



OPEN Intra-gastric administration of short chain fatty acids greatly reduces voluntary ethanol intake in rats

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Alcohol use disorder (AUD) represents a public health crisis with few FDA-approved medications for its treatment. Growing evidence supports the key role of the bidirectional communication between the gut microbiota and the central nervous system (CNS) during the initiation and progression of alcohol use disorder. Among the different protective molecules that could mediate this communication, short chain fatty acids (SCFAs) have emerged as attractive candidates, since these gut microbiota-derived molecules have multi-target effects that could normalize several of the functional and structural parameters altered by chronic alcohol abuse. The present study, conducted in male alcohol-preferring UChB rats, shows that the initiation of voluntary ethanol intake was inhibited in 85% by the intra-gastric administration of a combination of SCFAs (acetate, propionate and butyrate) given before ethanol exposure, while SCFAs administration after two months of ethanol intake induced a 90% reduction in its consumption. These SCFAs therapeutic effects were associated with (1) a significant reduction of ethanol-induced intestinal inflammation and damage; (2) reduction of plasma lipopolysaccharide levels and hepatic inflammation; (3) reduction of ethanol-induced astrocyte and microglia activation; and (4) attenuation of the ethanol-induced gene expression changes within the nucleus accumbens. Finally, we determined that among the different SCFAs evaluated, butyrate was the most potent, reducing chronic ethanol intake in a dose–response manner. These findings support a key role of SCFAs, and especially butyrate, in regulating AUD, providing a simple, inexpensive, and safe approach as a preventive and intervention-based strategy to address this devastating disease.

Keywords Alcohol abuse, Gut microbiota-brain axis, SCFAs, Butyrate, Alcohol use disorder treatment

Alcohol is the most consumed addictive substance in the world, often leading to the development of alcohol use disorders (AUD)¹. Alcohol dependence has traditionally been considered a brain disorder involving the alteration of various neurotransmitters and their receptors in specific areas such as the brain reward circuit². These alterations play a major role in the positive and negative reinforcement processes driving the motivation for ethanol seeking². However, pharmacological approaches targeting these neurotransmitter systems have generated only small therapeutic effects³, suggesting the involvement of other biological processes that could also be driving the addictive behavior.

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Over the past few years, several studies have supported the role of the gut microbiota, a large community of microorganisms colonizing the intestine, as an important component mediating the development of neuropsychiatric disorders⁴, including addictive behaviors^{5,6}. Alterations in gut microbiota composition, have been observed both in male and female patients presenting AUD^{7,8} and in animal models characterized by high alcohol intake^{9,10}. This disturbance in bacterial community could communicate with the central nervous system (CNS) through the gut brain axis, influencing brain function and behavior, thus accelerating the cycle of addiction^{5,9,11}.

In animal models exposed to alcohol^{12,13} and in alcohol-dependent individuals^{14,15}, it has been reported that alcohol and its first metabolite, acetaldehyde, induce intestinal damage and inflammation, altering the intestinal epithelial permeability, a phenomenon known as “leaky gut”⁸. This hyper-permeable gut mucosa allows lipopolysaccharides (LPS) and other pro-inflammatory/pathogenic microbial products to easily reach the systemic circulation and activate the Toll-like receptor 4 (TLR4) of immune cells in target organs, including the liver and other peripheral tissues. Remarkably, microglia and astrocytes, the main immunological cells in the brain, also express TLR4 and consequently respond to pathogen-associated molecular patterns by producing pro-inflammatory cytokines¹⁶. This inflammatory response promotes neuroinflammation and epigenetic changes¹⁷, reducing glutamate transport¹⁸, thereby increasing extracellular glutamate levels that potentiate drinking behavior^{19,20}. Conclusively, the use of TLR4-knockout mice demonstrates that activation of TLR4 in astrocytes and microglia following ethanol exposure is crucial for inducing neuroinflammation²¹. Moreover, intraperitoneal injection of LPS induces a long-lasting increase in ethanol intake in mice²², indicating a major role of increased gut permeability in alcohol addictive behavior.

A decrease in beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* has been shown in animals exposed to alcohol and in alcohol-dependent patients^{9,15}; therefore, restoring these anti-inflammatory bacteria could represent a potential therapeutic approach for improving alcohol-related diseases and reduce alcohol dependence. Accordingly, in animal models of alcohol-induced liver disease, modulation of the gut microbiota by *Lactobacillus GG* supplementation reduces gut leakiness, endotoxemia, and liver inflammation¹³. Moreover, *Lactobacillus GG* supplementation also reduces both (1) the acquisition of high ethanol intake when administered before ethanol exposure to the ethanol-drinking UChB rat strain⁵ and (2) ethanol relapse when administered to chronically ethanol-drinking UChB animals⁹. Similarly, six months of *Lactobacillus GG* supplementation to alcohol-dependent patients attenuates liver injury and reduces heavy alcohol intake²³, indicating that probiotic supplementation could be a therapeutic alternative for AUD treatment.

The mechanisms underlying the communication between the administered probiotics and the CNS mediating the behavioral effects have not yet been elucidated. However, the three main short-chain fatty acids (SCFAs; acetate, propionate, and butyrate), beneficial lipid metabolites generated by colonic dietary fiber fermentation by some anti-inflammatory gut bacterial strains²⁴, have gained attention due to their anti-inflammatory properties and the ability to improve intestinal barrier integrity^{25,26}. Additionally, SCFAs have neuroactive properties due to their role as histone deacetylase (HDAC) inhibitors²⁷, directly influencing brain function and behavior^{28–30}. Interestingly, alcohol consumption directly decreases the abundance of SCFA-producing bacteria¹⁵ and lowers SCFA levels in stools and blood^{7,31}. Thus, oral SCFA supplementation could represent an attractive alternative for the treatment of alcohol use disorder.

SCFAs supplementation has several advantages compared with the administration of probiotics, including (1) direct and rapid effects; (2) dosage consistency; (3) independence from interindividual microbiota variability, and (4) lower risk of infections and adverse effects³². Thus, in this work, we evaluated whether the intragastric administration of the SCFAs acetate, propionate and butyrate given before or after ethanol exposure prevent the acquisition phase of ethanol intake or reduce a previously established chronic alcohol consumption, respectively. We also explored the mechanism behind the SCFAs therapeutic effects by evaluating intestinal architecture and inflammation, liver inflammation, microglial and astrocyte activation, and transcriptional dynamics in the nucleus accumbens, the main brain center controlling drug addiction. Finally, we determined the most potent SCFA in reducing chronic ethanol intake and its effects on the levels of histone acetylation.

Results

Intragastric co-administration of SCFAs prevents the acquisition of high voluntary alcohol intake in ethanol-naïve rats

To determine whether the intragastric administration of SCFAs prevents the acquisition of ethanol intake, ethanol-naïve male UChB rats were given a combination of three SCFAs containing 300 mg/kg sodium acetate, 300 mg/kg sodium propionate and 300 mg/kg sodium butyrate dissolved in distilled water. A second group (control) was treated with 544 mg/kg of NaCl to rule out the influence of sodium provided by the three SCFAs on the intake of ethanol solution and water in the SCFAs treated group. Another control group received the vehicle (distilled water). All treatments were administered via oral gavage twice-daily for six days before access to ethanol.

