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Targeted intestinal tight junction hyperpermeability alters the microbiome, behavior, and visceromotor responses

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Markedly increased intestinal permeability occurs in inflammatory bowel disease (IBD), graft-versus-host disease (GVHD), celiac disease, and multiple organ dysfunction. In these diseases, effectors of increased permeability include immune signaling,[1] microbiome,[2] and corticosteroids[3] that, in part, signal through epithelial myosin light chain kinase (MLCK). More modest permeability increases occur in other disorders, including irritable bowel syndrome (IBS), autism spectrum disorder (ASD), depression, and stress-related disorders. Data directly linking barrier loss to disease phenotypes, however, are lacking.

To define the impact of modestly-increased intestinal permeability, we studied transgenic mice with intestinal epithelial-specific constitutively-active myosin light chain kinase (CAMLCK) expression. This MLCK-dependent tight junction regulation increased intestinal permeability (Fig. S1A,B).[1] Nevertheless, postnatal growth (Fig. S1C), reproduction, intestinal transit (Fig. S1D), and intestinal histology, epithelial proliferation (a sensitive indicator of epithelial damage), and epithelial turnover are unaffected in CAMLCK transgenic (*CAMLCK^{Tg}*) mice.[1] In contrast, mucosal tumor necrosis factor-α, interferon-γ, IL-10, and IL-13 transcripts as well as numbers of lamina propria neutrophils, $CD4^+$ T cells, and IgA^+ plasma cells are modestly increased by CAMLCK expression.[1, 2] Subclinical inflammation is, therefore, present and, by microbiome-dependent, IL-17-mediated processes, affords partial protection from acute pathogen invasion.[2] Immune activation is nevertheless unlikely to amplify CAMLCK-driven permeability increases, as barrier function and ZO-1 anchoring are both acutely normalized by enzymatic MLCK inhibition.[1, 4]

We initially analyzed the gut microbiome of 31 WT and *CAMLCK^{Tg}* pups born to 8 WT dams. The microbiomes segregated by pup genotype but not dam (Fig. S1E) and included increased *Clostridium* and decreased Bacteroidetes, *Enterococcus spp,* and *Prevotella* in *CAMLCK^{Tg}* mice (Fig. S1F). Increased intestinal permeability can therefore cause dysbiosis-like microbiome shifts. Interestingly, maternal separation, which increases intestinal permeability, causes similar alterations and can be partially corrected by MLCK inhibitor-induced barrier restoration.[5]

Microbiome alterations overlapping with the above have been reported in IBS and ASD. We therefore asked if *CAMLCK^{Tg}* mice displayed anxiety-like behavior, as occurs in those disorders, using the open-field test (Fig. 1C). Both the percentage of distance traveled in the center and the fraction of time spent in the center of the open field were reduced in *CAMLCK^{Tg}* mice (Fig. 1C); this did not reflect reduced locomotor activity, as total distance traveled in the entire area was similar in *CAMLCK^{Tg}* and WT mice (Fig. 1C). These data are consistent with increased anxiety-like behavior in *CAMLCK^{Tg}* mice. Although the results cannot differentiate between

direct effects of increased permeability and those requiring intermediate mediators, these data demonstrate that intestinal permeability increases can influence behavior.

Stress and increased permeability have been associated with enhanced visceral sensitivity in humans and rodents. Surprisingly, *CAMLCK*^{Tg} mice displayed striking visceral analgesia to colorectal distension relative to WT littermates (Fig. 1D). Sensitivity was restored by enzymatic MLCK inhibition, water avoidance stress, or naloxone-mediated opioid receptor antagonism (Fig. 1D). Although this effect of increased permeability on visceral sensitivity was unexpected, it is remarkably similar to the naloxone-reversible visceral analgesia reported in chronically-stressed female rats[6] and naloxone-sensitive inhibition of nociceptive neurons by supernatants of colitic human and murine tissues.[7]

