

# Normal gut microbiota modulates brain development and behavior

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**Microbial colonization of mammals is an evolution-driven process that modulate host physiology, many of which are associated with immunity and nutrient intake. Here, we report that colonization by gut microbiota impacts mammalian brain development and subsequent adult behavior. Using measures of motor activity and anxiety-like behavior, we demonstrate that germ free (GF) mice display increased motor activity and reduced anxiety, compared with specific pathogen free (SPF) mice with a normal gut microbiota. This behavioral phenotype is associated with altered expression of genes known to be involved in second messenger pathways and synaptic long-term potentiation in brain regions implicated in motor control and anxiety-like behavior. GF mice exposed to gut microbiota early in life display similar characteristics as SPF mice, including reduced expression of PSD-95 and synaptophysin in the striatum. Hence, our results suggest that the microbial colonization process initiates signaling mechanisms that affect neuronal circuits involved in motor control and anxiety behavior.**

developmental programming | microbiome | basal ganglia | cognitive behavior | synapse

Early life environmental influences have a profound impact on the organism's later development, structure, and function. This phenomenon is called "developmental programming," a process whereby an environmental factor acting during a sensitive or vulnerable developmental period exerts effects that impact on structure and function of organs that, in some cases, will persist throughout life (1). One such environmental factor is the gut microbiota that, because of an evolutionary process, has adapted to coexist in commensal or symbiotic relationship with mammals (2). Immediately after birth, the newborn organism is rapidly and densely populated with complex forms of indigenous microbes. This process has been shown to contribute to developmental programming of epithelial barrier function, gut homeostasis, and angiogenesis, as well as the innate and host adaptive immune function (3, 4). Recent data indicate that gut microbiota have systemic effects on liver function (5–7), thus raising the possibility that gut microbiota can have developmental effects in other organs elsewhere in the body.

The functional development of the mammalian brain is of particular interest because it has been shown to be susceptible to both internal and external environmental cues during perinatal life. Epidemiological studies have indicated an association between common neurodevelopmental disorders, such as autism and schizophrenia, and microbial pathogen infections during the perinatal period (8, 9). These findings are supported by experimental studies in rodents, demonstrating that exposure to microbial pathogens during similar developmental periods result in behavioral abnormalities, including anxiety-like behavior and impaired cognitive function (10–12). In a recent study, it was shown that the commensal bacteria, *Bifidobacteria infantis*, could modulate tryptophan metabolism, suggesting that the normal gut microbiota can influence the precursor pool for serotonin (5-HT) (13).

Here, we tested the hypothesis that the "normal" gut microbiota is an integral part of the external environmental signals that modulate brain development and function.

## Results

**Germ Free (GF) Mice Display Increased Motor Activity and Reduced Anxiety-Like Behavior.** In the first set of experiments, we subjected adult GF and specific pathogen free (SPF) mice with a normal gut microbiota to a battery of tests for exploratory activity and anxiety. GF and SPF mice were placed in a novel, open-field activity box. Their spontaneous motor activity, including locomotor and rearing activities, were measured for 60 min. GF mice showed greater total distance traveled and more exploration of the center of the open field ( $P < 0.05$ ; Fig. 1A). There was also a trend for GF to display higher levels of rearing activity compared with SPF (GF vs. SPF,  $489 \pm 43$  vs.  $369 \pm 50$ ,  $P = 0.088$ ). Both GF and SPF mice displayed similar locomotor activity (Fig. 1B) during the initial open field exposure, indicating that the increased locomotor activity in GF mice was not triggered by novelty. Instead, significant differences between groups were detected in habituation over time (repeated measures ANOVA, main effect  $F_{(1, 70)} = 6.28$ ,  $P < 0.05$ ). Thus, GF mice traveled a significantly longer distance (Fig. 1B and C) and spent significantly ( $P < 0.05$ ) more time in both slow and fast locomotion (Fig. 1D) during the 20- to 60-min interval of testing.