Voluntary alcohol preference was subsequently evaluated daily for seven days while maintaining the two daily administrations of SCFAs or vehicle. Animals were offered a two-bottle-choice access between a 10% ethanol solution (v/v) and water. Data indicated that SCFAs administration induced an 85% reduction ($p < 0.0001$) in voluntary ethanol intake (Fig. 1A) and in ethanol preference ($p < 0.0001$) (Fig. 1B) compared with control animals treated with NaCl or the vehicle. The inhibition in alcohol intake induced by the SCFAs administration was fully compensated by an increase in water intake ($p < 0.0001$) (Fig. 1C), normalizing the animal's hydric homeostasis (Fig. 1D). The finding that no increase in water intake was observed in control rats treated with NaCl rules out a possible role of the sodium provided by SCFAs in the increase in water intake observed in the SCFAs-treated group (Fig. 1C). Additionally, the finding that SCFAs administration did not induce changes in total fluid intake or body weight (Fig. 1D; top and bottom, respectively) compared with the NaCl- and vehicle-treated groups

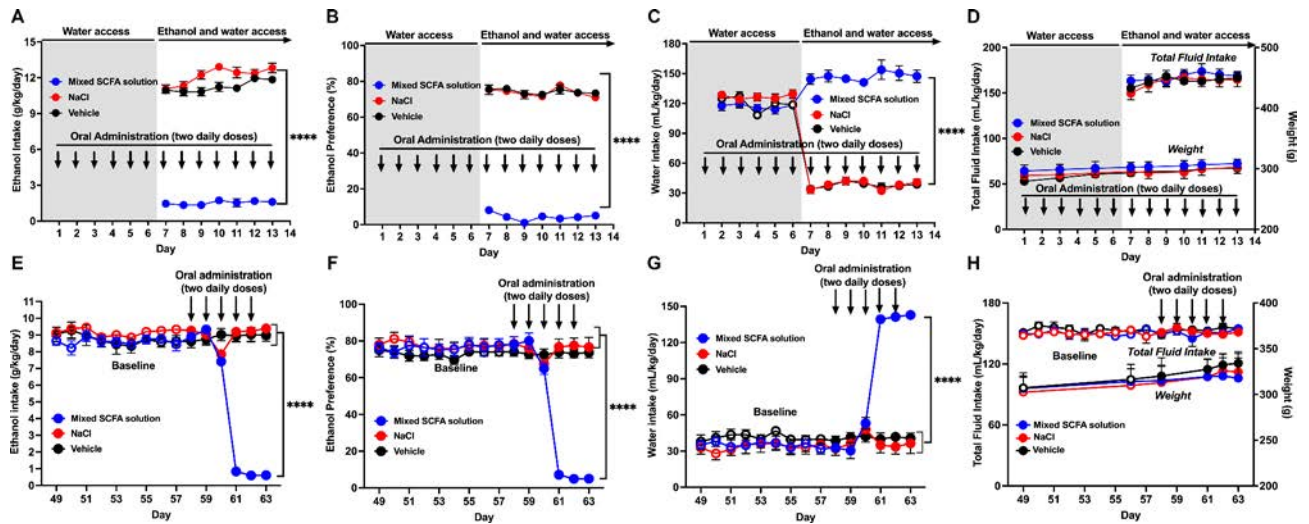


Fig. 1. Intra-gastric administration of SCFAs prevents voluntary alcohol intake and alcohol preference in ethanol-naïve animals and reduces voluntary alcohol intake and preference in animals chronically drinking ethanol. Ethanol-naïve male UChB rats were treated with either (1) a combination of SCFAs (300 mg/kg each sodium acetate, sodium propionate, and sodium butyrate), 554 mg/kg NaCl solution, or vehicle, by oral gavage twice-daily for six days. Next, animals were exposed to 10% ethanol solution and water. SCFAs or vehicle administration was maintained for additional seven days. **(A)** Voluntary ethanol intake is expressed in g/kg body weight/day. We observed a significant effect of treatment [$F_{\text{treatment}(2,15)} = 662.1, p < 0.0001$], day [$F_{\text{day}(3613, 54.19)} = 10.88, p < 0.0001$] and a significant treatment \times day interaction [$F_{\text{interaction}(12,90)} = 3.335, p < 0.0005$] compared to the vehicle group. The administration of SCFAs induced a significant reduction of ethanol intake compared to both the NaCl and the vehicle groups. **(B)** Ethanol preference was expressed as a percentage of total fluid intake, calculated as the sum of daily alcohol and water consumption. Two-way ANOVA (treatment \times day) revealed significant effect of treatment [$F_{\text{treatment}(2,15)} = 3008, p < 0.0001$], day [$F_{\text{day}(3927, 58.91)} = 3.382, p < 0.01$], but not significant interaction compared to the vehicle group. Tukey post hoc indicate that the administration of SCFAs induced a significant reduction of ethanol preference. **(C)** Water intake is expressed in mL/kg body weight/day. We observed a significant effect of treatment [$F_{\text{treatment}(2,15)} = 401.0, p < 0.0001$] compared to the vehicle group. The administration of SCFAs induced a significant increase in water intake compared to the vehicle group. **(D)** Total fluid intake is expressed in mL/kg body weight/day, and weight is expressed in grams. The administration of SCFAs did not induce a significant change in total fluid intake [$F_{\text{treatment}(2,15)} = 0.2688, p:0.76$] or in body weight [$F_{\text{treatment}(2,15)} = 0.3229, p:0.72$] compared to the vehicle group. Note that body weight did not differ among the three groups of rats before alcohol access (Mean \pm SEM: SCFA group 299 ± 9.34 g, NaCl group 291 ± 3.4 g, and vehicle group 285 ± 1.85 g; one-way ANOVA $F_{\text{treatment}(2,45)} = 1.448, p:0.2458, \text{N.S.}$). Male UChB rats voluntarily consuming a 10% ethanol solution for 58 days were treated with the same combination of SCFAs, 554 mg/kg NaCl solution, or vehicle, by oral gavage twice-daily for five days. **(E)** Ethanol intake is expressed in g/kg body weight/day. We observed a significant effect of treatment [$F_{\text{treatment}(2,15)} = 8.811, p < 0.01$], day [$F_{\text{day}(5202, 78.02)} = 81.86, p < 0.0001$] and a significant treatment \times day interaction [$F_{\text{interaction}(28,210)} = 94.80, p < 0.0001$] compared to the vehicle group. The administration of SCFAs induced a significant reduction in ethanol intake compared to both the NaCl and the vehicle groups. **(F)** Ethanol preference was expressed as a percentage of total fluid intake, calculated by adding alcohol and water consumption. Two-way ANOVA (treatment \times day) revealed significant effect of treatment [$F_{\text{treatment}(2,15)} = 67.17, p < 0.0001$], day [$F_{\text{day}(2625, 39.37)} = 115.7, p < 0.0001$] and significant interaction [$F_{\text{interaction}(8,60)} = 146.8, p < 0.0001$], compared to the vehicle group. Tukey post hoc indicate that the administration of SCFAs induced a significant reduction of ethanol preference compared to the vehicle and NaCl groups. **(G)** Water intake is expressed in mL/kg body weight/day. We observed a significant effect of treatment [$F_{\text{treatment}(2,15)} = 61.50, p < 0.0001$], day [$F_{\text{day}(2757, 41.36)} = 103.9, p < 0.0001$] and a significant treatment \times day interaction [$F_{\text{interaction}(8,60)} = 128.1, p < 0.0001$] compared to the vehicle group. The administration of SCFAs induced a significant increase in water intake compared to the vehicle group. **(H)** Total fluid intake is expressed in mL/kg body weight/day, and weight is expressed in grams. The administration of SCFAs did not induce a significant change in total fluid intake [$F_{\text{treatment}(2,15)} = 0.8664, p:0.44$] or in body weight [$F_{\text{treatment}(2,15)} = 0.06547, p:0.93$] compared to the vehicle group. Arrows indicate intra-gastric administration of SCFAs, NaCl, or vehicle. Data are presented as mean \pm SEM. $N = 6$ animals per group. Two-way ANOVA followed by Tukey *post-hoc* tests, **** $p < 0.0001$.

suggests that the observed behavioral effect on ethanol intake is attributable to SCFAs administration rather than to any nonspecific discomfort. Note that no significant differences were found in body weight among the three groups of rats before ethanol access (Fig. 1D).