Studies of female IBS patients have linked increased permeability to altered functional and structural brain connectivity.[8] Thus, although responses to colorectal distension can be mediated by spinal reflexes and sensory, limbic, and paralimbic regions of the brain,[9] we asked if neuronal activation was modified by CAMLCK-induced permeability increases. C-Fos immunolabeling, an indicator of neuronal activity, was significantly greater in the paraventricular nucleus of the thalamus, the paraventricular nucleus of the hypothalamus, and the hippocampus, but not the medial prefrontal cortex, nucleus accumbens, or amygdala, of *CAMLCK*^{Tg} , relative to WT, mice (Figs. 2, S2). Increased intestinal permeability may therefore increase basal neuronal activity in areas of the brain that regulate responses to visceral pain or stress[9] but not those associated with conscious visceral sensation.

These results demonstrate that increased intestinal permeability can impact i) gut microbiome composition; ii) behavior; iii) visceral pain responses; and iv) neuronal activation within the brain. Critically, these changes are all results, rather than causes, of intestinal barrier loss, as the latter was induced by targeted CAMLCK expression.

The sites of neuronal activation in *CAMLCK*^{Tg} mice support the hypothesis that increased intestinal permeability can activate the hypothalamic-pituitary-adrenal axis.[10] Conversely, hypothalamic-pituitary-adrenal axis activation by exogenous stress can induce intestinal permeability increases.[3] Thus, as has been proposed in IBD and GVHD, a self-amplifying cycle may ultimately direct the diverse phenotypes induced by MLCK-dependent, intestinal permeability increases. Further study is needed to define the complex relationships between intestinal permeability, stress, behavioral alterations, visceromotor responses, microbiome composition, and other abnormalities.

These data are the first to assess behavior in a model where a targeted increase in intestinal tight junction permeability is the only direct perturbation. The results demonstrate, unequivocally, that modest tight junction permeability increases induced via a physiologically- and pathophysiologically-relevant mechanism are sufficient to trigger local and systemic microbial, behavioral, and neurosensory changes. This provides new perspective with which to understand previously hypothesized cause-effect relationships that have been proposed on the basis of correlative data.

Figure legends:

Figure 1: Increased intestinal permeability modifies behavior and visceral sensitivity. A. Videotracking paths of representative WT and *CAMLCK*^{Tg} mice in the open field test. Percent distance traveled in the center (dashed lines), percent time in the center, and overall distance traveled in the entire field are shown. *CAMLCK*^{Tg} (blue circles, n=8) and WT (red squares, n=9) littermates were tested. mean±SEM. *, p<0.05; **, p<0.01, Mann-Whitney U test. **B.** Stepwise colorectal distension-induced visceromotor responses in *CAMLCK*^{Tg} (blue circles, n=7) were reduced relative to WT (red squares, n=7) littermates. Genotype-specific differences were eliminated by MLCK inhibition, water avoidance stress, or naloxone treatment. n = 5-9 per condition; for each treatment (vehicle control *CAMLCK*^{Tg} and WT mice from the same experiment are shown with pale symbols in the last three graphs). mean±SEM; **, p<0.01, 2-way ANOVA.

Figure 2: Increased intestinal permeability induces increased C-Fos immunolabelling in selected brain regions. *CAMLCK*^{Tg} (blue circles, n=5-6) and WT (red squares, n=5-6) littermates. Representative images of C-Fos immunolabeled brains from *CAMLCK*^{Tg} and WT mice. Bars = 200 µm; mean±SEM; *, p<0.05, t-test.

