal Pain in Children With Autism Spectrum Disorder. *Cell Mol Gastroenterol Hepatol*. 3:218-230.

26.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.

27.Tomova A, Husarova V, Lakatosova S, Bakos J, Vlkova B, Babinska K, et al. (2015): Gastrointestinal microbiota in children with autism in Slovakia. *Physiol Behav*. 138:179-187.

28.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.

29.Gabriele S, Sacco R, Altieri L, Neri C, Urbani A, Bravaccio C, et al. (2016): Slow intestinal transit contributes to elevate urinary p-cresol level in Italian autistic children. *Autism Res*. 9:752-759.

30.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.

31.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.

32.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.

33.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.

34.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.

35.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.

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

37.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.

38.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.

39.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.

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

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

42.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.

43.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.

44.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.

45.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.

46.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.

47.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.

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

49.MacFabe DF, Cain NE, Boon F, Ossenkopp KP, Cain DP (2011): Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: Relevance to autism spectrum disorder. *Behav Brain Res*. 217:47-54.

50.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.

51.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.

52.Persico AM, Napolioni V (2013): Urinary p-cresol in autism spectrum disorder. *Neurotoxicol Teratol*. 36:82-90.

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

54.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.

55.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.

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

57.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.

58.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.

59.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.

60.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.

## FIGURES & TABLES

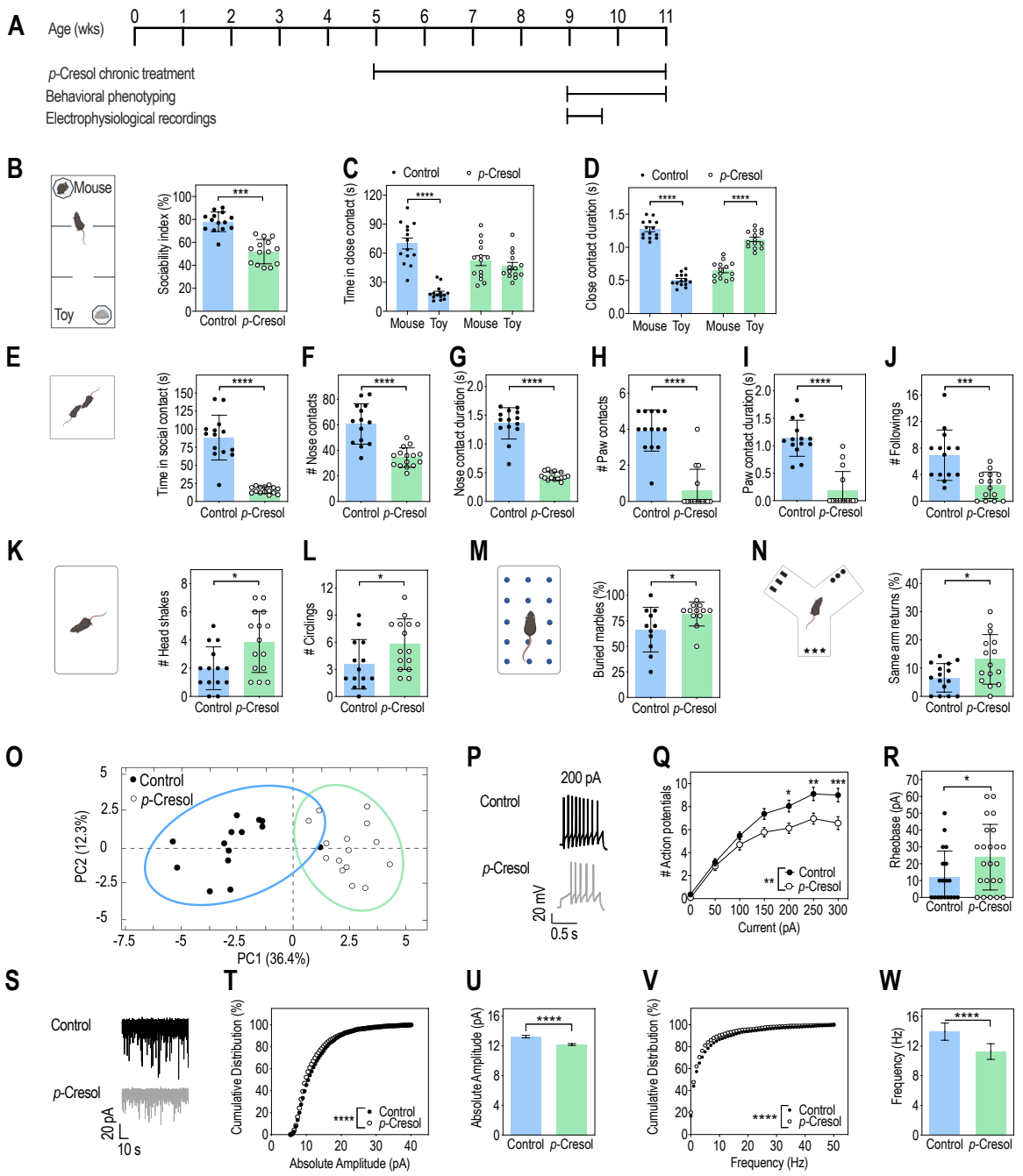


**Figure 1. *p*-Cresol chronic treatment selectively promotes autistic-like behaviors and alters VTA dopamine neurons excitability**

(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 Preference)=p<0.0001, Šidák's post hoc tests: ***p<0.001.

(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 Preference)=p<0.0001, Šidák's post hoc tests: ****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: % 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 (E-L and Supplementary Figure 1C, D); n = 14 Control, n=15 *p*-Cresol; ellipses of the 95% confidence intervals are indicated for each group; PC, principal component.

(P-W) Electrophysiological recordings of dopaminergic neurotransmission in the VTA:

(P) Representative traces of dopaminergic 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, 20 cells recorded for *p*-Cresol); 2-way ANOVA: p(Treatment)=0.0066, p(Current)<0.0001, p(Treatment x Current)=0.0006, followed by Šidák's post hoc test, *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=20 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, 2662 events for Control, 2593 events for *p*-Cresol); 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, 2662 events for Control, 2593 events for *p*-Cresol;

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.