Chronic voluntary alcohol intake is markedly inhibited by intragastric co-administration of three SCFAs

Subsequently, we evaluated whether intragastric co-administration of SCFAs would also reduce voluntary ethanol intake, but now in animals that had been drinking alcohol chronically. In these studies, male UChB rats were maintained with free access to 10% ethanol solution and water for 58 days to induce chronic ethanol dependence, leading to a voluntary ethanol intake of 9 g/kg/day (Fig. 1E). At that time, animals were divided into three experimental groups and treated with (1) a combination of SCFAs containing 300 mg/kg sodium acetate, 300 mg/kg sodium propionate and 300 mg/kg sodium butyrate; (2) NaCl at a dose of 554 mg/kg containing the same molar concentration of sodium as that received by the animals treated with SCFAs; or (3) distilled water. All treatments were administered by oral gavage twice-daily for five days. The administration of SCFAs led to a very strong reduction in chronic ethanol intake (~93%, $p < 0.0001$) (Fig. 1E) and ethanol preference ($p < 0.0001$) (Fig. 1F) compared to the control groups. This effect did not appear immediately after SCFAs treatment but occurred with a lag time of about three days following the first SCFAs dose administration. Once again, SCFAs administration induced a significant increase in water intake ($p < 0.0001$) (Fig. 1G), normalizing animal's hydric homeostasis without affecting animal body weight (Fig. 1H).

Intragastric co-administration of three SCFAs reduces ethanol-induced intestinal and hepatic inflammation and restores normal intestinal architecture

Then, we evaluate whether SCFAs administration could reduce ethanol-induced intestinal alterations. For this, samples of proximal colon were obtained 24 h after the last SCFAs or vehicle administration. At histological level, we observed that chronic alcohol intake induced a significant increase ($p < 0.0001$) in the length of the intestinal crypts (Fig. 2A and B), one of the common markers of intestinal damage³³, and a significant increase ($p < 0.01$) in lamina propria cell infiltration in the colonic mucosa (Fig. 2C and D) compared to ethanol-naïve animals. However, SCFAs administration completely normalized the length of the intestinal crypts ($p < 0.0001$) (Fig. 2A and B), and lamina propria cell infiltration ($p < 0.0001$) (Fig. 2C and D) compared to vehicle-treated animals.

Additionally, we evaluated the ratio of proliferating cells in the lamina propria of the proximal colon samples. Proliferating cell nuclear antigen (PCNA) positivity was significantly reduced ($p < 0.01$) in animals chronically drinking ethanol and treated with SCFAs compared to animals chronically drinking ethanol and treated with the vehicle (Supplementary Fig. 1A and B). Then, we evaluated if PCNA-positive areas in the lamina propria correlated with cell infiltrate density, as a surrogate for proliferation rate. We observed a direct association between cell infiltrate density and PCNA-positive areas (Supplementary Fig. 1C), suggesting that infiltrating cells are actively proliferating and potentially contributing to epithelial barrier disruption, as previously reported³⁴.

Ethanol-induced intestinal inflammation was further corroborated measuring the mRNA levels of pro-inflammatory cytokines by RT-qPCR in samples of proximal colon. Chronic ethanol intake induced a significant increase in the expression levels of IL1 β , IL8 and TNF- α ($p < 0.01$) compared to ethanol-naïve animals, while SCFAs administration restored these levels to normal levels ($p < 0.05$) (Fig. 2E).

We further evaluated whether ethanol-induced intestinal alterations could lead to an increased intestinal LPS permeability and the concomitant increase in liver inflammation. For this, plasma LPS levels were measured by ELISA, while hepatic inflammation was evaluated by RT-qPCR. We observed that chronic ethanol intake induced a significant increase ($p < 0.01$) in plasma LPS levels (Fig. 3A), which was associated with a significant increase in the hepatic expression levels of the LPS-binding protein (LBP) ($p < 0.001$) and the pro-inflammatory cytokines IL1 β ($p < 0.0001$), and TNF- α ($p < 0.001$) (Fig. 3B) compared to ethanol-naïve animals. However, SCFAs administration completely abolished the ethanol-induced increase in plasma LPS levels ($p < 0.01$) (Fig. 3A) and reduced the ethanol-induced increase in hepatic LBP ($p < 0.01$), IL1 β ($p < 0.05$) and TNF- α ($p < 0.05$) expression levels (Fig. 3B).

Intragastric co-administration of three SCFAs reduces ethanol-induced neuroinflammation

To assess ethanol-induced neuroinflammation, astrocyte and microglia morphology was evaluated by immunofluorescence in the hippocampus. As previously reported³⁵, chronic alcohol intake induced a significant increase in astrocyte and microglial activation, evidenced by a significant increase in astrocyte surface area ($p < 0.05$) (Fig. 4A, B and E), total length of astrocytic processes ($p < 0.05$) (Fig. 4A, B and F), the astrocytic ramification index ($p < 0.01$) (Fig. 4A, B and G), and the microglial solidity ($p < 0.05$) (Fig. 4C, D and H) compared to ethanol-naïve animals. In contrast, SCFA administration significantly reduced ethanol-induced increase in astrocyte surface area ($p < 0.01$) (Fig. 4A, B and E), total length of astrocytic processes ($p < 0.01$) (Fig. 4A, B and F), and the microglial solidity ($p < 0.0001$) (Fig. 4C, D and H), while no effects were observed at the level of astrocytic ramification index (Fig. 4A, B and G).

Intragastric co-administration of three SCFAs reduces alterations in the transcriptional dynamics of the nucleus accumbens induced by chronic ethanol intake

The nucleus accumbens has repeatedly been demonstrated to be a key component of the circuitry associated with excessive alcohol consumption³⁶. To explore the gene regulatory mechanisms in UChB animals chronically drinking ethanol, and how this pattern changes upon SCFAs administration, we performed an RNA-Seq analysis. In the comparison of ethanol-naïve vs chronic ethanol intake, we found 367 differentially expressed genes (DEGs) at the adjusted $p < 0.05$, with 206 up-regulated and 161 down-regulated (Fig. 5A). For chronic ethanol intake vs SCFAs condition, 63 DEGs were found, with 5 up-regulated and 58 down-regulated ($p < 0.05$; Fig. 5B), while