Given that certain microbial pathogens have been reported to induce anxiety-like behavior in animal models (10–12), we assessed whether the nonpathogenic gut microbiota could also affect anxiety-like behavior. For this purpose, we used two rodent tests of anxiety: the light–dark box test and the elevated plus maze (14). In the light–dark box test, GF mice spent significantly ( $P < 0.05$ ) more time in the light compartment of the box than control SPF mice (Fig. 2A). In the elevated plus maze test, GF mice spent significantly ( $P < 0.05$ ) more time in the open arm than SPF mice (Fig. 2B and Movies S1 and S2). The GF mice also engaged in riskier behavior than SPF as indicated by the great number of visits (GF vs. SPF:  $3.9 \pm 0.6$  vs.  $1.4 \pm 0.69$ ,  $P < 0.05$ ) to the ends of the open arms. There were no significant differences in the number of entries (GF vs. SPF:  $13.14 \pm 0.9$  vs.  $14.4 \pm 1.5$ ,  $P > 0.1$ ) and time spent (Fig. 2B) in the closed arm between GF and SPF mice.

To test whether conventionalization in early life of GF mice could "normalize" the increased motor activity and alter the anxiety behavior, we conventionalized a new set of GF mice with microbiota obtained from SPF mice 30 d before mating and allowed the progeny to mature in an isolator with bacteria. Adult conventionalized offspring (CON) were behaviorally tested as described above. The rearing activity of the CON mice was sig-

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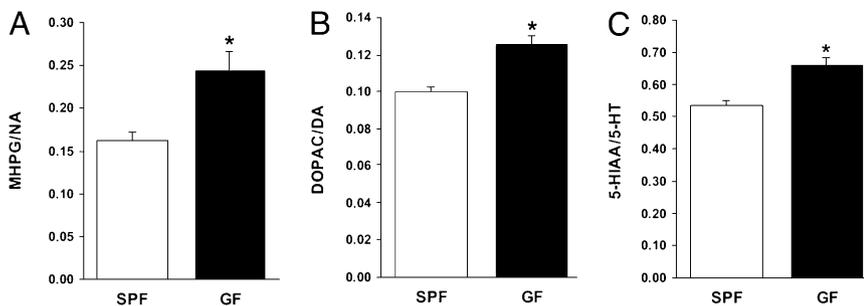
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**Fig. 3.** GF mice show elevated NA, DA, and 5-HT turnover in the striatum. The histograms depict the mean ratios ( $\pm$  SEM;  $n = 6$  per group) for MHPG/NA (A), DOPAC/DA (B), and 5-HIAA/5-HT (C) in the striatum of male GF and SPF mice. Asterisks denote where GF mice differ significantly ( $P < 0.01$ ) from SPF mice.

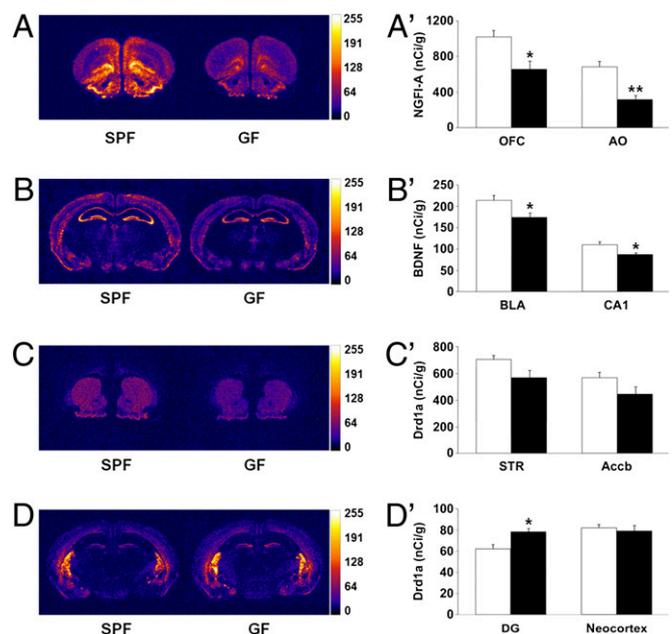
brain-derived neurotrophic factor (BDNF), in the development of anxiety-like behavior (15–17). We therefore studied the expression of these genes in the frontal cortex, striatum, amygdala, and hippocampus of GF and SPF mice, by means of in situ hybridization technique. In GF mice, NGFI-A mRNA expression was significantly lower in various subregions of the prefrontal cortex, including the orbital frontal cortex (Fig. 4 A and A'); as well as in the striatum (GF vs. SPF:  $329 \pm 33$  vs.  $586 \pm 18$ ,  $P < 0.0001$ ), hippocampus (CA1 region, GF vs. SPF:  $258 \pm 15$  vs.  $499 \pm 22$ ,  $P < 0.0001$ ; CA3 region, GF vs. SPF:  $166 \pm 13$  vs.  $236 \pm 6$ ,  $P < 0.001$ ; dentate gyrus, GF vs. SPF:  $76 \pm 4$  vs.  $113 \pm 5$ ,  $P < 0.0001$ ) and amygdala (GF vs. SPF:  $126 \pm 17$  vs.  $212 \pm 19$ ,  $P < 0.01$ ) compared with SPF mice. Similarly, GF mice had significantly lower BDNF mRNA expression in the hippocampus, amygdala (Fig. 4 B and B'), and cingulate cortex (GF vs. SPF:  $162 \pm 6$  vs.  $193 \pm 10$ ,  $P < 0.05$ ), which are key components of the neural circuitry underlying anxiety and fear (18).