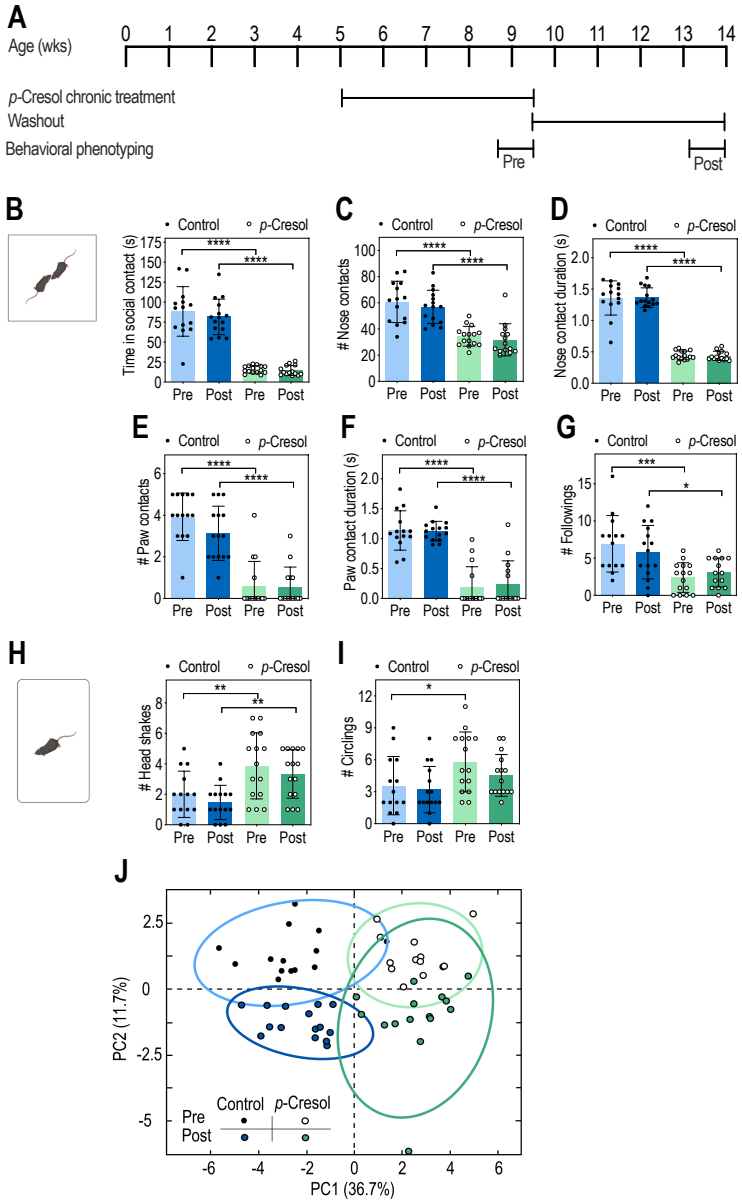


**Figure 2. The effects of *p*-Cresol chronic treatment persist after a 4-week washout period**

(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: ****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: ****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: ****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: ****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: ****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: ***p<0.001, *p<0.05.

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

(H) Number of headshakes; 2-way ANOVA: p(Treatment) <0.0001, p(Washout) = 0.2185, p(Treatment x Washout) >0.9999; Šidák's post hoc