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Supplementary Materials for

The gut microbiota influences blood-brain barrier permeability in mice

Viorica Braniste,* Maha Al-Asmakh, Czeslawa Kowal, Farhana Anuar, Afrouz Abbaspour, Miklós Tóth, Agata Korecka, Nadja Bakocevic, Lai Guan Ng, Parag Kundu, Balázs Gulyás, Christer Halldin, Kjell Hultenby, Harriet Nilsson, Hans Hebert, Bruce T. Volpe, Betty Diamond, Sven Pettersson*

*Corresponding author. E-mail: viorica.braniste@ki.se (V.B.); sven.pettersson@ki.se (S.P.)

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The PDF file includes:

Material and methods:

Qualitative analysis of the penetration of the antibody into the fetal brain

The infrared-labeled IgG2b antibody MPC-11 (200 μg/mouse) was injected into pathogen-free and germ-free time pregnant dams 1 hour before harvesting the embryos. In order to retain the signal in the embryo for visualization, the umbilical vessels were cauterized using the Gemini cautery system (SouthPointe Surgical Supply, Coral Springs, FL). The penetration of the antibody into the brain of the embryos was visualized using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Quantitative analysis of the penetration of the maternal antibody into the fetal brain

The MPC-11 antibody (200 μg/mouse) was injected intravenously into female pathogen-free mice during pregnancy. Twenty four hours after injection, the pathogen-free dams were sacrificed and the placental sac containing the embryos was surgically removed. The embryos were anesthetized on ice cold PBS. Each embryo was kept in its placental sac until ready for perfusion. Cardiac perfusion was performed under a surgical microscope (Universal S2, ZEISS, Germany) using glass capillaries and a high precision infusion pump (PHD 2000, Harvard Apparatus, Holliston, MA). The volume used for perfusion was 120 μl for 13.5 days old embryos (E13.5), which corresponds to the three times the volume needed for observed removal of blood. The volume used for perfusion was gradually increased to 500 μl for E17.5 embryos. The flow rate was 0.05 ml/minute for E13.5-E14.5 embryos and 0.1 ml/minute for E15.5- E17.5 embryos. After perfusion, the brains were extracted from each embryo and placed in a tube filled with lysis buffer. The volume of the lysis buffer was 10 times the weight of the brain. The brain tissues were sonicated on ice using the Sonicator 3000 (Misonix, Farmingdale, NY). The measurement of the concentration of the infrared-labeled

antibody present in the embryonic brain was performed in duplicate using the Odyssey Infrared Imaging System (LI-COR). The original labeled antibody was included as a standard. The serum of the pregnant dam was used for calculation of the initial antibody concentration. When possible, multiple animals were analyzed on the same membrane to minimize variation. The concentration of antibody in embryonic brain was calculated per milligram of perfused brain tissue.

Evans blue assay

Evans blue perfusion was performed as previously described (52). Briefly, the mice were anaesthetized with isofluorane, the thoracic cavity was opened, and the cardiac perfusion was performed using 50 ml of PBS (pH 7.2) followed by 50 ml of the cocktail containing 1% Evans blue (Sigma–Aldrich) dissolved in 4% paraformaldehyde (PFA, Histolab). The dissected brains were post-fixed 4 h in 4% PFA, cryoprotected in 30% sucrose overnight at 4°C, and frozen in OCT medium on dry ice.

Cryotome 20-µm-thick coronal brain sections were mounted on Superfrost^{+} slides with Entellan mounting media (Merck Millipore) and visualized using fluorescent microscope (Leica Leitz DMRB Fluorescence Microscope) by excitation with 543-nm laser beams (green zone) and visualized as red fluorescence.

In vivo positron emission tomography (PET) imaging of ^{11}C-Raclopride

The pathogen-free and germ-free male mice (8-10 weeks old) were anaesthetized with isofluorane, controlled by E-Z anaesthesia vaporizer (initial 5%, then 1.5% to maintain anesthesia, blended with 7:3 air/O_2, delivered through Microflex non-rebreather mask (Euthanex Corporation, Palmer, PA, U.S.A.). Fifteen minutes before injection, the mice were placed on a heating pad (37°C) with a scanner bed, with most of the body in the field-of-view.

