Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Absence of gut microbial colonization attenuates the sympathoadrenal response to hypoglycemic stress in mice: implications for human neonates

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BACKGROUND: Gut microbiota plays an important role during early development via bidirectional gut–brain signaling. Catecholamines provide a survival advantage allowing adaptation to common postnatal stressors. We aimed to explore the potential link between gut microbiota/gut-derived metabolites and sympathoadrenal stress responsivity.
METHODS: The effect of insulin-induced hypoglycemia was compared in mice with (control, adapted control) and without microbiome (germ-free, GF). Counter-regulatory hormones were analyzed in urine and plasma. Adrenal gene expression levels were evaluated and correlated to cecal short chain fatty acids (SCFA) content.
RESULTS: There was a significant association between absent microbiota/SCFA and epinephrine levels at baseline and after stress. Corticosterone (hypothalamic-pituitary-adrenal axis) and glucagon release (parasympathetic signaling) were similar in all groups. Hypoglycemia-induced c-*Fos* (marker of trans-synaptic neuronal activation) in both conditions. Delayed increases in adrenal tyrosine hydroxylase and neuropeptide Y messenger RNA were observed in GF mice. Transcriptome analysis provided insight into underlying mechanisms for attenuated epinephrine production and release.
CONCLUSION: Lack of microbiome selectively impaired adrenal catecholamine responses to hypoglycemia. We speculate that absent/delayed acquisition of flora (e.g., after antibiotic exposure) may compromise sympathoadrenal stress responsivity. Conversely, controlled manipulation of the intestinal microflora may provide a novel therapeutic opportunity to improve survival and overall health in preterm neonates.

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INTRODUCTION

During the birthing process, diverse classes of organisms from the maternal vaginal vault and the immediate postnatal environment colonize newborns as the antecedents of their individualized mature microbial bacterial signature.[1] The establishment of a life-long host–microbe symbiosis[2] is now recognized to serve essential functions in human health and disease[3] via bidirectional communication between the gastrointestinal tract and the central nervous system.[4–8] Disruption of these interactions during critical developmental windows can affect health throughout life, including increased risk of neurodevelopmental disorders.[9,10]

Precisely how initial gut colonization affects the microbiome–host interaction in preterm neonates has not been completely elucidated. However, since they are predominantly born by cesarean section, over 90% receive antibiotics plus all are handled with "sterile" technique, the issue of intended or incidental disruption of postnatal acquisition of gut flora is of interest.[11,12] Complicating this process is the immaturity of gastrointestinal and innate immune barrier systems.[13] Together, delayed colonization with symbiotic maternal species and reduced overall diversity[14] predisposes the neonate to a higher occurrence of adverse outcomes.[15]

The complex microbiota–gut–brain axis interconnects via direct and indirect routes involving neural (enteric nervous system, vagus nerve and spinal afferents),[16] humoral signaling (through gut hormones, neurotransmitters, and microbial metabolites),[3] and immune pathways (including pro-inflammatory cytokines and prostaglandins).[17] How gut microbes affect the adrenal component of the sympathetic nervous system[18] is not known and the evidence relating abnormal gut function to maladaptive responses to stressors is limited.[19] The mutually beneficial relationship between the host and gut microorganisms arises in part from short chain fatty acids (SCFAs)—byproducts of bacterial fermentation of some proteins, dietary carbohydrates and oligosaccharides.[20,21] In addition to their direct effects on gastrointestinal physiology, SCFA can alter brain and behavior by multiple epigenetic mechanisms via histone deacetylase inhibition, by enhanced 3′ RNA degradation or by altered transcription of neurotransmitter-related genes.[22,20,23] For example, our prior work identified a novel upstream 5′ regulatory element (−509 to −504 5′-GCCTGG) in the tyrosine hydroxylase (*TH*) gene that accelerated catecholamine biosynthesis via an interaction with a downstream cAMP response element and its binding proteins.[24] We also showed that SCFA/butyrate can increase TH messenger RNA (mRNA) and TH protein, either in vitro or in vivo.[23] In addition, butyrate regulates expression of other genes involved in catecholamine biosynthesis, suggesting a potential yet compelling role of diet-derived SCFA in regulating

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catecholaminergic systems during postnatal adaptation.[23] In support of the notion of neurohumoral–gut communication, experiments with germ-free (GF) adult mice showed increased activity of the hypothalamic-pituitary-adrenal (HPA) axis in response to restraint stress.[25]

In the current report, we sought to determine whether the absence of microbiome would alter the response to metabolic stress (insulin-induced hypoglycemia). The results presented provide a proof of concept that gut microflora and perhaps microbial metabolites (e.g., SCFA) are essential for establishing postnatal epinephrine homeostasis under basal and stimulated conditions.

METHODS

Animals

C57Bl/6 N mice were purchased from Taconic Farms Inc. (Germantown, NY, USA). Three main experimental groups were included in the study: conventionally raised control (C) and adapted control (AC) mice and sterile GF mice. AC mice were kept for 1 week in sterile conditions with GF at the Massachusetts Host–Microbiome Center, Boston in GF flexible film isolators (Class Biologically Clean) throughout the study. Sterility tests for each isolator (bacterial culture and PCR) were done on a bi-weekly basis. Control mice were housed in temperature and humidity-controlled rooms and allowed access to food and water ad libitum at the NYMC animal facility. They remained in the housing for 1 week of acclimatization and diet adaptation.