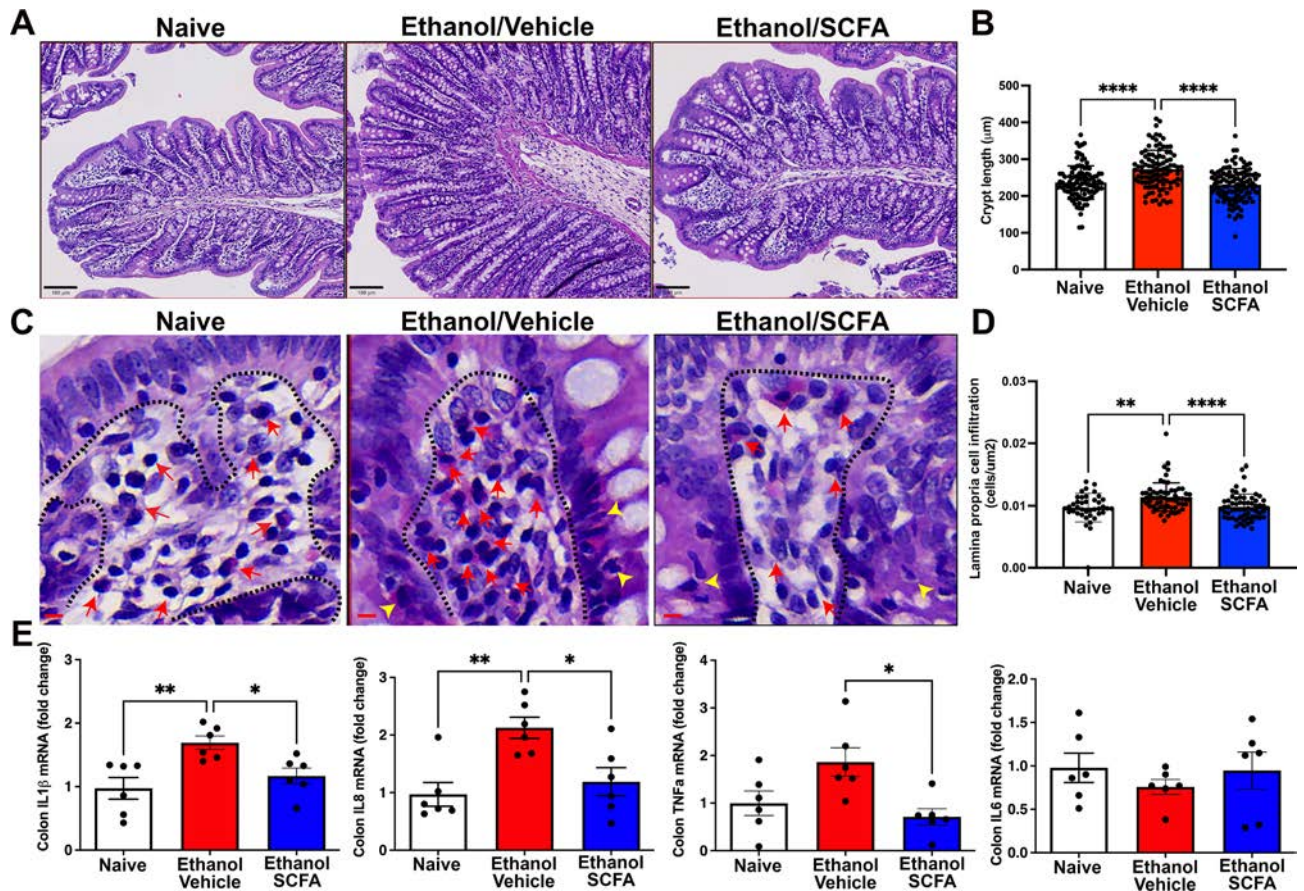


Fig. 2. Intra-gastric administration of SCFAs to animals chronically consuming ethanol restores normal intestinal architecture and reduces ethanol-induced inflammation in the proximal colon. Male UChB rats, voluntarily consuming a 10% ethanol solution for 58 days, were treated with either a combination of SCFAs (300 mg/kg each sodium acetate, sodium propionate and sodium butyrate), or 554 mg/kg NaCl solution (vehicle), by oral gavage twice-daily for five days. An additional group of untreated animals drinking only water (naïve) was used as a control. **(A)** Representative haematoxylin & eosin staining of proximal colon tissue showing crypts length. **(B)** Quantification of crypt length. **(C)** Representative hematoxylin & eosin staining of proximal colon tissue showing lamina propria cell infiltration, with dashed black line separating epithelial lining from lamina propria compartments. Red arrows point to polymorphonuclear and eosinophil leukocytes, plasma cells and macrophages, based on morphological identification. Yellow arrows point to intraepithelial polymorphonuclear cell infiltration. Scale bar 10 μm . **(D)** Quantification of lamina propria cell infiltration in the colonic mucosa. **(E)** Quantification of mRNA levels of the pro-inflammatory cytokines IL-1 β , IL-8, TNF- α and IL-6 determined by RT-qPCR in samples of proximal colon. Data are presented as mean \pm SEM. N = 6 animals per group. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$, One-way ANOVA followed by Bonferroni *post-hoc* test.

for the ethanol-naïve vs SCFAs conditions, 105 DEGs were found, with 59 up-regulated and 46 down-regulated ($p < 0.05$; Fig. 5C). The clustering heatmap of DEGs presented the differences in the transcriptomic profiles between the different experimental conditions (Supplementary Fig. 2A, B, and C). In the two most important comparisons (Ethanol-Naïve vs. Chronic Ethanol and Chronic Ethanol vs. SCFAs), we identified a total of 35 common differentially expressed genes. The expression of these genes was altered in the ethanol condition, but their expression levels were restored when chronic ethanol drinking animals were treated with SCFAs (Fig. 5D). The full list of all significantly regulated genes in all comparison is presented in Supplementary Table 2.

The differentially expressed genes underwent Gene-Ontology (GO) pathway enrichment analysis, classifying the up- and down-regulated genes. In the GO terms analysis for the Ethanol-Naïve vs chronic ethanol samples, we found up-regulated genes involved in biological processes such as “Response to endoplasmic reticulum stress” and “Regulation of cytosolic calcium ion concentration”. Importantly, genes involved in “Synapse organization”; “Response to toxic substances” and “Negative regulation of neuron apoptotic process”, were found to be down-regulated (Fig. 5E). Conversely, for the chronic ethanol vs SCFAs conditions, down-regulated genes were associated with “Regulation of behavior” and “Central nervous system development” (Fig. 5F). For the ethanol-Naïve vs SCFAs conditions, we found up-regulated genes involved in processes such as “Positive regulation of protein kinase B signaling” and “Response to glucocorticoid” (Fig. 5G). Altogether, these data demonstrated that chronic ethanol intake leads to marked transcriptional changes, and that SCFAs treatment attenuates some of the molecular phenotypes.

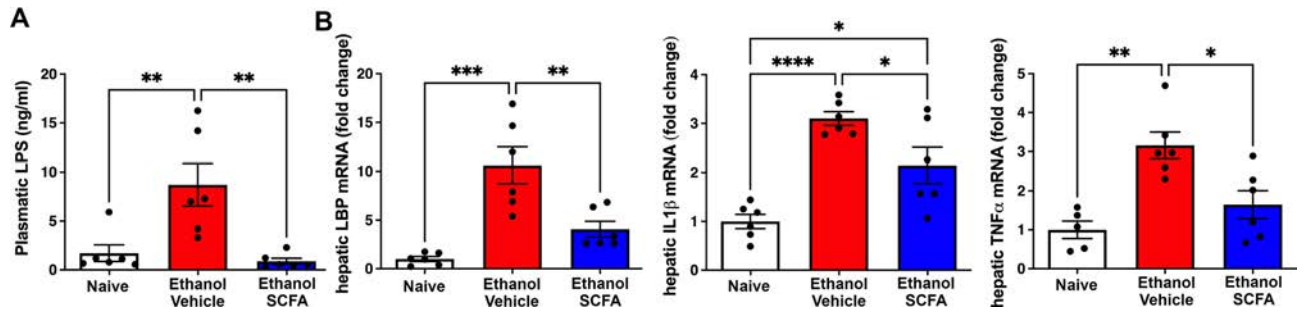


Fig. 3. Intra-gastric administration of SCFAs reduces both plasma LPS levels and liver inflammation induced by chronic ethanol consumption. Male UChB rats, voluntarily drinking a 10% ethanol solution for 58 days were treated with either a combination of SCFAs (300 mg/kg each sodium acetate, sodium propionate and sodium butyrate), or ii) 554 mg/kg NaCl solution (vehicle), by oral gavage twice-daily for five days. An additional group of untreated animals drinking only water (naïve) was used as a control. **(A)** Plasmatic levels of LPS were determined by ELISA. **(B)** mRNA levels of the LPS binding protein (LBP) and the pro-inflammatory cytokines IL-1 β and TNF- α were determined by RT-qPCR in hepatic samples. Data are presented as mean \pm SEM. N = 6 animals per group. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001, One-way ANOVA followed by Bonferroni *post-hoc* test.

Sodium butyrate is the most potent SCFA in reducing chronic voluntary ethanol intake

Then, we wanted to know which of the SCFAs is the most potent in reducing chronic voluntary ethanol intake. For this, a new group of male UChB rats was maintained with free access to 10% (v/v) ethanol solution and water for 45 days, and at that time, animals were divided into five groups and treated with either (1) a combination of SCFAs containing 300 mg/kg sodium acetate, 300 mg/kg sodium propionate and 300 mg/kg sodium butyrate; (2) 300 mg/kg sodium butyrate; (3) 300 mg/kg sodium propionate; (4) 300 mg/kg sodium acetate or (5) distilled water. All treatments were administered by oral gavage twice-daily for five days. When administered alone, all SCFAs were able to significantly reduce chronic voluntary ethanol intake compared to the vehicle-treated animals (Fig. 6A). However, the most potent therapeutic effect was achieved by sodium butyrate, which induced a ~72% reduction in ethanol intake compared to the vehicle-treated rats (p < 0.0001), an effect that was significantly more potent than the ~48% reduction induced by sodium propionate and sodium acetate (p < 0.001) (Fig. 6A). Once butyrate was identified as the most potent SCFA, we performed a dose–response curve to determine which is the optimal butyrate dose to reduce voluntary alcohol intake. For this, a new group of male UChB rats was given free access to 10% ethanol solution and water for 64 days, and at that time, animals were divided into four groups and treated either with (1) 300 mg/kg sodium butyrate; (2) 30 mg/kg sodium butyrate; (3) 3 mg/kg sodium butyrate or (4) distilled water. All treatments were administered by oral gavage twice-daily for five days. We observed that intra-gastric administration of 300 mg/kg sodium butyrate induced the strongest therapeutic effect, leading to an ~89% reduction in voluntary ethanol intake (p < 0.0001) compared to vehicle-treated animals, while intra-gastric administration of 30 mg/kg sodium butyrate induced a ~51% reduction in voluntary ethanol intake (p < 0.0001), and the administration of 3 mg/kg sodium butyrate had no therapeutic effect (Fig. 6B). For both the 300 mg/kg and 30 mg/kg sodium butyrate doses, the therapeutic effect was completely lost three days after treatment discontinuation (Fig. 6B).