Given that DA is an important regulator of motor and cognitive functions (19), we examined the expression of dopamine receptors (D1 and D2 receptors) and intracellular signaling mechanisms (i.e., DARPP-32) in the above regions. In GF mice, DA D1 receptor mRNA was significantly ( $P < 0.05$ ) higher in the hippocampus (Fig. 4 D and D'), while lower in the striatum and nucleus accumbens, albeit without significance (Fig. 4 C and C') compared with SPF mice. There were no significant differences in the expression of DARPP-32 or DA D2 receptors between GF and SPF mice.

**GF Mice Show Alterations in Genes Involved in Four Canonical Pathways.** Having established that the normal gut flora could modify behavior and gene expression in key brain regions, we applied the gene expression profiling technique to assess whether other genes were subject to modulation by gut microbiota. The gene expression patterns of five brain regions of 11 mice (5 GF mice and 6 SPF mice) were profiled. Genes differentially expressed between GF and SPF mice (with significant fold changes  $>2$ ) were used to generate heat maps by using Cluster and Tree View software. The gene expression profile was very consistent across independent tissue samples of the same brain region (see patterns of green or red color). In the hippocampus, we found 50 genes to be significantly differentially expressed (45 genes were higher and 5 were lower in GF mice compared with SPF mice; Fig. 5A). Moreover, 20 and 23 genes were significantly, differentially expressed in the cortex and striatum, respectively (Fig. 5B and C). In addition, 84 genes were differentially expressed in the cerebellum and only 1 gene in hypothalamus. For quantitative real-time PCR (Q-PCR) confirmation of microarray data, we selected 44 genes of the modulated genes described above and 6 candidate genes implicated in the behavioral phenotype of GF mice. We confirmed 38 of these 50 genes to be differentially expressed (35 in hippocampus, 11 in cortex, and 4 in striatum), with three genes common to all regions (Indo, Tlr1, and Gna1) (Table S2–S4). These gut microbiota-regulated genes were characterized into four statistically significant canonical pathways (Fig. 5D).

**Gut Microbiota Colonization of GF Mice Reduces Protein Expression of Synaptophysin and PSD-95 in Striatum.** In an attempt to identify

whether specific proteins connected to synaptogenesis would be subject to gut microbiota regulation and, thereby, provide a possible mechanism for the observed alteration of brain function, we investigated the protein expression of PSD-95 and synaptophysin in the frontal cortex, striatum, and hippocampus of GF, SPF, and CON mice. As can be seen in Fig. 6 and Table 1, the expression of both synaptophysin and PSD-95 in the striatum was significantly lower ( $P < 0.05$ ) in SPF and CON mice compared with GF mice. However, there were no differences in the expression of synaptophysin or PSD-95 in frontal cortex and hippocampus.



