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The microbiota shapes the development of the mouse hypothalamic paraventricular nucleus☆

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Abstract

Microbes massively colonize the mammalian newborn at birth. We previously reported that the microbiota influences key neurodevelopmental events, e.g., when compared to their conventionally colonized (CC) counterparts, sterile newborn mice ("germ-free" or GF) show higher cell death in the hypothalamic paraventricular nucleus (PVN). Here, we tested the hypothesis that the microbiota, perhaps via cell death mechanisms, shapes PVN development. To this aim, we used a cross-fostering approach that also allowed us to test whether any potential effects are influenced by microbial colonization at birth or programmed prenatally via the maternal microbiota. Specifically, we cross-fostered GF pups to CC dams (GF → CC) immediately after birth and compared them to control groups cross-fostered within microbial status (CC → CC, GF → GF). At postnatal day 7, GF → GF and GF → CC newborns had fewer PVN cells than did CC → CC newborns, without affecting PVN volume. In a follow-up experiment, we confirmed a reduction in PVN cell number with no change in PVN volume in adult GF mice. Thus, the greater cell death previously observed in the PVN of newborn GF mice is associated with a permanent reduction in cell number. Because the deficit is not altered by introducing a microbiota at birth, our findings also suggest that the maternal microbiota shapes development of the PVN starting in utero.

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CRediT authorship contribution statement

Yvonne C. Milligan: Methodology, Formal analysis, Conceptualization. **Nicole V. Peters:** Methodology, Conceptualization. **Gabby West:** Methodology, Formal analysis. **Laura R. Cortes:** Methodology, Formal analysis. **Benoit Chassaing:** Conceptualization, Funding acquisition, Methodology. **Geert J. de Vries:** Funding acquisition, Conceptualization. **Alexandra Castillo-Ruiz:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

Nothing to declare.

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Keywords

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1. Introduction

The collection of microorganisms living in/on our bodies, the microbiota, influences diverse body systems in health and disease (Hou et al., 2022). In the last two decades, a vast number of studies has highlighted that the microbiota also influences the mammalian brain (Lynch et al., 2023). Moreover, strategies that eliminate or deplete the microbiota, including sterile (germ-free (GF)) models and antibiotic treatment, demonstrate that the microbiota affects behavioral modalities that are integral for well-being: sociality, stress responsivity, and anxiety (Desbonnet et al., 2014; Diaz Heijtz et al., 2011; Sudo et al., 2004). Notably, the hypothalamic paraventricular nucleus (PVN) plays a role in these behaviors via the various neurohormones that it produces, including vasopressin, oxytocin, and corticotropin releasing hormone (Rasiah et al., 2023; Rigney et al., 2022). Remarkably, microbial manipulations alter the production of these neurohormones in the adult mouse PVN (Buffington et al., 2016; Tofani et al., 2025; Wu et al., 2021). Thus, the PVN is sensitive to microbial signaling. It is unclear, however, whether the microbiota acts early in life to guide the formation of the PVN.

The first direct exposure to microbes occurs at birth, when maternal and environmental microbes colonize the newborn. This colonization occurs at a time when the brain is undergoing remodeling via key neurodevelopmental processes, including neuronal cell death. We previously reported that the microbiota may modulate this process, as neonatal GF mice have increased neuronal cell death in the PVN compared to their conventionally colonized (CC) counterparts (Castillo-Ruiz et al., 2018). Here we first tested whether this affects PVN structure by assessing total cell number and volume in the PVN of GF and CC mice at one week after birth (i.e., the end of the cell death period). Second, we used a cross-fostering approach to test whether effects are influenced by microbial colonization at birth or are programmed prenatally via the maternal microbiota. Lastly, we tested whether early microbial effects endure by examining PVN structure in adult GF and CC mice.