The scans were performed in a microPET Focus 120 scanner (CTI Concorde Microsystems, Knoxville, TN, USA). The PET tracer (maximum volume 200 µl) was administered by bolus injection via the tail vein. PET data were acquired in fully 3-dimensionally mode, and images were reconstructed by standard 2-dimensionally filtered back projection using a ramp filter. The matrix size of reconstructed images was 128×128×95 with a spatial resolution of 1.3 mm. The data were normalized and corrected for random, dead time and decay. The amounts of the injected [^{11}C]raclopride ranged from 6.7 to 11 MBq. The PET data acquisition times were 30 min with scanning started at tracer injection. The whole brain 'Regions of Interest' (ROI) were manually delineated on PET images for each animal using PMOD 3.0 (Zurich, Switzerland). The %SUV represents the regional tissue radioactivity concentration normalized for injected dose and body weight:

$$\%SUV = \frac{\text{radioactivity in the target organ (Bq/g)}}{\text{total injected radioactivity (Bq) / body weight (g)}} \times 100$$

R4A antibody injection, tissue staining and quantitative analysis

The germ-free and pathogen-free male mice (8-10 weeks old) received an intravenous injection of either R4A dissolved in PBS (100µg/mouse or 250 µg/mouse) or only PBS as a control. Forty eight hours later the mice were anaesthetized with isofluorane and received intracardiac perfusion with 0.9% NaCl followed by 4% PFA (Histolab). The harvested brains were post-fixed for 2 hours in 4% PFA. The frozen embedded brains were sectioned at 40-µm-thick on a freezing stage microtome. The sections were collected in 0.1 M phosphate buffer (PB, pH7.4).

We modified our published techniques for unbiased stereology of the hippocampus to include the automated procedures in developing a systematic-random-sampling grid and optical dissector dimensions (Stereologer Programs, MBF, Williston, Vt) (53, 54). Essentially for each animal, neurons in the right and left stratum pyramidale were sampled from comparable regions of the anterior dorsal hippocampus (Bregma, -2.06mm) stained with cresyl violet. Four coronal sections (each cut at 40μ) from each animal separated by 120μ were visualized and stored using a z-stack (1μ separation) mosaic program (100x, oil, Axio-Vison 4.0, Zeiss, Thornwood, NY). The images were analyzed and there was comparable planimetry across groups of animals (data available but not presented), and the coefficient of error (Gundersen) was ≤ 0.07 for all analyses.

Transmission electron microscopy

The pathogen-free and germ-free adult mice were transcardiacally perfused with a fixative containing 2.5% glutaraldehyde (Sigma), 1% PFA in 0.1M sodium cacodylate buffer (Sigma), pH 7.4. The brains were removed and the areas of interest were dissected and stored in refrigerator in the same fixative. Specimens were rinsed in 0.1 M PB, pH 7.4 and postfixed in 2% osmium tetroxide 0.1 M PB, pH 7.4 at 4°C for 2 hours, dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, Vermont, USA). Semi-thin sections were cut and stained with toluidine blue O and used for light microscopic analysis. Ultrathin sections (approximately 50-60 nm) were cut by a Leica EM UC 6 (Leica, Wien, Austria) and contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 12 Spirit Bio TWIN transmission electron microscope (FEI company, Eindhoven, The Netherlands) at 100 kV. Digital images were taken by using a Veleta camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany).

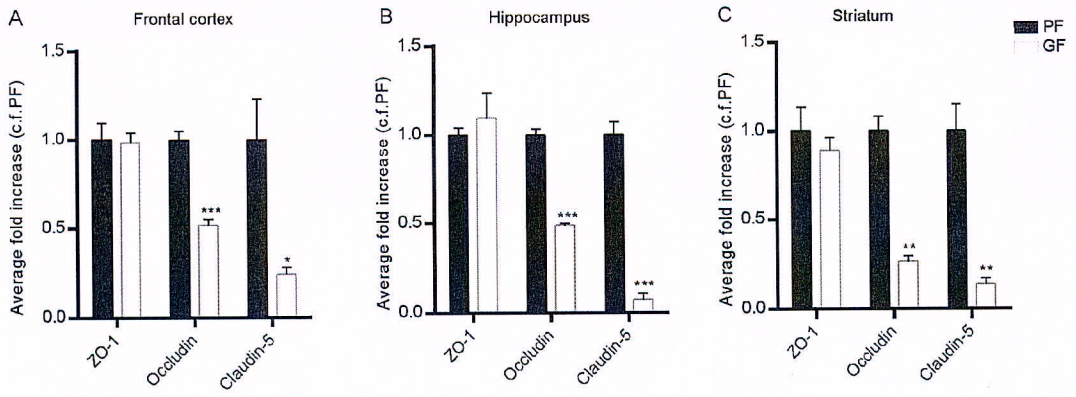

Fig. S1. Expression of tight junction proteins in the brains of germ- and pathogen-free adult female mice.

Densitometric analysis of the western blots analysis demonstrating significantly low expression of the TJPs, occludin and claudin-5, in the frontal cortex, striatum, and hippocampus of female germ-free mice (GF) compared with female pathogen-free mice (PF). Data are normalized for GAPDH expression and represented as fold change. Values are expressed as ± S.E.M. (4-5 mice/group). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by Student's *t*-test.