**Fig. 4.** GF mice show altered expression of anxiety and synaptic plasticity-related genes. (A) Representative autoradiograms showing NGFI-A mRNA expression at the level of the frontal cortex of SPF and GF mice (OFC, orbital frontal cortex; AO, anterior olfactory region). (A') Bars show expression of NGFI-A mRNA (nCi/g) in the OFC and AO of SPF and GF mice. (B) Representative autoradiograms showing BDNF mRNA expression at the level of amygdala and dorsal hippocampus of SPF and GF mice (BLA, basolateral amygdala; CA1, CA1 region of the dorsal hippocampus). (B') Bars show expression of BDNF mRNA (nCi/g) in the BLA and CA1 region of SPF and GF mice. (C) Representative autoradiograms showing dopamine D1 receptor (Drd1a) mRNA expression at the level of the striatum and nucleus accumbens of SPF and GF mice (STR, striatum; Accb, nucleus accumbens, shell region). (C') Bars show expression of Drd1a mRNA (nCi/g) in the STR and Accb of SPF and GF mice. (D) Representative autoradiograms showing Drd1a mRNA expression at the level of the dorsal hippocampus of SPF and GF mice (DG, dentate gyrus; PtCx, parietal cortex, somatosensory area). (D') Bars show expression of Drd1a mRNA (nCi/g) in the DG and PtCx of SPF and GF mice. All data (A'–D') are expressed as means  $\pm$  SEM,  $n = 8$  per group. Filled bars represent GF mice. Open bars represent SPF mice. \* $P < 0.05$ , \*\* $P < 0.001$  compared with SPF mice.



**Table 1. Expression of synaptophysin and PSD-95 in various brain regions of GF, SPF, and CON mice**

Mice	Frontal cortex		Striatum		Hippocampus	
	Synaptophysin	PSD-95	Synaptophysin	PSD-95	Synaptophysin	PSD-95
GF	0.989 ± 0.058	1.039 ± 0.085	<b>1.803 ± 0.037*</b>	<b>2.171 ± 0.042*</b>	0.982 ± 0.046	1.025 ± 0.136
SPF	1.000 ± 0.062	1.000 ± 0.145	1.000 ± 0.015*	1.000 ± 0.036*	1.000 ± 0.051	1.000 ± 0.075
CON	0.992 ± 0.022	1.011 ± 0.090	1.013 ± 0.028*	1.001 ± 0.063*	0.973 ± 0.039	1.045 ± 0.076

Expression of synaptophysin and PSD-95 in the frontal cortex, striatum, and hippocampus of GF ( $n = 4$ ), SPF ( $n = 4$ ), and CON ( $n = 6$ ) mice were quantified and calculated against their respective actin expression (Fig. 6). The values were then compared against the SPF mice values and expressed as average fold increase. Values are expressed as means ± SEM.

\* $P < 0.05$  compared with SPF mice.

to affect later-life brain and behavior. Interestingly, nonhuman primate studies have demonstrated that administered glucocorticoids reduce synaptophysin expression in the fetal brain (25). Hence, modulation of stress hormones (e.g., ACTH and corticosterone) by the gut microbiota is another potential mechanism that could explain the present findings.

Another possible mechanism mediating the gut-brain communication, proposed here, may be via established neuronal circuits. Gut microbiota can elicit signals via the vagal nerve to the brain and vice versa (26, 27). Modulation of transmitters (e.g., serotonin, melatonin, gamma-aminobutyric acid, histamines, and acetylcholine) within the gut is yet another possible mechanism of action that could mediate the effects of the gut microbiota. For example, metabolic profiling of GF and conventionalized mice have revealed that conventionalization of GF mice by gut microbiota results in a 2.8-fold increase in plasma serotonin levels (28). This increase may arise from peripheral serotonin pools stored in the colon that, upon exposure to the incoming gut microbiota during early postnatal life, are released (29). Thus, this postnatal developmental change induced by the gut microbiota creates system conditions by which periphery pools of serotonin are carefully monitored and tightly regulated in early postnatal development. It is intriguing that the same neurotransmitter pathway is involved in the regulation of both food intake (30) bone remodeling (31) and behavioral brain functions (32). Recent data have demonstrated similar cross-talk between food intake, innate immunity, and G-coupled receptors expressed in the nervous system of *Caenorhabditis elegans* (33).