Mice aged 8 weeks were used in the study. Animal care and all experimental procedures were approved by the Institutional Animal Care and Use Committee at NYMC and MHMC.

Experimental design

On the test day, mice were transferred to new cages and fasted for 3 h prior to the experiment (water was provided ad libitum). The animals in each experimental group were randomly assigned to one of two subgroups ($n \geq 6$ per time point, per group): saline treated and insulin treated. Hypoglycemia was induced by injecting 2 IU/kg regular human insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA) as described.[26] Equivalent volume of saline was injected to the respective saline controls. Glucose was monitored every 30 min by tail bleed using a handheld glucometer (AlphaTrak, Abbott Laboratories, Chicago, IL, USA). Urine was collected when possible to measure epinephrine levels. The samples were acidified immediately by the addition of an equal volume of 0.01 M HCl and stored at −80 °C for analysis. The animals were sacrificed 60 or 90 min after injection with an overdose of ketamine–xylazine cocktail, followed by cardiac puncture for blood collection and decapitation (Fig. 1a). All tissue samples were collected at the same time of the day and either snap frozen and stored at −80 °C for further molecular analysis, or fixed overnight at room temperature in 4% paraformaldehyde for immunohistochemistry.

Hormone analyses

All samples for hormonal assays were measured in duplicates using commercially available immunoassay kits for detection of urinary epinephrine[26] (Rocky Mountain Diagnostics, Colorado Springs, CO, USA), urinary creatinine and plasma corticosterone (Arbor Assays, Ann Arbor, MI, USA), and glucagon (Millipore Sigma, Burlington MA, USA).

SCFA analysis

Cecum samples were kept in sealed containers at −80 °C until ready for SCFA gas chromatography analysis, performed at the Gnotobiotics, Microbiology, and Metagenomics Center (Boston, MA, USA). The chromatographic analysis was carried out using an Agilent 7890B system with a flame ionization detector and OpenLab ChemStation software (Agilent Technologies, Santa Clara, CA, USA). A volatile acid mix (10 mM of acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic, and heptanoic acids) was used for standard solution (Supelco

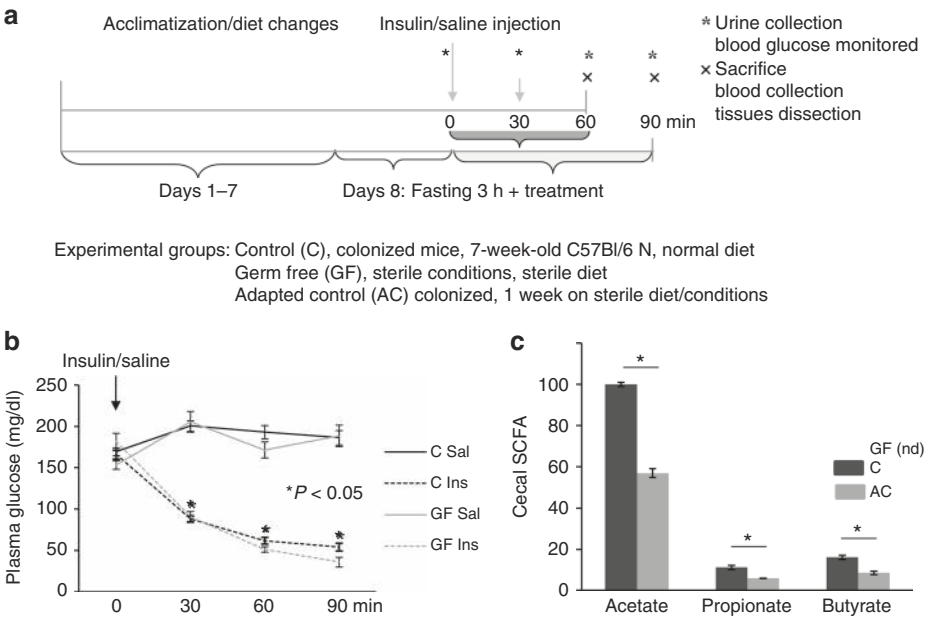

Fig. 1 **a** Schematic representing the basic protocol and the timeline of the experiments. **b** Blood glucose concentrations. Glucose values (mg/dl) for saline (Sal) treated (control (C) and germ free (GF), Sal—solid lines) and insulin treated (C and GF, Ins—dash lines) are shown as mean ± SEM, $n \geq 6$ for each experimental group. Data are summarized from four independent experiments, $^*P < 0.05$ vs. corresponding saline control at given time point. **c** Cecal short chain fatty acid (SCFA) content is microbiome- and environment-dependent. Concentration of SCFAs in cecal samples from C, adapted control (AC), and GF mice were determined as described in Methods. Data are summarized from individual samples ($n = 3$) for each experimental group and presented as % of the acetate in C, taken as 100%. $^*P < 0.05$ vs. corresponding C values. *ND* not detected

CRM46975, Bellefonte, PA, USA). An internal standard control (1% 2-methyl pentanoic acid, Sigma-Aldrich St Louis, MO, USA) was used for the volatile acid extractions.