2. Methods

2.1. Animals

GF and CC Swiss Webster mice were obtained from our breeding program at Georgia State University. All GF mice were kept under sterile conditions. For experiments in neonates, mice were kept as described in (Castillo-Ruiz et al., 2023). For experiments in adults, GF mice were kept in a Park Bioservices isolator, and CC mice in ventilated transparent Optimouse cages (Animal Care Systems, Centennial, CO, USA). Offspring were weaned on postnatal day (P) 21 and housed with same sex littermates. Mice were maintained in a 12:12 light dark cycle with ad libitum access to food and water. All procedures were approved by

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the Institutional Animal Care and Use Committee at Georgia State University and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Cross-fostering experiment

We leveraged access to brain tissue from our previous cross-fostering study to test for pre- vs. post-natal effects of the microbiota (see Castillo-Ruiz et al., 2023 for further details). Briefly, immediately upon observing the birth of a litter, cages were thoroughly sprayed with a sterilizing solution and placed within a sterile biosafety cabinet. Offspring were transferred to a sterile container before being assigned to a foster dam. We cross-fostered GF pups to CC dams (GF → CC group), and, to control for the cross-fostering procedure, CC and GF pups to dams within the same microbiota status (CC → CC and GF → GF groups). In two additional cases, foster mothers were unavailable for control litters and these pups were sham cross-fostered (i.e., returned to the birth mother after undergoing all cross-fostering procedures). These mice did not differ from pups fostered to an unrelated mother for any dependent variable tested (determined by *t*-tests within microbial status), and are therefore included in the analyses below and identified on Fig. 1. The total number of experimental animals and litters (cross-fostered and biological) represented in each group was: CC → CC group: $n = 16$ (14 females, 2 males), 5 litters (from 5 birth mothers); GF → GF group: $n = 5$ (3 females, 2 males), 2 litters (from 2 birth mothers); GF → CC group: $n = 7$ (5 females, 2 males), 3 litters (from 3 birth mothers). All offspring were euthanized on P7 via rapid decapitation.

2.3. Adult experiment

We collected adult CC ($n = 14$ (7 females, 7 males)) and GF (n = 16 (7 females, 9 males)) mice at 8 weeks of age. Mice were exposed to 5 % (*v*/v) isoflurane and, upon anesthesia onset, cervically dislocated, and then rapidly decapitated.

2.4. Brain collection

Brains were removed, preserved (as described in Castillo-Ruiz et al., 2023), and frozen-sectioned coronally into four series at 40 μm for neonates or three series at 30 μm for adults. One series was used for thionin staining.

2.5. Stereological analysis and forebrain size

All analyses were performed by investigators blinded to experimental condition. Analyses of total cell number, volume, and cell density in the PVN were performed using Stereo Investigator (MBF Biosciences, Williston, VT, USA). Because neonates had fewer PVN representative sections than adults, the PVN was traced in both hemispheres for neonates and in one hemisphere for adults. Counts of cells with a neuronal morphology were made using the optical fractionator function. The counting frame for both ages was 16 μm × 16 μm and the sampling grid was 65 μm × 70 μm for neonates and 45 μm × 65 μm for adults. The coefficient of error (Gundersen, m = 1) was ≤0.11. For adults, volume and neuronal counts were multiplied by two to estimate bilateral PVN volume and total cell number, respectively. Cell density was calculated by dividing total cell number by volume for each animal.

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We also outlined the left side of the forebrain of adult mice on every fourth section of the thionin-stained tissue, following criteria described previously (Castillo-Ruiz et al., 2018, 2023). The sum of areas across all sections was multiplied by two and then by section thickness to obtain overall forebrain volume in mm^3 for each animal. Four CC and six GF mice did not have enough sections for the assessments and therefore were not included in the analysis below.

2.6. Statistics

For all analyses of neonates, we pooled the sexes as the low number of males (n = 2/group) prevented us from testing sex as a factor. For analyses of adults, we also pooled sexes as the effect of sex was not significant, with the exception of forebrain size. For neonates, we used one-way ANOVA to test for cross-fostering effects on cell number, volume, and cell density. For adults, independent samples t-tests (two-tailed) were used to evaluate microbiota status effects on cell number, volume, and cell density. For forebrain size in adults, two-way ANOVA was used to test effects of microbiota status and sex. Post-hoc comparisons were performed following significant main effects using Fisher's LSD. Partial eta squared (η^2) and Cohen's d (d_s) were used to calculate effect sizes for overall ANOVAs and pair-wise comparisons, respectively.