Although the mechanisms outlined above may help to explain how gut microbiota could modulate brain development and functions, they do not, however, explain the region-specific changes in brain observed in the present study, i.e., regulation of synaptic-associated proteins and neurotransmitter turnover specifically in the striatum. Within this context, it is worth mentioning that some bacteria (e.g., *Clostridium botulinum* and *Clostridium tetani*) are known to produce neurotoxins that specifically target a group of proteins that enable synaptic vesicles to dock and fuse with pre-synaptic plasma membranes. In the present study, we also found that the normal gut microbiota target two key synaptic proteins, namely synaptophysin and PSD-95 in the striatum. Recent data indicate that pattern recognition receptors expressed on the microglia surface characterize one of the primary, common pathways by which neurotoxin signals affect neuronal tissues (34). Interestingly, there seems to be a great diversity in microglia phenotype and function (35). Therefore, microglia cells could be involved in the signaling pathways induced by normal gut microbiota-derived metabolites.

Our results suggest that during evolution, the colonization of gut microbiota has become integrated into the programming of brain development, affecting motor control and anxiety-like behavior. It is tempting to speculate that the differences that we observe between GF and SPF mice are mediated by signaling initiated soon after birth at a time when the newborn mice become exposed to gut microbiota. This suggestion does not exclude the possibility that exposure to gut microbiota metabolites, generated by the flora of the pregnant mother, could also influence brain development during embryogenesis.

Gut microbiota may also be able to modify expression of risk genes (16, 36) or be part of mechanisms that alter cognitive functions observed in patients with gastrointestinal diseases (37, 38). Finally, the observed behavioral changes imposed by the presence of the gut flora in rodents, reported in this paper, may have wider implications when considering psychiatric disorders in humans.

### Experimental Procedures

**Animals.** Newborn litters of GF and SPF NMRI mice were placed and raised in special plastic isolators until they reached 8 to 10 wk of age (Core Facility for Germ Free Research, Karolinska Institutet). Only male animals were used in these experiments. Animals were maintained on autoclaved R36 Lactamin Chow (Lactamin) and kept in 12-h light cycles. All procedures were approved by the Local Research Ethics Committee.

**Behavioral Studies.** Testing took place between 0900 and 1500 hours under low illumination to reduce stress. On the day of testing, animals were brought in sterile filtered cages to an adjacent testing room and allowed to rest for 1 h before testing. Test chambers were cleaned first with disinfectant and then with 70% ethanol and water after each animal.

**Open Field Test.** Animals were placed individually in the center of an open field box (48 cm × 48 cm; Acti-Mot detection system; TSE), and their spontaneous motor activity was recorded as described (39). The computer program automatically recorded the following parameters: distance traveled in the center, periphery and total (entire box), a count of rearing activity (vertical infrared photo beam breaks), and time spent in slow (>5 cm/s, with an upper range of 20 cm/s) or fast (>20 cm/s) locomotion.

**Light-Dark Box Test.** In this test, the mouse was placed into the dark compartment and allowed to freely explore the apparatus (48 × 48 cm; with two zones of equal areas) for 5 min. Time spent in the dark and light compartments were measured by using photocells (TSE).

**Elevated Plus Maze Test.** The elevated plus maze, made of dark gray glacial polyvinyl chloride, consisted of four arms (each 30 × 5 cm) and a central area (5 × 5 cm) elevated 50 cm above the floor. Two arms were open and two were closed with 10-cm-high walls made of the same material. Mice were individually placed in the center facing an open arm and allowed to explore for 5 min. The behavior of the animal was recorded with a video camera and later scored by two independent observers that were blind to the identity of the animals. The following behaviors were scored: open and closed arm entries, time spent in the closed and open arms and in the center, and exploration of open arm ends.

**Behavioral Analysis.** The behavioral data from the open field test were analyzed by using either repeated measures analysis of variance (ANOVA; phenotype and time as main factors) or factorial ANOVA when appropriate. The behavioral data from the light-dark box and elevated-plus maze tests were analyzed by using one-way ANOVA. All post hoc comparisons were made with Bonferroni/Dunn test in the presence of significant ANOVA effects. The threshold for statistical significance was set as  $P \leq 0.05$ .