Isolation of RNA, real-time RT-PCR, and microarray analysis

Total RNA was isolated from adrenal medullae using RNeasy kit (Qiagen, Hilden, Germany). RNA quality and quantity was assessed using a NanoDrop® Spectrophotometer ND-2000c (ThermoFisher, Waltham, MA, USA). Transcript levels were determined by a two-step quantitative reverse transcription-PCR (qRT-PCR) method: first-strand complementary DNA (cDNA) was synthesized by the use of 0.5–1 µg of RNA, oligodT primers, and Transcriptor high fidelity cDNA synthesis kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Diluted cDNA was used as a template for qPCR reaction with pre-designed Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for the genes of interest and TaqMan® Fast Advanced Master Mix. All reactions were carried out in duplicates using QuanStudio5™ (Applied Biosystems) instrument. Transcripts were normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase and quantified using the efficiency corrected ΔΔCt method.[27]

Genome-wide transcriptome profiling (three individual samples per experimental group, Clariom S Mouse, Applied Biosystems) was performed at Yale Center for Genome Analysis (New Haven, CT, USA). The data were normalized by robust multi-array average and quantile normalization using Parteck Genomics Suite 6.6. Quality control was performed by principal component analysis. Differentially expressed genes (DEGs) were selected with adjusted *P* value (Benjamini–Hochberg procedure) ≤0.05 and fold-change ≥1.5. DEGs between selected pairs was tested using Limma R package. Functional enrichment analysis was conducted using MetaCore platform (Thomson Reuter). Raw and quantile-normalized microarray data and an associated project metadata file are available through the NCBI-GEO repository, accession number GSE122754.

Immunohistochemistry

Adrenals were dissected, washed in phosphate-buffered saline (PBS), fixed overnight at 4 °C in 4% paraformaldehyde in PBS, and then cryoprotected in sucrose in PBS buffer as described.[28] Tissue blocks were made in optimal cutting temperature compound (O.C.T., Sakura, Japan). Cryosections (18 µm) were permeabilized (PBS/0.3% Triton X- 100/5% immunoglobulin G (IgG)-free bovine serum albumin (BSA)) for 2 h and then transferred to PBS/50 mM glycine for 60 min before incubation in blocking solution (PBS/0.05% Triton X-100/5% IgG-free BSA) for 30 min. Sections were incubated overnight at 4 °C in primary antibody (rabbit anti-TH and anti-c-Fos antibodies, 1:1000, Novus Biologicals, Littleton, CO, USA), then washed with PBS, and finally incubated in secondary antibody (1:1000 donkey anti-rabbit Alexa Fluor594, Jackson ImmunoResearch, West Grove, PA, USA) for 90 min at room temperature and mounted on white frosted, positive charged microscope slides (Denville Scientific, Metuchen, NJ, USA).

Image analysis

Images were obtained using a Nikon Eclipse 90i microscope, ×10, ×20, and ×40 objectives, a Nikon DS-Qi1Mc camera, and NIS-Elements AR 4.20 (Nikon) software. ImageJ (Rasband WS, ImageJ, US NIH, Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997–2016) was used for most analysis. To quantify staining in slices, a region of interest was drawn around the medulla (minus any cell-free areas) and the integrated pixel intensity was calculated and compared across the experimental groups. In most experiments >3 sections from each animal (two animals per group) were used to calculate the mean. All slides were blinded until the analysis was complete.

Statistics

Statistical analysis was performed with Sigma STAT/Plot software; version 12 (San Jose, CA, USA). All data were expressed as means ± standard error of the mean (SE). All differences were considered to be significant at $P < 0.05$. Comparisons of basal and hypoglycemic responses were made using the one-way analysis of variance followed by a Neuman–Keuls post hoc analysis.

RESULTS

To investigate the role of the microbiome in sympathoadrenal stress responsivity, we looked at the differences in the responses to hypoglycemic stress between GF (no microbiome) and colonized mice (C and AC). The experimental design is illustrated on Fig. 1a. Each experimental group was divided randomly into two subgroups and given intraperitoneal (i.p.) injection of insulin or saline. There was no significant difference in baseline blood glucose levels between the three experimental groups (Fig. 1b, Supplemental Table S1). As expected, blood glucose levels gradually decreased following insulin administration and remained at the target values (50–60 mg/dl) until the end of the study. There were no significant differences in the level of hypoglycemia that was achieved between the C, GF, and AC groups. Animals from the saline-treated subgroups were also subjected to the same handling to avoid differences in any additional stress exposures. There were no significant differences in blood glucose levels between individual animals ($n \geq 6$) in each experimental subgroup, or between the saline- or insulin-treated subgroups at any time point tested (Fig. 1b). The metabolic activity of the gut microbiome was determined by measuring the cecal SCFA. As expected, no SCFA were detected in the cecum of GF mice (Fig. 1c). After housing colonized mice in sterile conditions and sterile diet for 1 week, significantly lower levels of cecal SCFA were detected in the AC group.

Colonized and GF mice display similar plasma corticosterone and glucagon levels following acute hypoglycemia

There were no statistically significant differences in plasma glucagon levels between the saline-injected experimental subgroups (C, GF, and AC, Fig. 2a). In response to hypoglycemia, glucagon concentrations increased significantly from euglycemic values in all groups. Corticosterone responsiveness was also evaluated. Saline-injected GF mice displayed higher baseline plasma corticosterone levels (Fig. 2b), similar to what has been reported previously by others.[17] During the hypoglycemic episode, corticosterone levels increased in all insulin-treated groups and did not display significant differences between the groups.