3. Results

3.1. The microbiota shapes the development of the neonatal PVN prenatally

We found a significant effect of group on cell number ($F_{2,25}$ = 11.24, p = 0.0003, η^2 = 0.47), with the GF → GF group having fewer PVN cells than the CC → CC group (p = 0.006, d_s = 1.44) (Fig. 1A). Importantly, introduction of a microbiota at birth was not sufficient to change this phenotype as the GF → CC group did not differ from the GF → GF group (p = 0.46, d_s = 0.44) but differed from the CC → CC group (p = 0.0002, d_s = 2.09). This occurred despite a normalized microbiota in the GF → CC group (demonstrated in Castillo-Ruiz et al., 2023). Overall, the mice gestated GF had a 15 % reduction in cell number in comparison to the group gestated CC. Because changes in cell number could result in changes to PVN volume and/or cell density, we next assessed these measures. PVN volume was not affected by microbial status ($F_{2,25}$ = 0.24, p = 0.79, η^2 = 0.02) (Fig. 1B). Instead, cell density within the PVN was altered ($F_{2,25}$ = 4.36, p = 0.02, η^2 = 0.26), with the mice gestated GF having lower cell density than the mice gestated CC. This was reflected in a significant difference between the CC → CC and GF → CC groups (p = 0.01, d_s = 1.16) and a trend between the CC → CC and GF → GF groups (p = 0.07, d_s = 0.93). The GF → GF and GF → CC groups were no different from each other (p = 0.71, d_s = 0.29) (Fig. 1C).

3.2. The microbiota shapes the PVN long-term

To test whether microbiota effects on cell number are long-term, we assessed the same parameters as above in the PVN of adult CC and GF mice. Similar to what we observed in neonates, adult GF mice had 17 % fewer cells in the PVN than CC mice (t_{28} = 2.31, p = 0.03, d_s = 0.84) (Fig. 2A), with no changes in PVN volume (t_{28} = 0.12, p = 0.91, d_s = 0.04) (Fig. 2B) but significantly reduced cell density (t_{28} = 4.20, p = 0.0002, d_s = 1.54) (Fig. 2C).

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In addition to effects on the perinatal PVN, we previously reported that P7 GF mice have 6 % larger forebrains than CC mice (Castillo-Ruiz et al., 2023), so here we also examined whether this effect lasts into adulthood. As before, we found significant effects of microbiota status ($F_{1,16} = 6.30$, $p = 0.02$, $\eta^2 = 0.28$), with adult GF mice having a 6 % larger forebrain (Fig. 3). We also found an effect of sex ($F_{1,16} = 8.67$, $p = 0.0095$, $\eta^2 = 0.35$), with females having a 6 % larger forebrain, but no microbiota status-by-sex interaction ($F_{1,16} = 0.79$, $p = 0.39$, $\eta^2 = 0.05$).

4. Discussion

Here we demonstrate that mice gestated GF, irrespective of acquiring a microbiota at birth, have lower cell numbers in the PVN neonatally and in adulthood. This suggest that effects of the microbiota begin in utero, which may occur via effects on maternal physiology (e.g., production of cytokines, hormones) and/or on structures that support pregnancy; all of these in turn can affect fetal brain development. For example, the microbiota promotes growth and vascularization of the placental labyrinth (Pronovost et al., 2023), which is a key site for exchange of nutrients and other chemicals between mother and fetus. Alternatively, effects on fetal neurodevelopment may occur directly via maternal microbiota metabolites that can cross the placenta, and to date, at least two studies have identified some candidate metabolites. Vuong et al. (2020) determined that trimethylamine-N-oxide and imidazole propionate are produced by the maternal microbiota and translocate to the fetal mouse brain to promote axonogenesis (Vuong et al., 2020). Similarly, Kimura et al. (2020) reported that short-chain fatty acids produced by the maternal microbiota influence the development of the sympathetic nervous system (Kimura et al., 2020). The effects of maternal microbial metabolites on the development of the PVN merits further investigation.

The higher neuronal cell death previously observed in the PVN of perinatal GF mice (Castillo-Ruiz et al., 2018) is potentially the mechanism underlying the lower cell counts in the PVN reported here. Interestingly, we previously reported higher microglial number in the PVN of GF neonates (Castillo-Ruiz et al., 2018, 2023). Divergent microbiota effects on numbers of neurons vs. microglia therefore could explain why PVN volume was no different between GF and CC mice. For the future, it will be important to determine whether specific neuronal phenotypes are affected. Candidate populations are vasopressin and oxytocin neurons as they are prominent residents of the PVN and are sensitive to microbial manipulations in adult mice (Buffington et al., 2016; Tofani et al., 2025).