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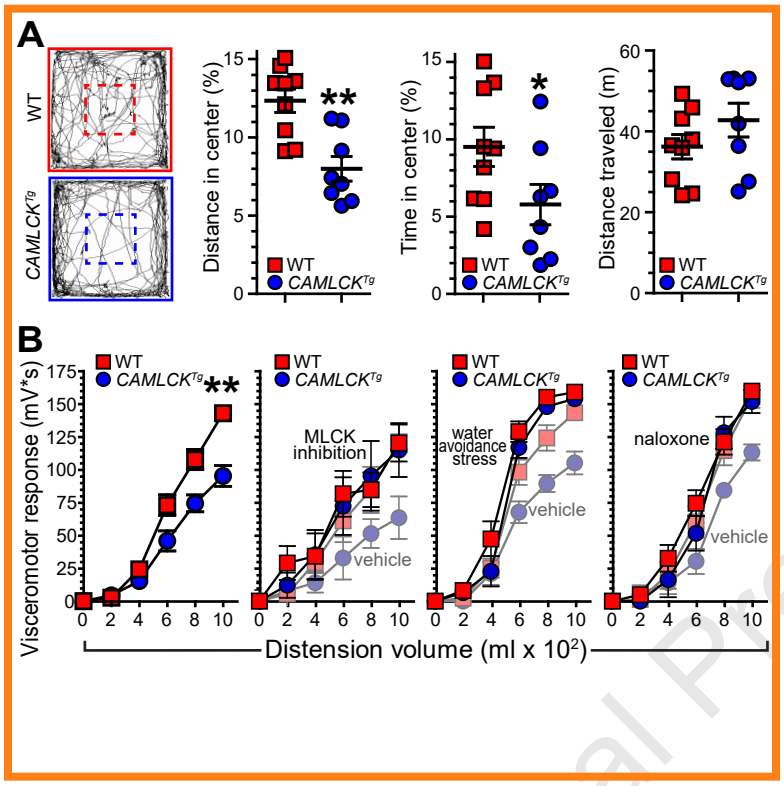