In the absence of microbiome (GF mice) baseline and hypoglycemia-induced urine epinephrine levels are reduced

To evaluate the catecholamine responses during hypoglycemia, urine samples were collected from each mouse throughout the study as described in Methods. This approach is minimally invasive and informative, given that urine epinephrine levels are known to correlate with whole-body catecholamine turnover.[29] Compared to C and AC, baseline urinary levels of epinephrine (collected before injection) in GF animals were significantly lower (Fig. 3a). Exposure to hypoglycemia resulted in increased release of epinephrine, but the levels in GF group remained much lower than in the corresponding C and AC groups (Fig. 3b, data shown for the 90 min time point).

Trans-synaptic activation of the adrenal stress response in GF mice

To determine whether insulin-induced hypoglycemia activated adrenal chromaffin cells in the absence of a microbiome, adrenal tissue sections from all experimental groups were stained for c-Fos. No increases in c-Fos immunoreactive cells in the adrenal

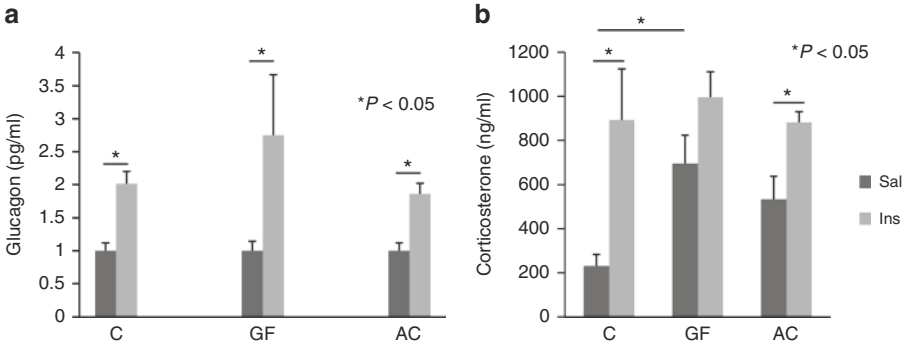

Fig. 2 Effect of microbiota/environment on glucagon and corticosterone release following insulin-induced hypoglycemia: Glucagon responses (**a**) and corticosterone levels (**b**) were measured in plasma samples from saline- and insulin-treated mice from each group as described in Methods. Data are summarized from two independent experiments, $n \geq 6$ animals per group. Results for 90 min time point are presented as mean ± SE. $^{*}P < 0.05$ vs. corresponding saline levels for each group

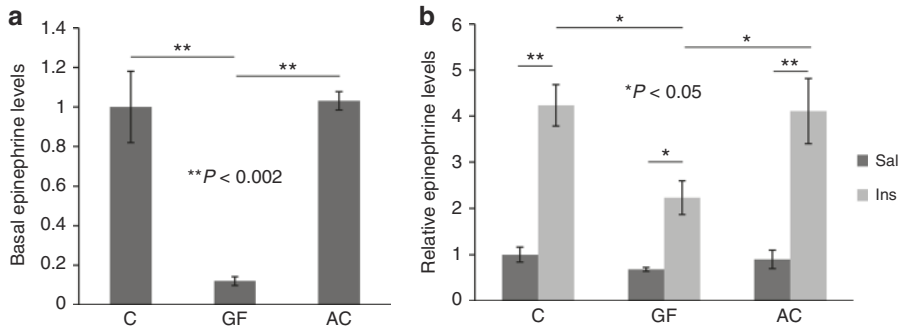

Fig. 3 Reduced basal and hypoglycemia-induced epinephrine levels in germ-free (GF) mice: Urine epinephrine levels measured before (**a**) and 90 min after insulin/saline injection (**b**) for control (C), adapted control (AC), and GF mice: hypoglycemia (gray bars), saline injected (black bars). Data are normalized to urinary creatinine and expressed relative to the saline values in control (C). Results are summarized from two independent experiments, $n \geq 6$ animals per group. Values are shown as mean ± SE, $^{*}P < 0.05$ or $^{**}P < 0.002$