In our study, we were not able to determine whether the introduction of a microbiota at birth normalizes PVN cell numbers beyond P7. However, since neurogenesis in the PVN is completed prenatally (Shimada and Nakamura, 1973) and cell death in the PVN is over by P7 (Mosley et al., 2017), cell numbers are unlikely to change by microbial conventionalization at birth.

Our finding that forebrains were larger in adult GF mice suggest that the microbiota may shape additional brain regions long-term in a region-dependent manner. Indeed, we previously reported that in contrast to the PVN, microbiota absence reduced cell death in the hypothalamic arcuate nucleus (Castillo-Ruiz et al., 2018, 2023). We also found that sex

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affected forebrain size, irrespective of microbial status. This is unsurprising as our sampling included regions reported to be larger in females (Spring et al., 2007; Guma et al., 2024).

In sum, our findings demonstrate that the microbiota shapes PVN development. Moreover, our findings may help to explain the social, stress, and anxiety-like behavioral deficits reported in GF adult mice (Luczynski et al., 2016), as the PVN plays a central role on those behaviors (Rasiah et al., 2023; Rigney et al., 2022).

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Data availability

Data will be shared upon reasonable request addressed to the corresponding author.

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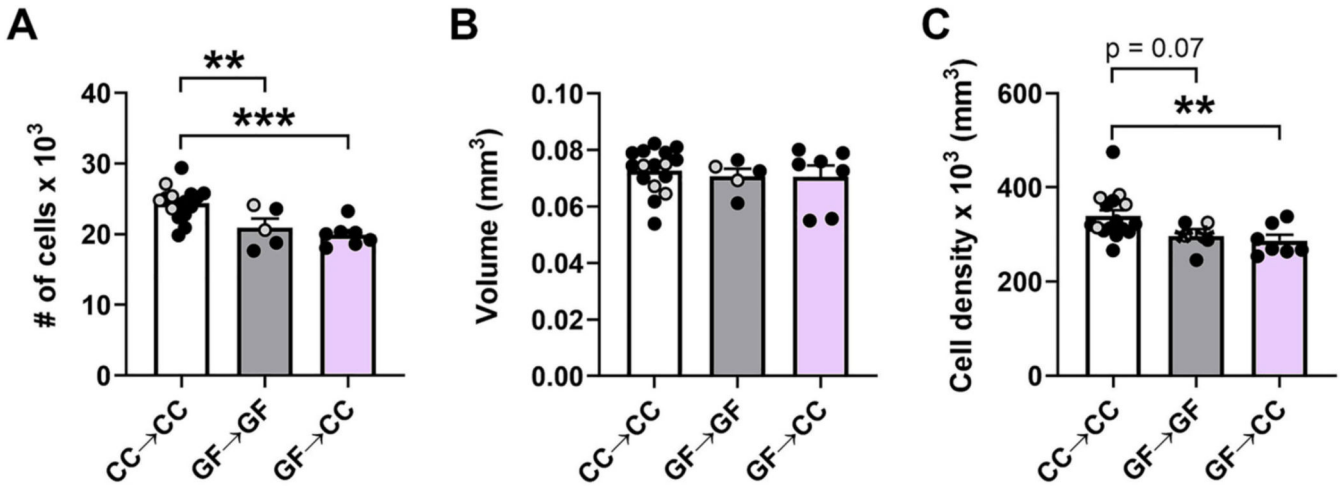

Fig. 1.

The microbiota programs PVN cell number prenatally. (A) Stereological counts of cells with a neuronal morphology indicate that cell number was lower in neonatal mice gestated GF, regardless of introduction to a microbiota at birth in the GF → CC group (**p = 0.006; ***p = 0.0002). (B) This effect did not result in changes to PVN volume, (C) but in decreased cell density (**p = 0.01). Statistics: in all cases one-way ANOVA, followed by Fisher's LSD post hoc tests. Mean + SEM and individual samples are depicted, with gray symbols representing sham cross-fostered mice in control groups. CC → CC group: n = 16; GF → GF group: n = 5; GF → CC group: n = 7.