Sodium butyrate increases histone acetylation

It has been reported that sodium butyrate can act as a potent inhibitor of the histone deacetylase activity, leading to histone hyperacetylation and changes in gene expression³⁷. Thus, we measured histone acetylation level by Western blot both in the striatum and the prefrontal cortex, since these brain areas play a key roles in mediating the acute and chronic effects of drugs³⁸. As expected, two daily doses of 300 mg/kg sodium butyrate for five days to animals that have been drinking ethanol chronically induced a significant increase in the levels of H3 histone acetylation in the striatum (p < 0.05) (Fig. 7A–C and Supplementary Fig. 3A) and in the prefrontal cortex (p < 0.05) (Fig. 7D–F and Supplementary Fig. 3B) compared to ethanol-drinking animals treated with the vehicle and ethanol-naïve animals, indicating that intra-gastrically administered sodium butyrate is able to reach the brain and induce an increase in histone acetylation.

Discussion

Increasing evidence suggests a role of altered gut microbiota as contributor to the development of gut, liver, and brain inflammation in the context of alcohol dependence³⁹. Thus, a healthy microbiota community is essential for the normal regulation of the microbiota-gut-brain axis. Amongst the most important and pleiotropic components of microbe-to-host signaling highlights SCFAs (acetate, propionate and butyrate)⁴⁰, and a reduction of SCFAs-producing bacteria associates with increased alcohol intake⁴¹. However, the underlying mechanisms through which SCFAs might influence brain physiology and behavior have not been fully elucidated.

Here we show that twice-daily intra-gastric administration of SCFAs for six days to male ethanol-naïve UChB rats (bred for generations for high ethanol intake), completely prevented the initiation phase of alcohol intake, evidenced by a ~85% reduction in voluntary ethanol intake compared with animals treated with NaCl or vehicle.

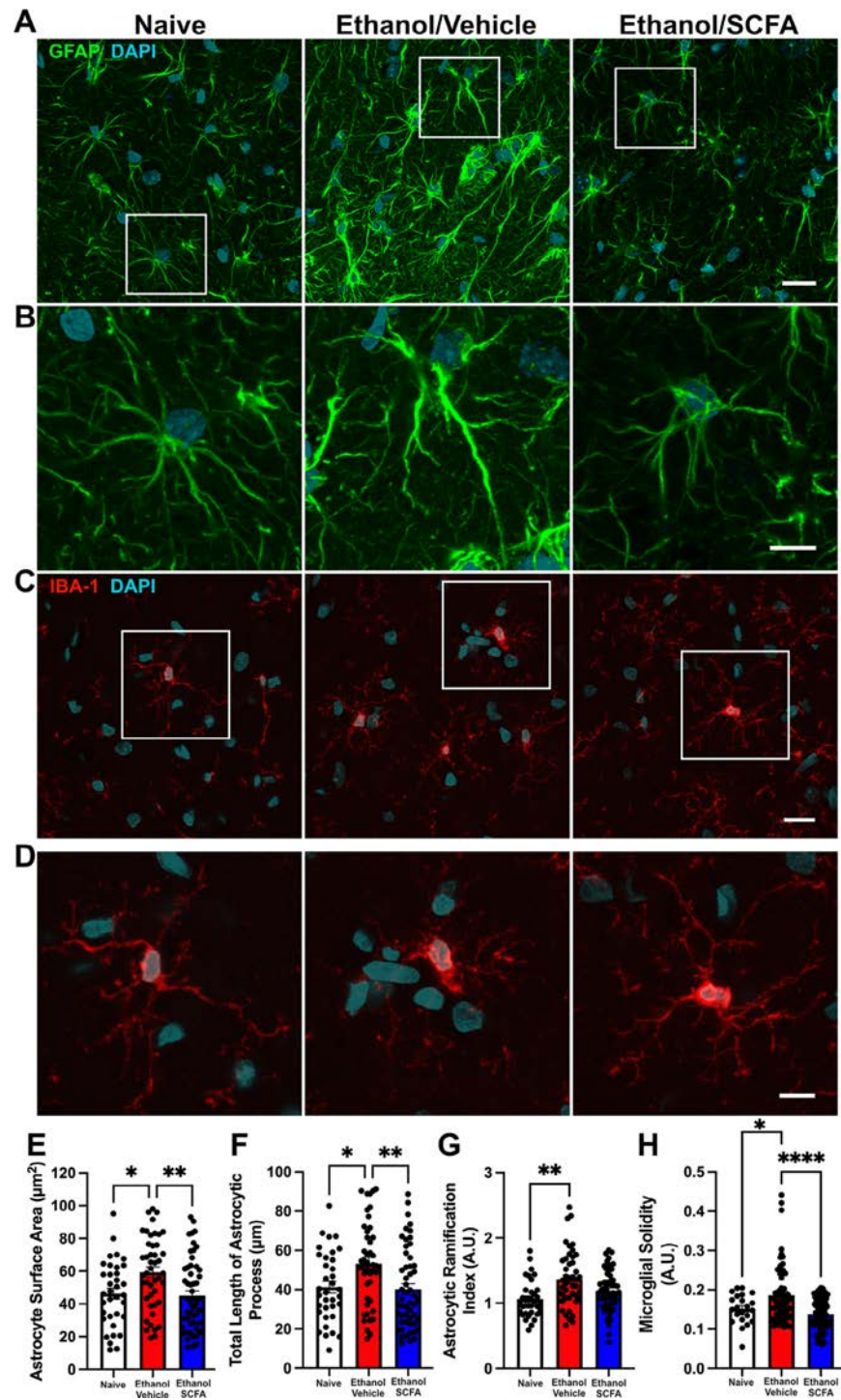


Fig. 4. Intra-gastric Administration of SCFAs Reduces Ethanol-Induced Neuroinflammation. Male UChB rats, voluntarily consuming 10% ethanol for 58 days, were treated with either: (1) a combination of SCFAs (300 mg/kg each of sodium acetate, sodium propionate, and sodium butyrate) or (2) a sodium chloride (NaCl) solution (554 mg/kg, vehicle) by oral gavage twice daily for five days. A third group of untreated animals drinking only water (naïve) was used as a control. (A) Representative confocal micrographs showing GFAP immunoreactivity (green) in the CA1 *stratum radiatum* of the hippocampus under the three experimental conditions. Nuclei are counterstained with DAPI (cyan). Scale bar: 25 μm . (B) Magnified view of the region highlighted by the rectangle in (A). Scale bar: 10 μm . (C) Microglial Iba-1 immunoreactivity (red) and DAPI (cyan). Scale bar: 25 μm . (D) Magnified view of the region highlighted by the rectangle in (C). (E) Quantification of astrocyte surface area. (F) Quantification of the total length of astrocytic processes. (G) Quantification of the astrocytic ramification index. (H) Quantification of microglial solidity. Data are presented as mean \pm SEM based on six microphotographs/hemisphere (N = 6 animals per group). Statistical significance was determined using one-way ANOVA followed by Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