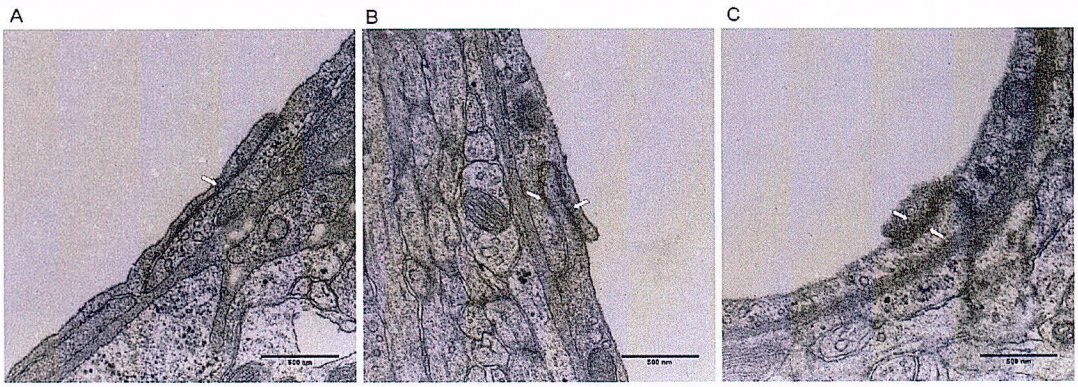

Fig. S2. Electron micrographs showing different tight junction structure (white arrows) in the brains of germ-free adult mice.

A. Perfect tight junctions, B. patches of blurriness, C. totally blurred tight junctions.

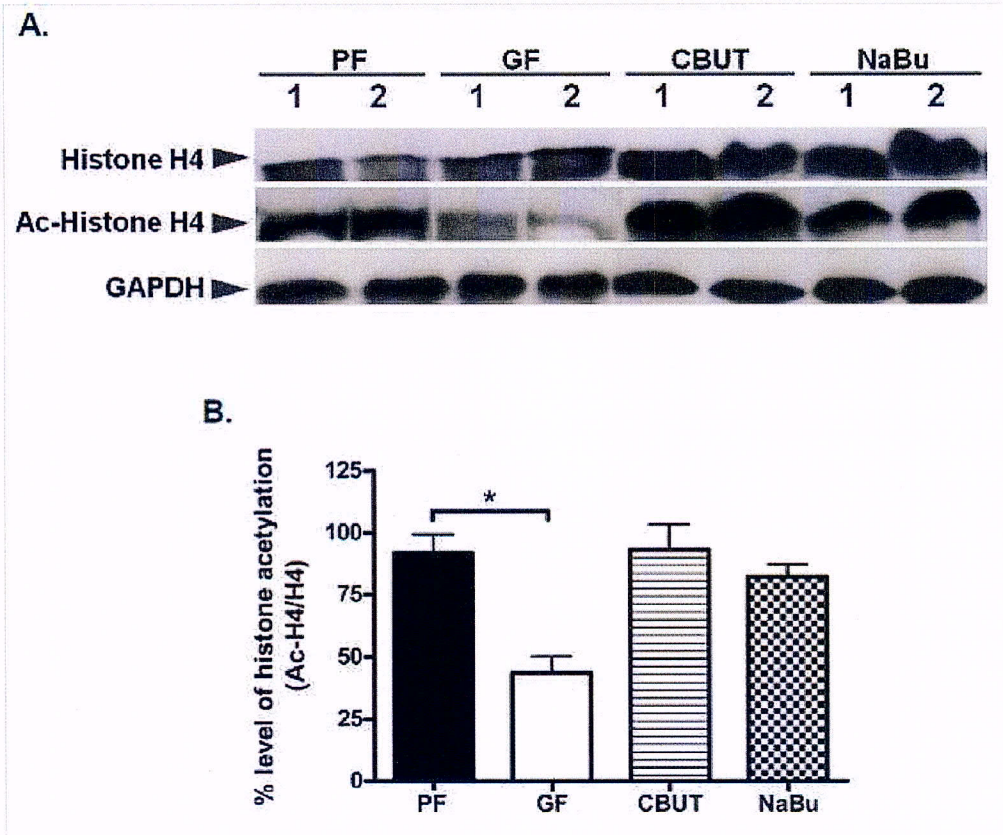

Fig. S3. The effect of oral treatment with the bacterial metabolite sodium butyrate or monocolonization with *C. tyrobutyricum* on histone acetylation in extracts of mouse brain frontal cortex.

A-B. Representative western blots and quantitative analysis showing the expression of Histone H4, Acetylated Histone H4 in the brain lysates extracted from frontal cortex region of following groups of mice: H2O treated pathogen-free mice (PF); H2O treated germ-free mice (GF); sodium butyrate treated GF mice (NaBu) or GF mice monocolonized with *C. tyrobutyricum* (CBut). Percentage level of histone acetylation was calculated for each of the treatment groups. Values are expressed as ± S.E.M. (4-5 mice/group). *P<0.05 compared to pathogen-free mice values.