**Neurochemical Analysis.** Brain samples were analyzed at Pronexus Analytical AB (Karolinska Institutet Science Park, Stockholm). Briefly, tissue concentration of NA, MHPG, DA, DOPAC, HVA, 5-HT, and 5-HIAA were determined by reversed-phase high-performance liquid chromatography with electro-

chemical detection as described (40). Data were analyzed by using one-way ANOVA. Post hoc comparisons were made with Bonferroni/Dunn test.

**In Situ Hybridization.** Brains were rapidly dissected, frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Coronal sections ( $14\ \mu\text{m}$ ) of various brain regions (frontal cortex, striatum, hippocampus, and amygdala) were prepared on a cryostat and stored at  $-80^{\circ}\text{C}$  until used. Fixation, prehybridization, and hybridization were performed as described (ref. 39; see *SI Experimental Procedures* for information about synthesis of riboprobes and quantification procedure).

**Illumina Expression Array.** Brain tissues (frontal cortex, striatum, hypothalamus, hippocampus, and cerebellum) were rapidly dissected and stored at  $-80^{\circ}\text{C}$  until used. RNA was extracted by using Qiagen RNA extraction kit. The RNA ( $0.5\ \mu\text{g}$ ) was amplified and labeled by using Illumina total prep RNA amplification kit (Ambion). cRNA ( $0.75\ \mu\text{g}$ ) were applied on Illumina chips (Mouse chips Ref.V1.1) according to the manufacturer's instruction. After hybridization and washing, each array was scanned by Illumina's scanning software (BeadScan) to produce an image in the Tagged Image File Format, along with files in a proprietary file format containing intensity and location information. Original data were extracted and normalized by using BeadStudio software. After normalization, probe signals were checked for detection against negative controls with a BeadStudio internal algorithm and missing values were introduced to replace signals under the detection limit. Probes that failed to detect signal in at least three mice were filtered out.

Further data analysis was applied on Gene Spring, where probes with low detection values ( $<50$ ) or low confidence detection ( $P < 0.99$ ) were further filtered out. Genes differentially expressed more than twofold were selected and used for statistical analysis (one-way ANOVA) to select for significance ( $P < 0.05$ ). Lists of statistically, differentially expressed genes were then analyzed by using Ingenuity Pathway Analysis, Cluster, and Tree View software.

**Western Immunoblotting.** Brain tissues were dissected on ice frozen by liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Tissue samples were homogenized in ice-cold RIPA lysis buffer [50 mM Tris at pH 7.4, 150 mM NaCl, 1% (wt/vol) Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, 1 mM EDTA, 0.1% (wt/vol) SDS] in the presence of protease inhibitors (1 mM PMSF; 10  $\mu\text{g}/\text{mL}$  chymostatin, leupeptin, antipain and pepstatin A; and 2  $\mu\text{g}/\text{mL}$  aprotinin). After centrifugation at  $4^{\circ}\text{C}$ , 25  $\mu\text{g}$  of protein were mixed with reducing sample buffer, denatured, and subjected to electrophoresis (7.5–15% SDS/PAGE gels) followed by electroblotting onto presoaked PVDF membranes (BioRad). Blots were blocked in 0.1% Tween-20, 5% milk in PBS, at room temperature. Primary antibodies synaptophysin and PSD-95 (Cell Signaling) were incubated with the blot o/n at  $4^{\circ}\text{C}$ . Incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia) was performed at room temperature. Protein bands were visualized by chemiluminescence by using Super Signal West Pico Chemiluminescent Substrate (Pierce) reagent. Protein expression of synaptophysin and PSD-95 were then quantified by using ImageJ software. Group differences were analyzed by using separate Student's *t* test from each protein.

**Q-PCR.** Expression of genes of interest, including genes from microarray results (Table S1–S3), were quantitatively measured by using TaqMan real-time PCR system as previously described (4). The unpaired Student's *t* test was used for statistical analyses. The level of statistical significance was set at  $P \leq 0.05$ .

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