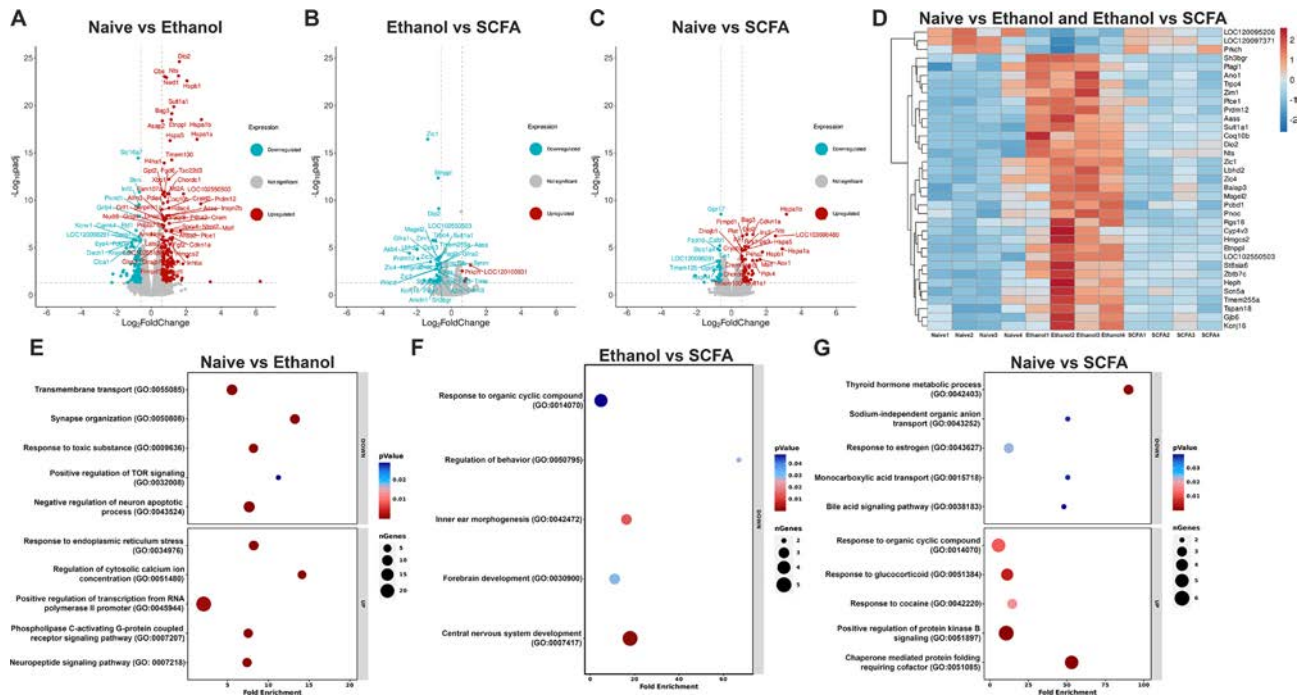


Fig. 5. Transcriptional dynamics in the nucleus accumbens of animals chronically drinking ethanol treated with the SCFAs combination or the vehicle. Male UChB rats, voluntarily consuming a 10% ethanol solution for 58 days, were treated with either a combination of SCFAs (300 mg/kg each sodium acetate, sodium propionate and sodium butyrate) or 554 mg/kg NaCl solution (vehicle), by oral gavage twice-daily for five days. An additional group of untreated animals drinking only water (naïve) was used as a control. After the last administration animals were euthanized and the nucleus accumbens was dissected for RNA-seq determinations. (A–C) Volcano plots of differentially expressed genes display the up-regulated (red dots) and down-regulated (light blue dots) genes. Gray dots indicate no significant changes among the different conditions for (A) Ethanol-Naïve vs. Chronic Ethanol conditions, (B) Chronic Ethanol vs. SCFAs conditions and (C) Ethanol-Naïve vs. SCFAs conditions. (D) Heatmap of the differentially expressed genes that were found common among Ethanol-Naïve vs. Chronic Ethanol and Chronic Ethanol vs. SCFAs conditions. (E–G) Bubble plots representing the functional enrichment analysis of differentially expressed genes. GO biological processes terms were classified based on up- and down-regulated expression changes. Only GO terms with a p-value < 0.05 were included in the analysis for (E) Ethanol-Naïve vs. Chronic Ethanol conditions, (F) Chronic Ethanol vs. SCFA conditions and (G) Ethanol-Naïve vs. SCFAs conditions. Data were obtained from four animals per experimental condition.

Similarly, twice-daily intragastric administration of SCFAs to UChB animals that had been voluntarily drinking ethanol for 58 days leads to a marked reduction in voluntary ethanol intake (~93%) compared to vehicle-treated animals. This indicates that SCFAs administration can prevent the initiation of alcohol intake and strongly reduce chronic alcohol intake once the addictive behavior is already established. These results agree with reports showing that ad libitum sodium butyrate supplementation in drinking water reduced ethanol preference in C57BL/6 J mice⁴², while intraperitoneal administration of sodium butyrate to alcohol-dependent rats decreased ethanol consumption in the operant ethanol self-administration paradigm⁴³. These therapeutic effects of SCFA administration have also been reported for animal models of morphine⁴⁴ and cocaine⁴⁵ dependence. Thus, highlighting the therapeutic potential of SCFAs administration to reduce the seeking behavior induced by the chronic use of several drugs of abuse.

The molecular mechanisms underlying alcohol-drinking behavior are multifaceted and shared between males and females⁴⁶. In our animal model, chronic alcohol intake alters the intestinal architecture, as evidenced by increased intestinal crypt length, and induces intestinal inflammation, as evidenced by an increase in colonic leukocyte infiltration and pro-inflammatory cytokines transcript levels. Indeed, increased intestinal levels of pro-inflammatory cytokines, including TNF- α , downregulate tight junction protein expression and cause disruption of the gut barrier⁴⁷. Furthermore, TNF- α is highly expressed in lamina propria macrophages from duodenal biopsies of alcoholics patients⁴⁸, possibly contributing to gut barrier alterations. Accordingly, in our animal model SCFAs administration completely restored normal intestinal structure and strongly reduced colonic inflammation. Many studies have shown that SCFAs, especially butyrate, can act as anti-inflammatory agents at the intestinal level^{26,39,49}. These studies, both in human and in animal models, have shown that the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, and IL-8 are inhibited in response to SCFAs administration^{26,39,49}. The mechanism underlying the anti-inflammatory effects of SCFAs involves, at least in part, NF- κ B inhibition^{39,50}, by rescuing the redox machinery that controls reactive oxygen species mediating NF- κ B activation²⁵.