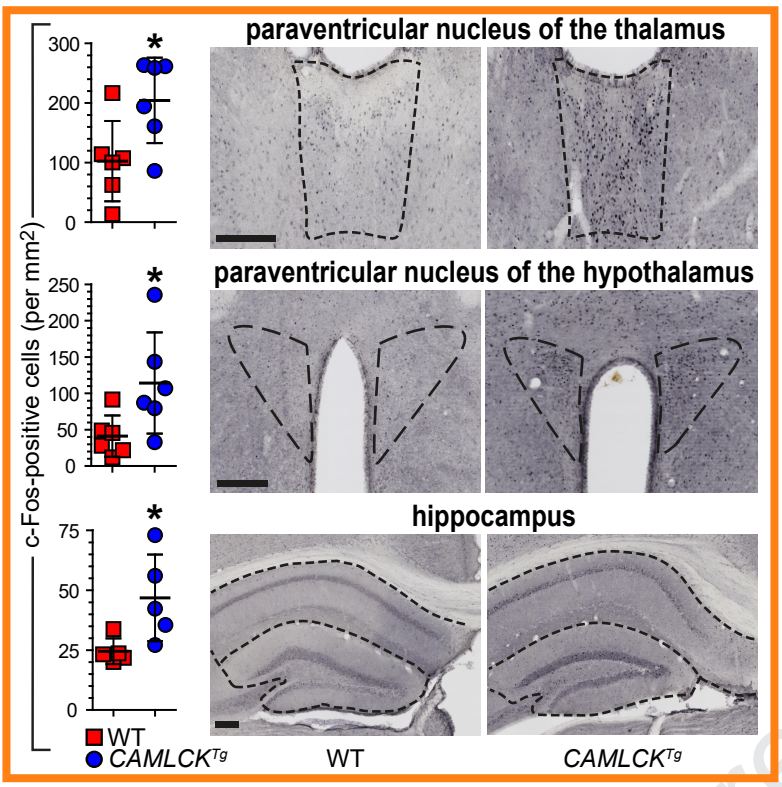

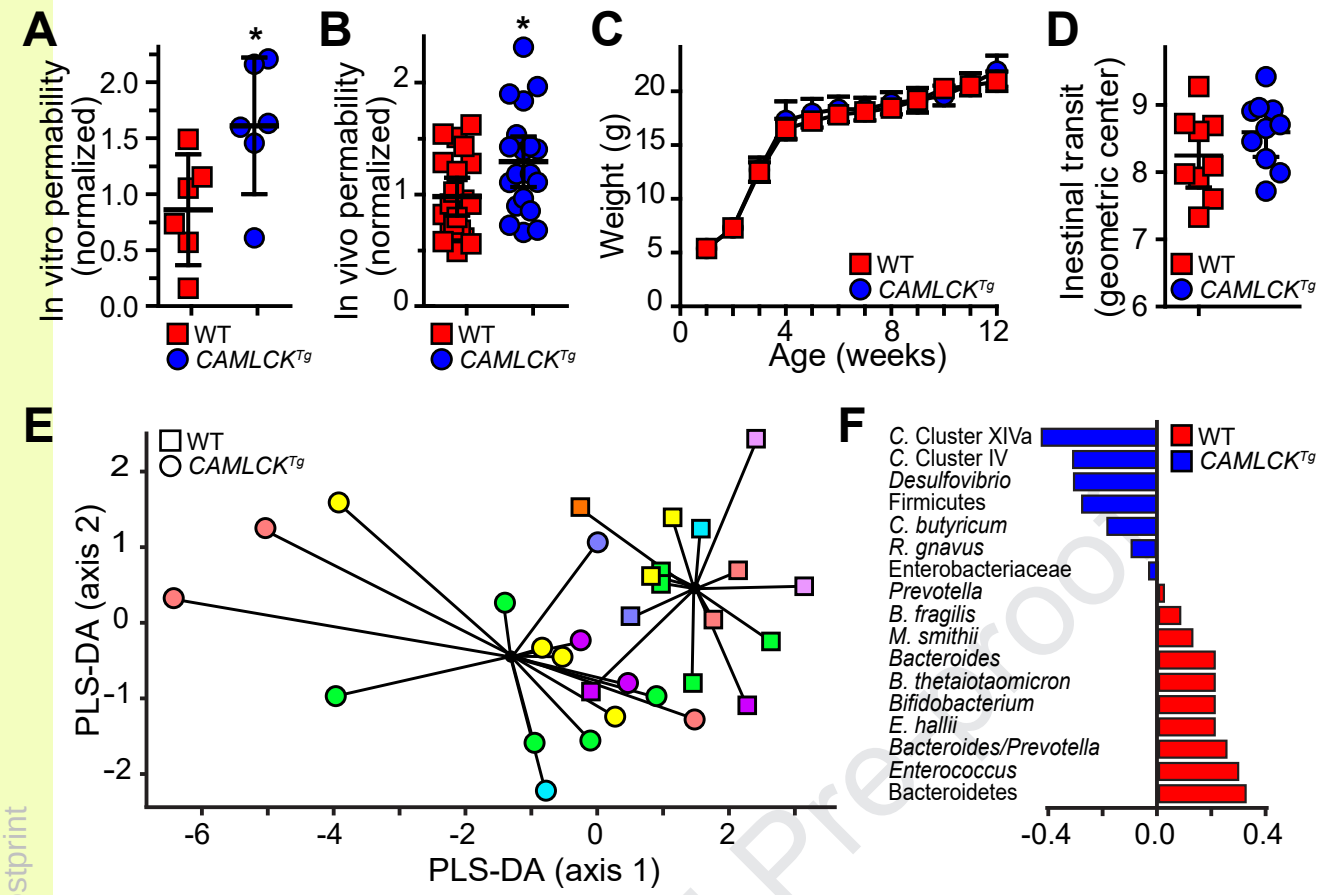

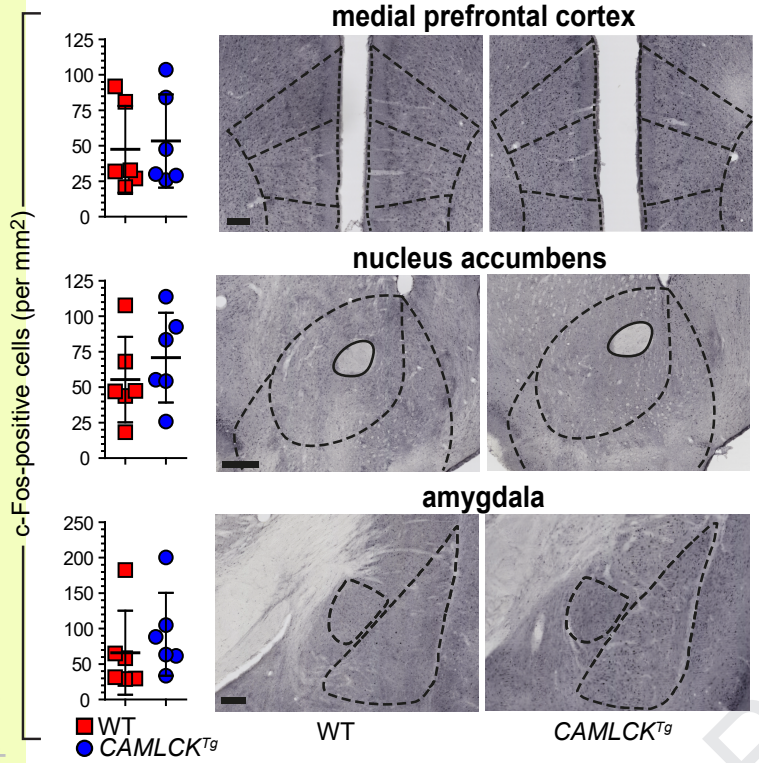

Supplemental Figure 1:

A. Trans-jejunal fluorescein flux was increased in *CAMLCK*^{Tg} (blue circles) relative to WT (red squares) littermates. mean±SD; *, p<0.05, Mann-Whitney U test.

B. In vivo analysis using FITC-4kDa dextran demonstrated increased permeability of *CAMLCK*^{Tg} (blue circles, n=19) relative to WT (red squares, n=20) littermates. mean±SD; *, p<0.05, t-test.

C. Weight gain was similar in WT (red squares, n=6) and *CAMLCK*^{Tg} (blue circles, n=6) littermates. mean±SD.

D. Intestinal transit was similar in WT (red squares, n=10) and *CAMLCK*^{Tg} (blue circles, n=9) littermates. mean±SD.

E. Partial least squares discriminant analysis (PLS-DA) score plot based on the relative abundances of 18 microbial taxa in gut contents of *CAMLCK*^{Tg} (circles, n=16) and WT (squares, n=15) born to 8 different dams (each color represents one dam).

F. Relative abundances of microbial communities in *CAMLCK*^{Tg} (blue) and WT (red) mice. Diagrams indicate regions analyzed.

Supplemental Figure 2:

CAMLCK^{Tg} (blue circles, n=5-6) and WT (red squares, n=5-6) littermates. Representative images of C-Fos immunolabeled brains from *CAMLCK*^{Tg} and WT mice. Bars = 200µm; mean±SEM; *, $p<0.05$, t-test.

Supplemental Methods

Animals

CAMLCK^{Tg} mice[1-4] (Tg(Vil-FLAG-CAMLCK)#Jrt) were maintained as male heterozygotes on C57BL/6J background. These were mated with WT C57BL/6J females to produce WT and *CAMLCK*^{Tg} littermates. At weaning, female mice were separated and housed at constant temperature (22±1℃) with a 12 hour light/dark cycl e. Food (Teklad 2018, Envigo) and water were available ad libitum. All experiments were performed at 8 weeks of age. Procedures were approved by the Ethical Committee CEEA-86, under the number APAFiS#4145.

Gut microbiota composition analysis

Gut microbiota were analyzed in two cohorts (15 WT and 16 *CAMLCK*^{Tg}) from 8 different WT dams. At sacrifice, colonic contents were stored at -80℃. DNA was extracted using the ZR fecal DNA MiniPrep kit (Zymo Research) and adjusted to 1 ng/µL. Changes in relative abundance of 24 microbial 16S rRNA gene targets were obtained by qRTPCR using an adapted Gut Low-Density Array platform.[5-7] A universal bacterial primer set was included as the reference gene. qRTPCR was performed in duplicate on a ViiA7 (Applied Biosystems).