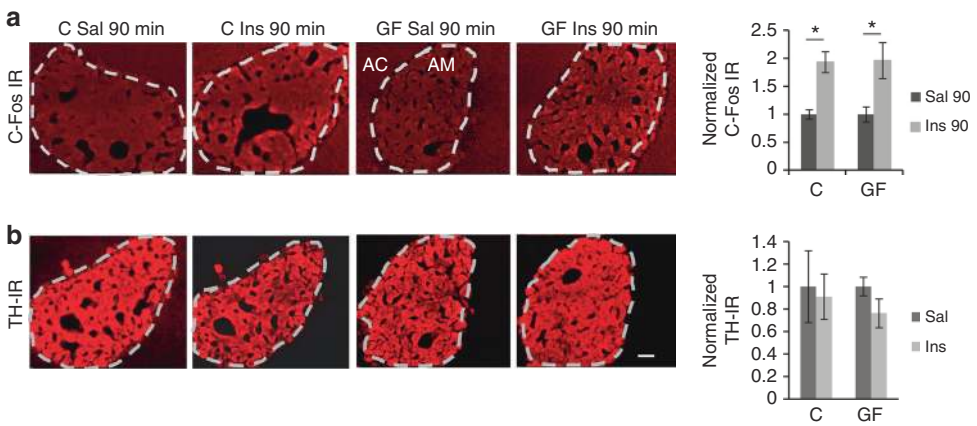

Fig. 4 Immunohistochemical studies: **a** c-Fos immunofluorescence is significantly increased 90 min following insulin injection in germ-free (GF) and control (C) mice. Results are presented as fold change from integrated intensity in the corresponding saline groups. Values are shown as mean ± SE, $^{*}P < 0.05$. AC adrenal cortex; AM adrenal medulla. **b** Strong immunostaining for tyrosine hydroxylase (TH) is limited to the adrenal medulla as expected. No significant differences in the integrated immunofluorescence intensity were observed between C and GF, saline- or insulin injected mice. Scale bars, 50 μM

medulla of both C and GF mice was observed when animals were sacrificed 60 min after insulin injection as compared to their respective saline controls. Longer exposure to hypoglycemia resulted in similar increases of c-Fos immunoreactivity in the adrenal medulla of GF and C mice consistent with a trans-synaptic activation of a stress response (Fig. 4a). In contrast to c-Fos staining, TH immunoreactivity was limited to the adrenal medulla as expected. For the examined time points (60 and 90 min after injection), no differences in the levels of TH-immunoreactivity in adrenal sections from stressed animals and their matched controls, or between mice with and without microbiome were detected (Fig. 4b).

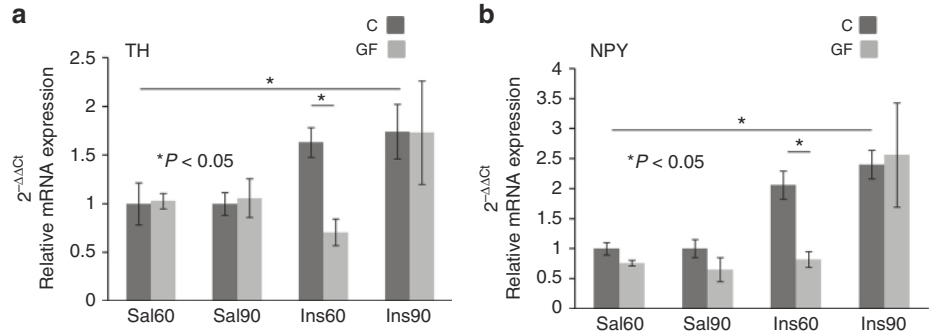

Fig. 5 Delayed rise in adrenal tyrosine hydroxylase (TH) and neuropeptide Y (NPY) messenger RNA (mRNA) levels in germ-free (GF) mice during acute insulin-induced hypoglycemia: total RNA isolation and quantitative reverse transcription-PCR (qRT-PCR) were performed as described in Methods. The data were quantified as $\Delta\Delta C_T$ relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values are shown as mean ± SE, $^*P < 0.05$

Stress-elicited induction of adrenal TH and NPY mRNA levels is delayed in the absence of microbiome

The lower urinary epinephrine levels observed here may indicate a reduction in adrenal catecholamine synthesis, re-uptake, storage, and release in the GF condition. To further explore this option, we performed qRT-PCR analysis of adrenal medullary RNA, isolated from C and GF mice exposed to glucoprivation, and their corresponding saline-treated controls. No significant differences were found in TH mRNA levels from saline-treated C and GF experimental groups 60 min or 90 min after injection (Fig. 5a). In response to insulin-induced hypoglycemia TH mRNA levels were increased at 60 min only in C group. Both C and GF groups displayed similar rise in TH mRNA levels with longer exposure to stress (90 min). The trans-synaptic regulation of neuropeptide Y (*NPY*) gene (encoding a neuropeptide co-released with catecholamines from adrenal chromaffin cells) followed the pattern described for *TH* (Fig. 5b).

Whole-genome transcriptome profiling provides insight into potential underlying mechanism(s)

Next, we aimed to establish global changes in adrenal gene expression caused by lack of microbiome. Microarray analysis revealed significant number of probe ID detected in the RNA samples from GF mice under basal or stress condition, which meet the criteria for DEGs (see Fig. 6a and Supplemental Table S2 for complete lists). Notably, the expression of the genes encoding other catecholamine biosynthetic enzymes (dopamine β-hydroxylase, aromatic L-amino acid decarboxylase, and phenylethanolamine *N*-methyltransferase) and nicotinic acetylcholine receptors was dysregulated in the absence of microbiome (Table 1). To delineate which biological processes/functionally related genes are affected in GF mice, we performed enrichment analysis. Shown are the top 10 process networks for the comparison C insulin vs. GF insulin (Fig. 6b), indicating that altered synaptic plasticity, neurotransmission machinery, and neuropeptide signaling networks can affect the capacity of the adrenal medulla to produce and/or release catecholamines under stress in GF mice.