tests: **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: *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 (n=14 Control; n=15 *p*-Cresol). Ellipses of the 95% confidence intervals are indicated for each group.

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

**Table 1. The abundance of specific discriminating OTU identified by LefSE analysis 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* | *# Headshakes* | *# 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 | | | |

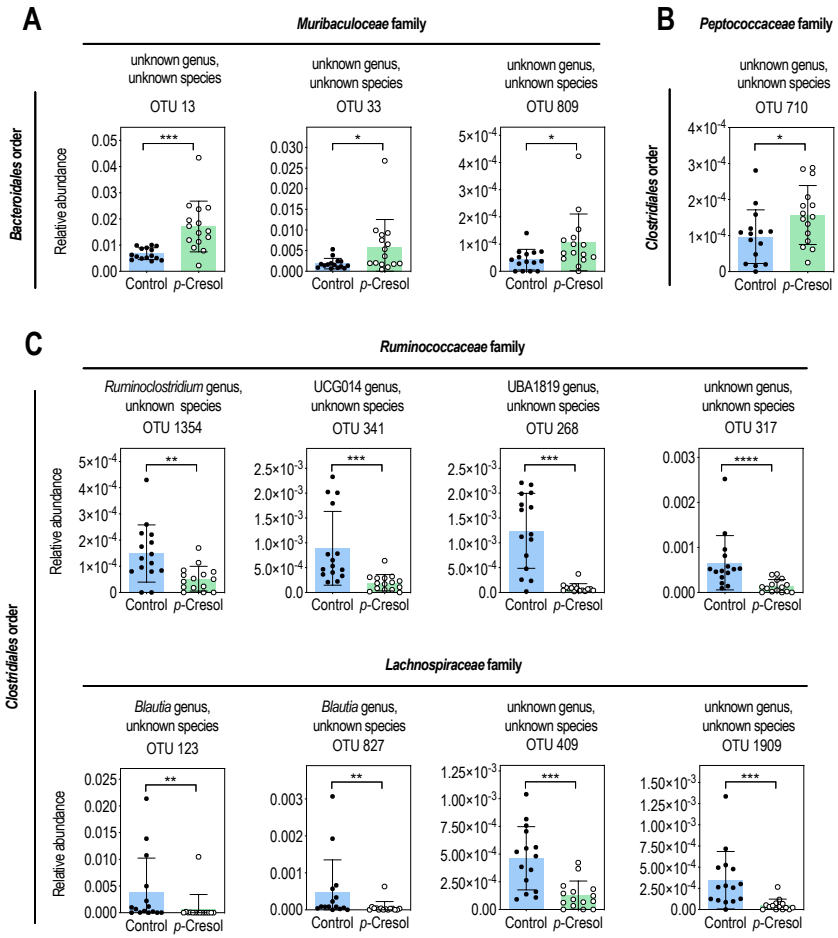


**Figure 3. Differential abundances of selected microbial OTU correlating with behavioral scores.**

(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.