Fluorescence data was imported into LinRegPCR to perform baseline corrections, calculate mean PCR efficiency per amplicon group. and calculate initial quantities. Among the 24 targeted amplicon groups, 6 were not detected in any fecal samples and were removed from the analysis (*B. vulgatus, Alistipes spp., Parabacteroidetes distasonis, Roseburia spp., E. coli and A. muciniphila*). Normalized N_0-values were $\log_{10}$-transformed and processed by MixOmics (v6.1.1) with RStudio (v1.0.44) to build a partial least-squares discriminant analysis (PLS-DA). This multivariate supervised approach projects samples (X) onto a low-dimensional space of latent variables to maximize separation between groups according (Y=genotype). Leave-one-out cross-validation was used to select the optimal number of latent variables for PLS-DA models.

Open field test

Mice explored a 50x50cm arena (illumination 300lux) for 10min. Exploration was automatically assessed using a video tracking system (Bioseb). The percentage of distance traveled and time spent and in the center area (20x20cm) and total distance traveled in the entire arena were assessed.

Colorectal distension (CRD)

Two 0.08mm diameter electrodes were implanted in the abdominal external oblique muscle and a third in the abdominal skin. On postoperative days 3-6, CRD was performed using a balloon catheter (Fogarty 4F catheter, 1.1cm length, tip 3.5cm from the anus)[8] in 10 sec periods with increasing volumes from 0.02 mL to 0.10 mL, with 5min rest between distensions. Abdominal electromyography activity was registered after the amplification (10000x) and analyzed (Powerlab Chart 5). Basal EMG activity was subtracted from EMG activity registered during distension. Some mice were treated with ML-7 (2 mg/kg i.p.) or naloxone sulfate (2 mg/kg i.p.) 1h before CRD. For others, water avoidance stress was induced on a floating platform (3cmx3cm) in the middle of a water-filled tank (40cmx40cm) for 1h daily over four days. Recovery (30min) preceded CRD.

Gastrointestinal transit

Animals received 70µL of 100mg/ml TRITC-70kDa dextran in tap water by gavage and were sacrificed 1 h later.[9] Stomach, small and large intestine were cut in 11 equal parts. Luminal contents of each segment were centrifuged and fluorescence determined. Transit was calculated as the geometric center of the values for each mouse.

Ussing chamber analysis

Jejunal sections were mounted in Ussing chambers (Physiologic Instruments) filled with Krebs buffer and continuously oxygenated (95% O2, 5% CO2). After 1 hour of equilibration, Fluorescein (1mg/mL) was added in the apical chamber and fluorescence intensity of the basolateral chamber was measured after 1 hour.

In vivo permeability analysis

Mice were fasted for 4 hours before gavage with 150µL of 100mg/mL FITC-4kDa dextran in tap water. Blood (200µL) was collected after 4h and plasma fluorescence determined.

C-Fos analysis

Vibratome sections (40µm) were stained using polyclonal rabbit anti-C-Fos (Santa Cruz) and secondary HRP-conjugated goat anti-rabbit antisera (Jackson ImmunoResearch). NDPI images (x20) were obtained (Nanozoomer, Hamamatsu Photonics) and converted into TIFF format using ImageJ (NDPI tools plugin). Regions of interest (ROI) were manually circumscribed using ROI tools and C-Fos-immunoreactive cells quantified automatically using the particle analysis function (size: 5-20 µm²; circularity: 0.5-1). For each animal, 3-6 sections of each brain area were assessed by a blinded observer.

Statistical analysis

Statistical significance was determined by two-tailed t-test, two-tailed Mann-Whitney U test, or 2-way ANOVA and set at $p < 0.05$. For microbial analyses, univariate analysis was realized in parallel to compare each amplicon separately using unpaired t-test followed by the Benjamini-Hochberg adjustment of *p*-values for multiple comparisons.

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