DISCUSSION

This study sought to determine whether any correlations existed between gut colonization/gut-derived metabolites and the host's ability to adapt to a common stressor such as hypoglycemia using a GF animal model. GF animals are a powerful scientific tool to study the global influence of the microbiome on nervous system development and function. The main findings of our report are: (1) significantly lower baseline epinephrine levels in the urine of GF compared to age-matched normally colonized mice; (2) attenuated adrenal epinephrine responses to insulin-induced hypoglycemia in GF mice; (3) glucagon responses (parasympathetic pathway) and corticosterone responses (hormonal pathway) similar to control mice; but (4) delayed trans-synaptic induction of adrenal TH mRNA levels in GF mice; and (5) markedly altered transcriptome profiles under basal and stress conditions in GF mice, including down-regulation of genes involved in synaptic transmission, nervous system development, and neuropeptide signaling pathways. This is the first report demonstrating imbalances of the sympathoadrenal system arising from the lack of microbiome/microbial metabolites.

Our simple, easily accessible peripheral nervous system model has the advantage of reliable and robust outcome measures such as release of epinephrine from a single sympathetic nervous system structure while simultaneously testing parasympathetic (glucagon) and hormonal (corticosterone) response systems. The GF data indicate that chemical messages arising from acquired microbial symbionts after birth are selectively relevant to postnatal sympathoadrenal maturation and stress adaptiveness.

Under the selected experimental conditions (see Fig. 1a), we did not observe differences in baseline glucose levels across the studied groups (C, AC, and GF mice). In another report, GF animals showed lower circulating glucose levels at baseline.[17] The observed differences are most probably due to a genetic drift in inbred sub-strains of C57Bl/6 mice.[30] Of note, target blood glucose levels (50–60 mg/dl) were achieved by 60 min after a single bolus injection of insulin and were maintained at similar levels in all groups through 90 min, indicating that all animals were exposed to identical glucose and insulin stimuli (Fig. 1b and Table S1).

It is well established that in control animals and healthy humans, the central nervous system (CNS) detects and orchestrates the hypoglycemia-induced hormonal and autonomic nervous system-mediated release of counter-regulatory hormones (i.e. glucagon, epinephrine, cortisol, and growth hormone) that act to stimulate endogenous glucose production, inhibit glucose uptake, and avert the progression of hypoglycemia.[31] Whether acute glucoprivation would affect these normal counter-regulatory responses under GF conditions had never been studied before. Here we report intact glucagon and corticosterone responses in the absence of a gut microbial ecosystem (Fig. 2a), suggesting functional CNS glucose-sensing mechanism(s) in the GF condition.

In addition to the removal of the passively restrained control exerted upon glucagon by circulating glucose and insulin (and other paracrine factors secreted by the neighboring islet β- and δ-cells), glucagon release from the pancreatic α-cells is subjected to three major autonomic inputs: (1) parasympathetic nerve impulses (acetylcholine); (2) epinephrine released from the adrenal medulla, and (3) sympathetic nerve impulses (norepinephrine). The progressive recruitment of each of these inputs depends on the severity of hypoglycemia.[32] Under our experimental conditions (mild to moderate acute hypoglycemia [26] and identical glucose