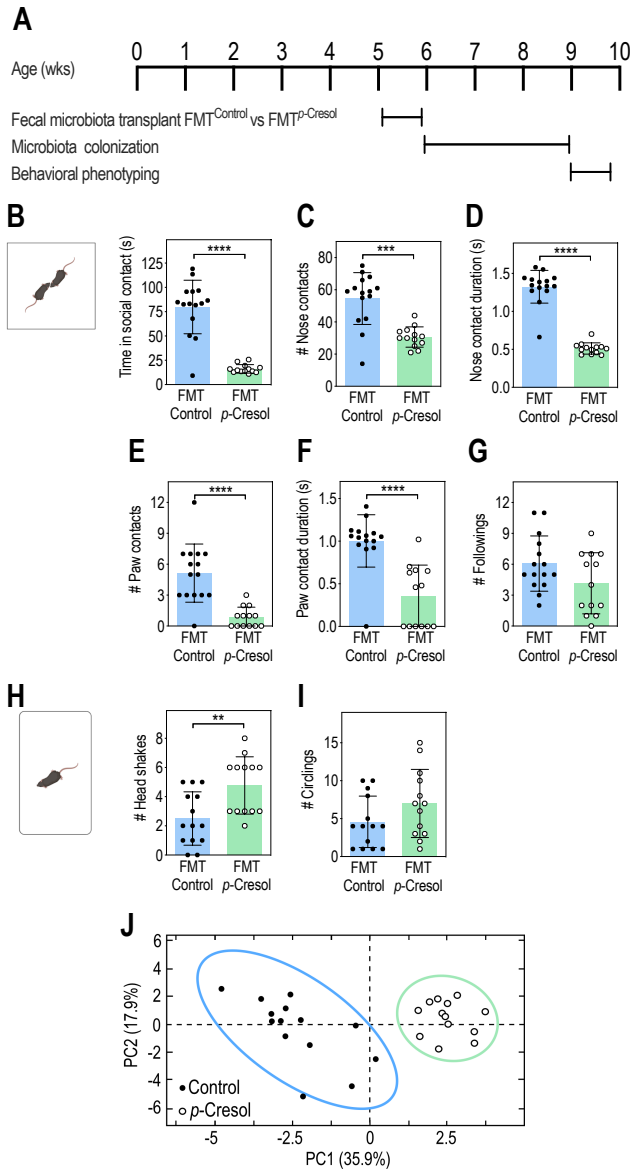


**Figure 4. Microbiome remodeling following *p*-Cresol exposure induces behavioral deficits.**

(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

(C) Number of nose contacts

(D) Mean duration of nose contact

(E) Number of paw contacts

(F) Mean duration of each paw contact

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

(H) Number of headshakes

(I) Number of circling events

(B-I) Mann-Whitney U-test: **p<0.01, ***p<0.001, ****p<0.0001:

(J) PCA plots of behavioral scores recorded in the dyadic social interaction and direct monitoring of motor stereotypies. 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.