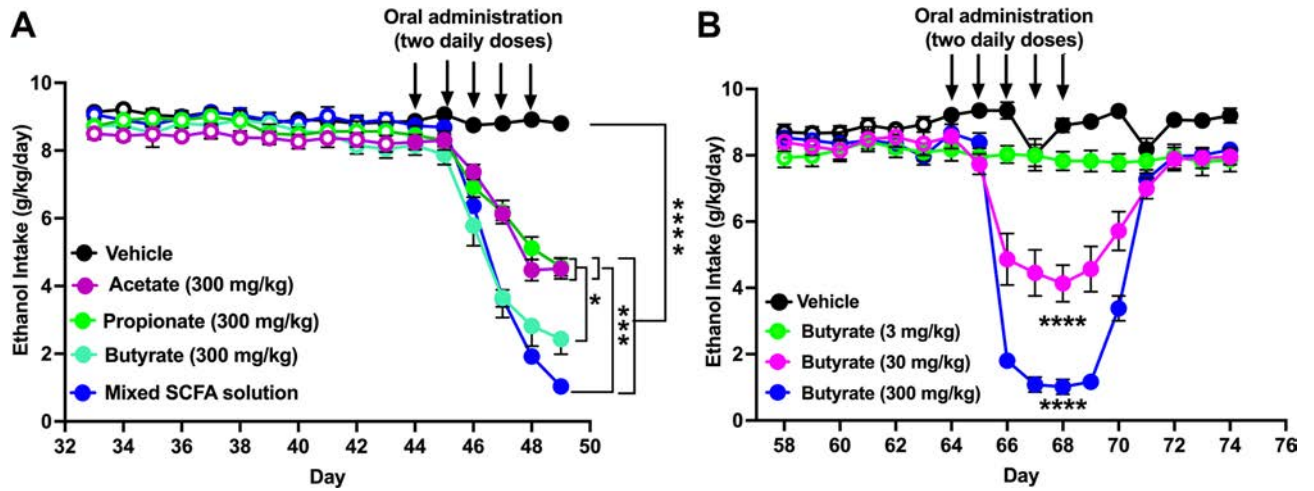


Fig. 6. Sodium butyrate is the most potent SCFA in reducing chronic voluntary ethanol intake, produced in a dose-dependent manner. **(A)** Male UChB rats, voluntarily consuming a 10% ethanol solution for 44 days, were treated with either (1) a combination of SCFAs (300 mg/kg each sodium acetate, sodium propionate and sodium butyrate), (2) 300 mg/kg sodium acetate; (3) 300 mg/kg sodium propionate; (4) 300 mg/kg sodium butyrate, or (5) vehicle by oral gavage twice-daily for five days. Ethanol intake was expressed as g/kg body weight/day. Arrows indicate intragastric administrations. We observed a significant effect of treatment [$F_{\text{treatment}(4,25)} = 8.216, p < 0.001$], day [$F_{\text{day}(3418, 85.46)} = 452.9, p < 0.0001$] and a significant treatment \times day interaction [$F_{\text{interaction}(64,400)} = 39.65, p < 0.0001$] compared to the vehicle group. Sodium butyrate administration produces a significantly greater reduction in ethanol consumption than sodium acetate ($*p < 0.05$), sodium propionate ($*p < 0.05$), and the vehicle ($****p < 0.0001$). **(B)** Male UChB rats voluntarily consuming a 10% ethanol solution for 63 days were treated with either (1) 300 mg/kg sodium butyrate; (2) 30 mg/kg sodium butyrate; (3) 3 mg/kg sodium butyrate; or (4) vehicle by oral gavage twice-daily for five days. Ethanol intake was expressed as g/kg body weight/day. We observed a significant effect of treatment [$F_{\text{treatment}(3,20)} = 46.42, p < 0.0001$], day [$F_{\text{day}(2339, 46.78)} = 128.8, p < 0.0001$] and a significant treatment \times day interaction [$F_{\text{interaction}(24,160)} = 48.54, p < 0.0001$] compared to the vehicle group. The reduction of ethanol intake induced by a dose of 300 mg/kg of sodium butyrate, compared to that of the vehicle group, is significantly greater than that induced by 30 mg/kg and by that 3 mg/kg sodium butyrate. Arrows indicate intragastric administrations. Data are presented as mean \pm SEM. $N = 6$ animals per group. Two-way ANOVA followed by Tukey *post-hoc* tests, $*p < 0.05$, $****p < 0.0001$.

Ethanol-induced intestinal inflammation and the concomitant alteration of intestinal structure results in increased gut permeability, facilitating the translocation of intestinal microbiota components, in particular LPS, to the portal circulation, inducing systemic and liver inflammation⁵¹. In the present study, chronic ethanol intake increased plasma LPS levels and the expression of pro-inflammatory molecules in the liver, which were restored to normal levels by SCFAs administration. It has been previously reported that in rats, the administration of SCFAs decreased ethanol-induced liver damage and hepatic inflammation⁵². Moreover, SCFAs play an important role in gut barrier integrity maintenance, blocking LPS translocation by increased mucin 2 expression, enhancing the protection of the mucosal layer⁵³. Furthermore, butyrate increases the expression of tight junction proteins to minimize paracellular permeability⁵⁴. Therefore, these effects could be responsible for the reduction of plasmatic LPS levels observed in SCFAs-treated animals, and the concomitant reduction in the expression level of pro-inflammatory molecules in the liver.

A limitation of our study is that we were unable to measure fecal SCFAs content, which would confirm SCFA delivery to the colon. However, a prior study by Xia et al.⁵⁵, using gas chromatographic analysis, demonstrated that rats treated with propionic acid by oral gavage—at a dose similar to that used in our study (300 mg/kg, twice daily)—had significantly higher levels of propionic acid in the proximal colon compared to the control group. Given that propionate, acetate, and butyrate were administered at the same dose and frequency in the present study as propionate in Xia et al., it is highly likely that all SCFAs reached the proximal colon in this study as well.

Since the small intestine expresses moderate levels of SCFA transporters compared to the colon⁵⁶ and based on the histological and molecular improvements observed in our model, we suggest that colonic SCFA levels were sufficient to exert protective effects against ethanol-induced colonic damage.

Microglia and astrocytes are the primary immune surveillance cells in the brain, and their activation, which can be evaluated by changes in their morphological shapes, is considered a hallmark of neuroinflammation^{57,58}. Several studies suggest that microglia and astrocytes play important roles in chronic ethanol intake, particularly in the hippocampus, as this brain area is the most pathologically affected by chronic ethanol consumption, both in humans^{59,60} and in animal models^{39,61–63}. Furthermore, increased levels of glial activation and inflammatory cytokines have been observed in the post-mortem human hippocampus of long-term alcohol users. This neuroinflammation, along with concomitant neuronal damage, has been proposed to be responsible for the loss of control over drinking and severe intoxication^{64,65}. Accumulating evidence suggests that SCFAs

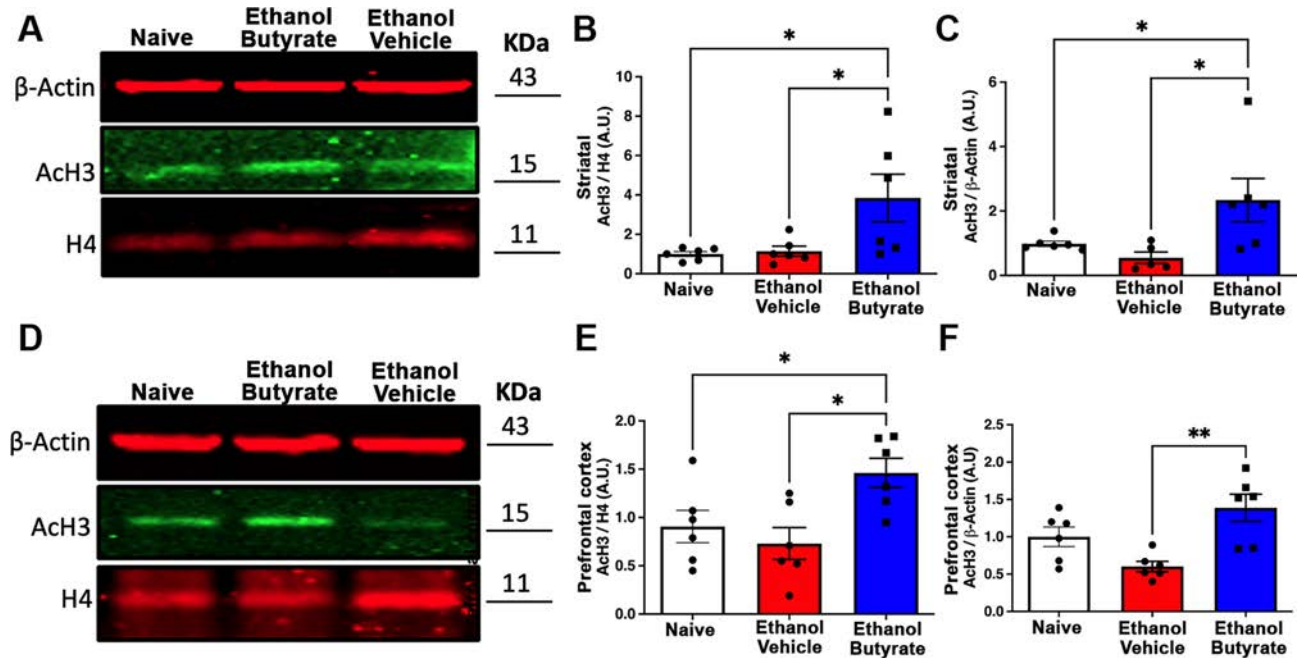


Fig. 7. Sodium butyrate increases H3 histone acetylation levels in the striatum and prefrontal cortex. Male UChB rats, voluntarily consuming a 10% ethanol solution for 44 days, were treated with either 300 mg/kg sodium butyrate or vehicle by oral gavage twice-daily for five days. An additional group of untreated animals drinking only water (naïve) was used as a control. (A) Representative Western Blot showing striatal levels of H3 histone acetylation (AcH3). Histone H4 and β -actin were used as housekeeping genes. (B) Quantification of striatal AcH3 levels normalized to the nuclear marker histone H4. (C) Quantification of striatal AcH3 levels normalized to the housekeeping gene β -actin. (D) Representative Western Blot showing prefrontal cortex levels of H3 histone acetylation (AcH3). (E) Quantification of prefrontal cortex AcH3 levels normalized to the nuclear marker histone H4. (F) Quantification of prefrontal cortex AcH3 levels normalized to β -actin. Data are presented as mean \pm SEM. $N = 6$ animals per group. * $p < 0.05$, ** $p < 0.01$. One-way ANOVA followed by Bonferroni *post-hoc* test.