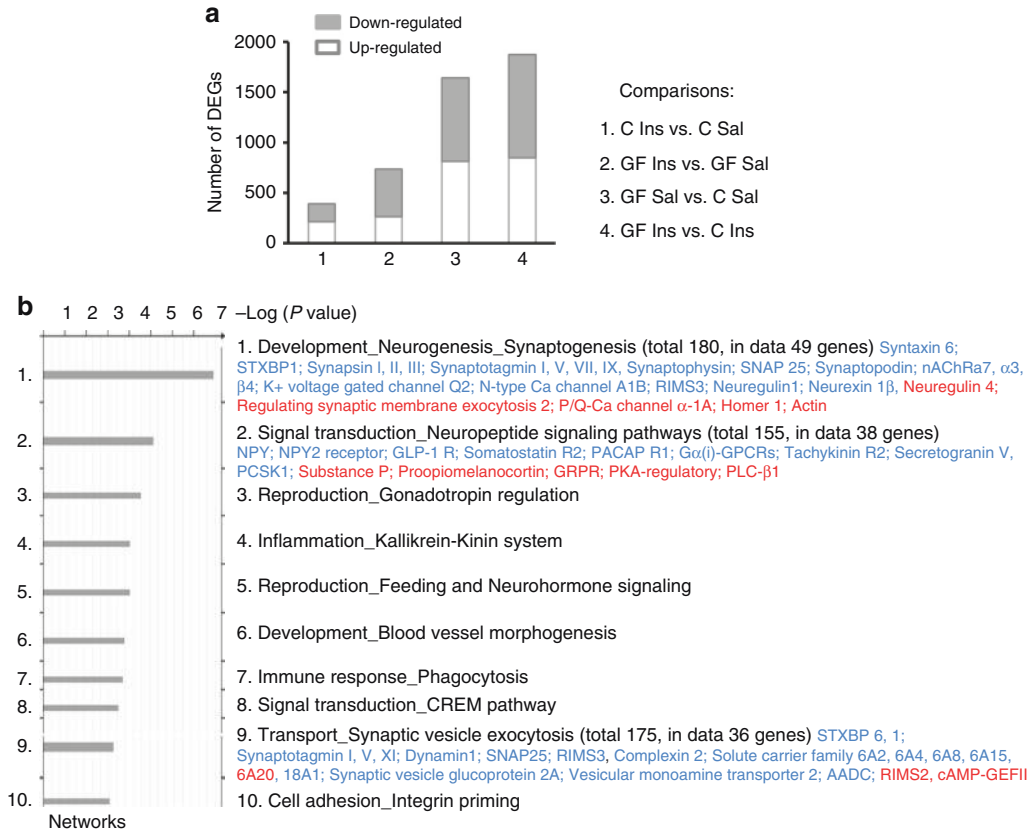

Fig. 6 Whole-genome transcriptome profiling: Total adrenal RNA was isolated and subjected to microarray analysis as described in Methods. **a** Summary of differentially expressed genes (DEGs), pair-wise comparisons; **b** enrichment analysis, shown is the distribution by process networks for control (C) insulin (Ins) vs. germ-free (GF) Ins comparison. The DEGs in the most relevant to the impaired epinephrine response in GF mice networks are included. Blue—down-regulated, red—up-regulated DEGs. Comments: No networks (1, 2 and 9) nor selected DEGs are shown in bold (unlike in the original figure submitted with the manuscript). We modified the legend to better describe the current (published) version of the figure

Table 1. Dysregulated expression of genes involved in catecholaminergic neurotransmission in GF mice

Gene	C Ins vs. C Sal	GF Sal vs. C Sal	GF Ins vs. GF Sal	GF Ins vs. C Ins
DBH	n/a	n/a	n/a	↓1.86
AADC	n/a	n/a	n/a	↓2.32
PNMT	n/a	n/a	↓1.71	↓2.38
POMC	n/a	n/a	↑2.09	↑1.92
Chrnb2	n/a	↓1.52	n/a	↓2.08
Chrna7	n/a	↓1.51	n/a	↓2.6
Chrnb4	n/a	n/a	↓1.92	↓2.88
Chrna3	n/a	n/a	↓1.52	↓1.81

DEGs in pair-wise comparisons are listed. The symbols ↑ and ↓ indicate up- and down-regulated genes, respectively. Numbers following the ↑ or ↓ symbols represent the fold-change for the gene expression level
DEG differentially expressed gene, C control, *Ins* insuin, *Sal* saline, GF germ free, n/a not available, *DBH* dopamine β-hydroxylase, *AADC* aromatic L-amino acid decarboxylase, *PNMT* phenylethanolamine *N*-methyltransferase, *POMC* proopiomelanocortin, *Chrnb2* cholinergic receptor nicotinic β-2 subunit, *Chrna7* cholinergic receptor nicotinic α-7, *Chrnb4* cholinergic receptor nicotinic β-4 subunit, *Chrna3* cholinergic receptor nicotinic α-3 subunit

and insulin stimuli among the experimental groups), the comparable parasympathetic activation (which occurs earliest at glucose levels between 85 and 75 mg/dl) is the major contributor to the observed similar glucagon release in C, AC, and GF mice. Since glucagon responses were similar after equivalent hypoglycemic exposure, it would appear reasonable to conclude that the GF condition does not affect parasympathetic effector tone.

Two main systems mediate the physiological responses to stress: the HPA axis and the sympathoadrenal system.[18] The adrenal gland, as part of both systems, serves a major role in maintenance of homeostasis by secreting hormones required for survival during acute stress: glucocorticoids from the adrenal cortex and catecholamines from the adrenal medulla. In this report, we also sought to determine whether the lack of a microbiome was associated with dysregulation of the HPA axis in response to insulin-induced hypoglycemia as reported for other type of stress.[25] Plasma corticosterone levels, a hallmark measure of the stress-triggered activation of the HPA axis were evaluated in all experimental groups at sacrifice (see Fig. 1a). While in C saline-injected male animals the values showed the expected normal diurnal levels for that time of day (~200 ng/ml), significantly higher values were observed in saline-injected GF mice (Fig. 2b). We interpret this to reflect an exaggerated response to handling/injection stress and increased sensitivity to pain.[33]

In response to hypoglycemia, corticosterone levels further increased to a similar extent in C, GF, and AC, indicating an intact hypothalamic-pituitary-adrenal cortical axis, arguably a response similar to early life, maternal separation-stress response in GF C57BL/6 mice.[17] Together, our data are consistent with the hypothesis that the observed by others dysregulation of the HPA axis in the absence of microbiome is stressor-specific: evident during restraint stress[25] but not during hypoglycemia (current report) or exposure to ether.[25]

This is the first study to evaluate baseline (Fig. 3a) and stress-induced urinary epinephrine levels (Fig. 3b) in animals lacking a microbiome. Our data revealed significantly reduced urinary epinephrine baseline levels in GF mice before any injections compared to C and AC mice. Furthermore, while exposure to hypoglycemia increased epinephrine release in all groups, the relative concentrations of urinary epinephrine remained attenuated in GF conditions (Fig. 3b), indicating a significant interaction between commensal microbiota (and/or microbial metabolites) and catecholamine homeostasis. Given that sensory information from peripheral glucosensors is integrated into a central glucoregulatory/sensory network to activate the counter-regulatory response (evident from the intact glucagon and corticosterone responses in GF mice reported here), the reduced epinephrine response to hypoglycemia may stem from altered trans-synaptic signaling at the splanchnic nerve fiber–adrenal chromaffin cell synapse.