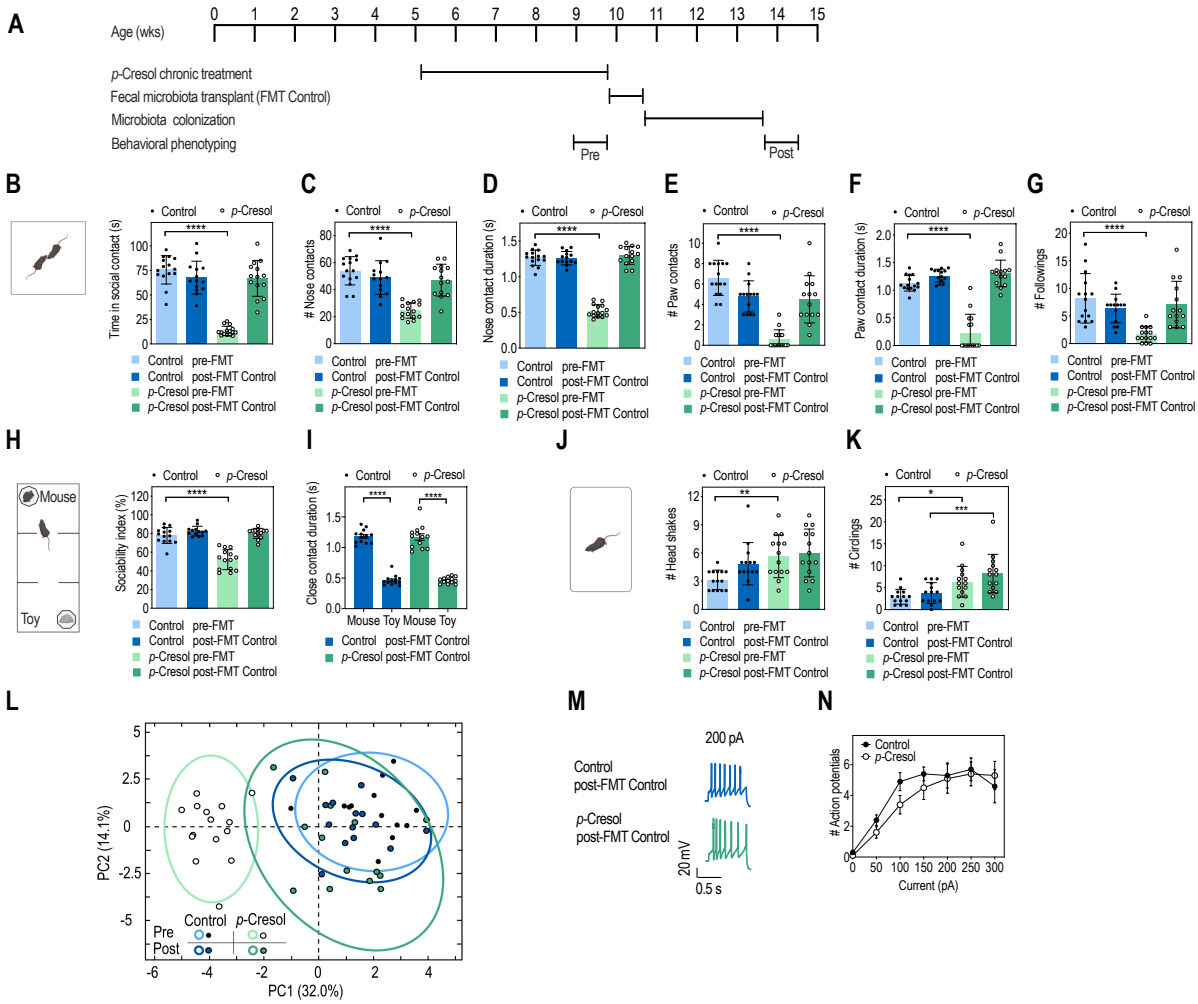


**Figure 5. Transfer of microbiota from control mice to *p*-Cresol-treated mice restores social interaction deficits and VTA dopamine neurons excitability**

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

(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: ****p<0.0001 pre-FMT Control, p>0.05 post-FMT Control

(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: ****p<0.0001 pre-FMT Control, p>0.05 post-FMT Control

(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: ****p<0.0001 pre-FMT Control, p>0.05 post-FMT Control

(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: ****p<0.0001 pre-FMT Control, p>0.05 post-FMT Control

(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: ****p<0.0001 pre-FMT Control, p>0.05 post-FMT Control

(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: ****p<0.0001 pre-FMT Control, p>0.05 post-FMT Control

(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: ****p<0.0001 pre-FMT Control, p>0.05 post-FMT Control.

(I) Mean duration of each nose contact with the mouse interactor or toy mouse; 2-way ANOVA: p(FMT Control) =0.9067, p(Preference) <0.0001, p(FMT Control x Preference) =0.8713; Šidák's post-hoc tests: ****p<0.01.

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

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

(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 pre-FMT Control, ***p<0.001 post-FMT Control.

(L) PCA plots of behavioral scores recorded in the dyadic social interaction and direct monitoring of motor stereotypies. 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 dopaminergic neurotransmission in the VTA post-FMT Control:

(M) Representative traces of dopaminergic 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: p>0.05.

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

(N) Data are presented as means ± SD.