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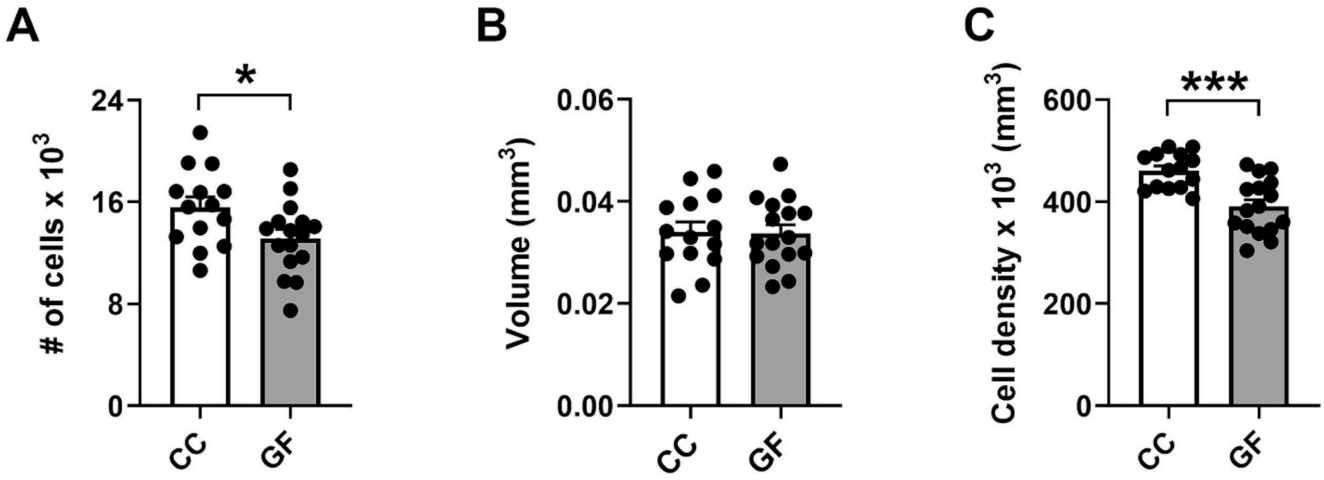

Fig. 2.

The microbiota shapes the PVN long-term. (A) Stereological counts of cells with a neuronal morphology indicate that cell number was lower in adult GF mice (**p* = 0.03). (B) This effect did not result in changes to PVN volume, (C) but in reduced cell density (***p = 0.0002). Statistics: in all cases independent samples two-tailed *t*-tests. Mean + SEM and individual samples are depicted. CC group: *n* = 14; GF group: *n* = 16.

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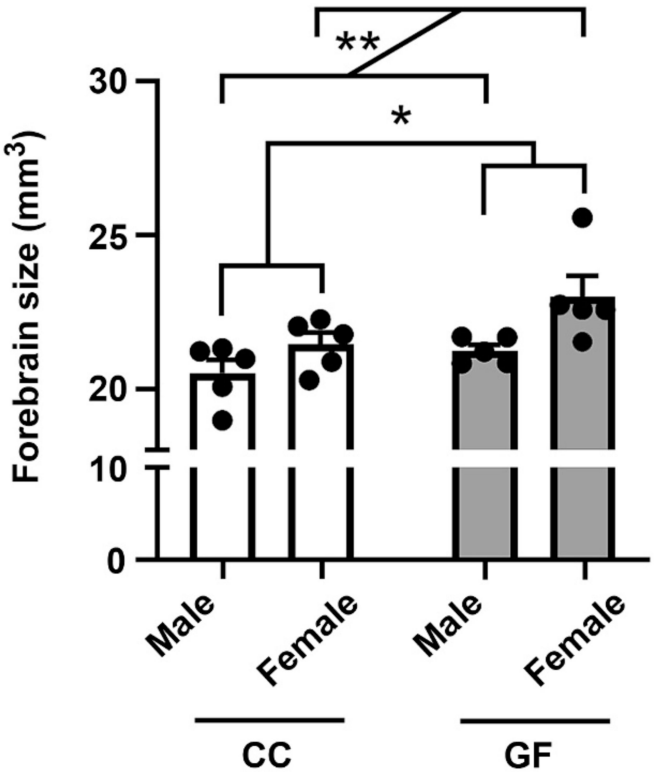

Fig. 3.

The microbiota and sex influence overall forebrain size in adult mice. Statistics: two-way ANOVA. Lower bracket indicates main effect of microbiota status (*$p = 0.02$) with larger forebrain size in GF animals; upper bracket indicates main effect of sex (**$p = 0.0095$), with larger forebrain size in females. There was no interaction between microbiota status and sex. Mean + SEM and individual samples are depicted. CC group: n = 5 females, 5 males; GF group: n = 5 females, 5 males.