Several mechanisms mediate epinephrine release during hypoglycemia acting pre-synaptically (changing synaptic strength), post-synaptically (by affecting catecholamine production and release), or at both sites to account for the impairment of epinephrine release in the absence of microbiome. Here we used c-Fos immunoreactivity as an indirect molecular correlate of neuronal activation[34] and observed no significant differences in adrenal medullary c-Fos immunoreactive protein in saline-injected C or GF mice (Fig. 4). Acute hypoglycemia increased c-Fos immunoreactive protein in both groups to a similar extent when compared to their respective saline controls. Thus, a reduction of the descending outflow in the absence of microbiome is unlikely to contribute to the impaired epinephrine responses.

The whole-genome expression profiling reported here indicated significant remodeling of the transcriptional landscape in the adrenals of GF mice when compared to normally colonized C mice, including both up-regulated and down-regulated genes (Fig. 6a). The number of DEGs were further increased by exposure to hypoglycemia (Table S2). Of note, we found significant enrichment of DEGs involved in neurogenesis and synaptogenesis (Fig. 6b, top scored process network), including cholinergic neurotransmission (Table 1, *nAChRα7*, *α3*, *β2*, and *β4*) that have been shown to be critical for the release (and production) of adrenal catecholamines in response to stress.[18] These results revealed that several pre- and post-synaptic components of the synaptic machinery and synaptic vesicle exocytosis are dysregulated in the absence of microbiome and may contribute to the observed marked decrease in stress-induced epinephrine release in GF mice. Our results are consistent with that reported by other's altered transcriptional profile and post-transcriptional regulation of several genes implicated in synaptic transmission, neural activity, and the development of the central nervous system of GF mice. [35]

The release of catecholamines from the adrenal medulla in response to stress is normally accompanied by compensatory mechanisms increasing TH mRNA, TH protein, and enzyme activity to maintain cellular catecholamine homeostasis.[18] Our study did not reveal significant differences in TH mRNA (Fig. 5a) or TH protein levels (Fig. 4) under baseline conditions in control and GF mice. However, insulin increased TH mRNA levels in GF mice only after longer exposure time. Further, the expression of genes encoding other enzymes in the catecholamine biosynthetic pathway were down-regulated by stress in GF mice (Table 1, Supplemental Table S2). Our results are inconsistent with recently published data[36] showing increased adrenal TH and PNMT mRNA levels at baseline and after stress in GF BALBC mice, but in good agreement with previous studies demonstrating dysregulated brain synthesis and degradation of catecholamines in the absence of microbiome (reviewed in ref. [17]). TH protein levels were not altered at any time point studied even in the C group, in agreement with previous reports indicating that a longer time period (>2 h) or chronic exposure to stress is needed to alter the steady-state levels of TH protein.[18,26]

In addition to the central regulation through the splanchnic nerve, autocrine/paracrine regulation by neuropeptides and cytokines[37,38] may also control the function of the adrenal gland. In this regard, neuropeptide signaling pathways were the second scored process network altered in GF mice in our study (Fig. 6b) and an impact on the blunted epinephrine response to hypoglycemia is likely.

Since the gut microbiota has an essential role in animal and human metabolic regulation,[21] the microbial ecosystem functionality was also addressed in our experiments by measuring the cecal concentration of SCFA (Fig. 1c). In the current study, the absence of a microbiome correlated with no detectable SCFA in the cecum (as expected) and the impaired baseline and insulin-induced epinephrine responses, reflecting dysregulation of sympathoadrenal stress responsivity. While a transient environmental change (AC group) altered the SCFA profile, indicating an altered microbiome composition, it had no effect on the production and release of adrenal catecholamines in response to acute hypoglycemia. These results suggest that stress-responsive systems are already "programmed" or that a more prolonged or chronic stress paradigm is needed to alter regulatory networks in the peripheral nervous system and in the brain. In the current study, we did not attempt to determine causality between absence of gut microbiota/SCFA and impaired epinephrine responses to stress using a recolonization strategy. On the other hand, other investigators showed that social and synaptic deficits[39] or abnormal responses to restraint stress[25] in adult GF mice can be reversed by microbial reconstitution or SCFA supplementation when performed during a critical neurodevelopmental window earlier in life. Testing these mechanisms was beyond the scope of this project.

There are some caveats of the study that should be considered. The mice were not cannulated to allow for stress-free plasma collection during the hypoglycemic episode. Plasma samples were collected at sacrifice (60 or 90 min after injection of insulin/saline) and the values for corticosterone and glucagon as well as changes in gene expression (which occur at differing time intervals following the onset of glucoprivation) could be off peak.

CONCLUSION

This is the first study to examine the potential interaction between gut microbiome/dietary factors (environmental signals) and the classical cholinergic-trans-synaptic signal transduction system regulating the response to hypoglycemia in vivo, in an animal model. Here we report imbalances of the sympathoadrenal system associated with the lack of microbiome or microbial metabolites (e.g., SCFA). Our data are in agreement with previous findings demonstrating that GF life profoundly disturbs brain neurotransmission systems and alters neuroendocrine/behavioral responses to acute stress.[40] Thus, early pre/postnatal gut colonization augments the maturation and function of sympathoadrenal responses to classic stressors during development, likely providing an evolutionarily preserved selective survival advantage in neonates (rodents as well as humans). Controlled manipulation of the intestinal microflora may provide a novel therapeutic approach to improve the adaptation to common stressors in premature babies, their survival, and overall health.

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ADDITIONAL INFORMATION

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