can cross the blood–brain-barrier, reaching the CNS⁶⁶, having neuroactive properties⁶⁷ not only in neurons but also in astrocytes and microglia. Here we showed that, as previously reported³⁵, chronic alcohol intake induced a significant increase in hippocampal astrocyte and microglial activation. However, these alterations were completely reversed in SCFAs-treated animals. Several studies have reported that SCFAs are able to decrease microglial and astrocyte activation and pro-inflammatory cytokine secretion^{39,68}. Additionally, butyrate treatment induces morphological and functional changes in microglia towards a homeostatic profile⁶⁹. Although the precise mechanism involved in SCFA effects on microglial and astrocytes remains unclear, it has been reported that these cells express the SCFA receptor GPR109a, which upon activation, can induce anti-inflammatory effects by downregulating NF- κ B activation⁷⁰.

While many brain areas contribute to drug reward and to the development of addiction, the nucleus accumbens is a key component of the circuitry associated with excessive alcohol consumption⁷¹, making it an attractive neurobiological area to evaluate the molecular effects associated to chronic alcohol intake. In agreement, we observed that chronic alcohol-drinking animals treated with the vehicle had the highest number of differentially expressed genes in this brain area, with 206 up-regulated and 161 down-regulated genes compared to ethanol-naïve animals. Among down-regulated genes it could be observed genes associated to synapse organization, response to toxic substances, and negative regulation of neuron apoptotic process, among others. Alteration in the expression levels of some of these genes, including genes associated to synaptic function, have also been reported in RNA-Seq data obtained from postmortem brains of alcoholic patients⁷² and from the nucleus accumbens of rhesus macaques drinking alcohol chronically³⁶. Interestingly, SCFAs administration reversed most of the transcriptional effects induced by alcohol intake, suggesting that SCFAs treatment leads to normalization of transcriptional regulation, counteracting specific neuro-adaptation underlying ethanol dependence.

Finally, we evaluated which of the administered SCFAs is the most potent for reducing chronic ethanol intake. Remarkably, we observed that acetate, propionate and butyrate are all capable of significantly reducing chronic alcohol consumption when administered separately. However, butyrate administration generated the most potent therapeutic effect compared to the other SCFAs, and it did so in a dose–response manner. It is interesting to note that when butyrate is no longer administered, UChB animals quickly recover their characteristic high ethanol intake, indicating that the anti-addictive effect is transient.

Increase evidence suggests that epigenetic modifications, in particular histone acetylation, are key mechanisms by which long-lasting changes on neural gene expression can occur, ultimately explaining the

persistence of alcohol addictive behaviors^{73,74}. In the rodent brain, different studies reported epigenetic alterations following ethanol exposure associated with an increase in HDAC expression, leading to alcohol tolerance and dependence^{73,74}. Among the SCFAs, butyrate is the most potent in inhibiting HDAC activities, both *in vitro* and *in vivo*³², promoting gene expression. In this study, we observed that sodium butyrate administration to chronically alcohol-drinking animals induced a significant increase in the levels of H3-histone acetylation, in both striatum and prefrontal cortex, which are brain areas playing a key role in mediating the chronic effects of drugs of abuse³⁸. These findings indicate that the intragastric administered sodium butyrate can reach the brain and induce a pharmacological effect, as SCFAs have the ability to penetrate the brain²⁴. The hyperacetylation in these brain regions has also been reported after intraperitoneal administration of butyrate⁴³. Nevertheless, further studies are needed to fully understand the mechanism behind the therapeutic effects, since SCFAs and butyrate may also directly influence the brain by reinforcing the brain-blood-barrier integrity, increasing tight junction protein expression⁷⁵, modulating neurotransmission and influencing levels of several neurotrophic factors^{76–78}, which are reduced after chronic ethanol intake⁷³. Additionally, chromatin remodeling and active gene transcription is crucial to the formation of long-term memories in the brain reward circuitry^{79,80}. Thus, SCFAs administration, through their HDAC inhibition activity, may play a role in the reduction of the persistence of memories associated with drug use.

Conclusion

Altogether, this study demonstrated that intragastric administration of SCFAs, and specially butyrate, can prevent the initiation of ethanol intake and to strongly reduce voluntary ethanol intake once chronic alcohol intake is already established. These therapeutic effects were associated with the reduction of ethanol-induced alteration of intestinal architecture, intestinal, liver and brain inflammation, and the increase in histone acetylation levels in striatum and prefrontal cortex. Thus, dietary butyrate supplementation is a highly appealing approach for the treatment of alcohol use disorder, as it represents a simple, inexpensive, and relatively low-risk method to improve the outcomes induced by chronic alcohol abuse. Though more research is needed to validate the effectiveness of this dietary intervention, it remains a promising strategy to be used in the future in conjunction with traditional pharmacological and psychological interventions.

Methods

Animals

Two-month-old male Wistar-derived rats, selectively bred for over 90 generations as ethanol consumers (University of Chile Bibulous; UChB)⁸¹, were used. Animals were housed individually and maintained on a 12-h light/dark cycle and regularly fed a soy protein, peanut-meal rodent diet (Cisternas, Santiago, Chile). All procedures were approved by the Ethics Committee for Experiments with Laboratory Animals at the Medical Faculty of the Universidad de Chile (Protocol CBA# 0994 FMUCH). All methods were performed in accordance with the ARRIVE guidelines, the SAGER guidelines, and with relevant institutional, national, and international guidelines and regulations for the care and use of laboratory animals.

Drugs

Ethanol solutions were prepared from absolute ethanol (Merck) diluted to 10% in tap water. Sodium acetate, sodium propionate and sodium butyrate (Sigma-Aldrich) were dissolved in distilled water, adjusted to pH 7.2, and administered by oral gavage in a volume of 6 mL/kg/day twice-daily. Previous studies have shown that administering moderate amounts of sodium can increase water consumption⁸². Thus, to rule out the influence of sodium contained in the SCFAs dose on ethanol consumption, an additional control group was generated which received a dose of 554 mg/kg of NaCl, corresponding to the same concentration of sodium (9.5 mmol/kg) as that received by the SCFAs-treated animals.

Effect of intragastric administration of SCFAs on alcohol intake in naïve animals

To evaluate the role of SCFAs on the acquisition phase of ethanol intake, two-month-old UChB ethanol-naïve animals were divided into three groups (n = 6 per experimental group). Animals were treated by oral gavage with either (1) a combination of SCFAs (300 mg/kg each sodium acetate, sodium propionate, and sodium butyrate); (2) 554 mg/kg NaCl solution; or (3) distilled water (vehicle). This SCFA dose was chosen based on previous studies in rats, where potent therapeutic effects, such as a reduction in neuroinflammation and depressive-like behavior, were reported following its administration⁸³. Treatments were administered twice-daily for six days before animals were given continuous free-choice access between 10% ethanol solution and tap water, as previously described⁵. SCFAs or vehicle administrations were maintained for an additional seven days. Ethanol and water intake were recorded daily. Ethanol intake was expressed in grams of ethanol consumed per kilogram of body weight per day, while ethanol preference was expressed as a percentage of total fluid intake, calculated as the sum of daily alcohol and water consumption.

Effect of intragastric administration of SCFAs on alcohol intake in chronically ethanol-consuming animals and sample collection

To evaluate the role of SCFAs in the maintenance phase of ethanol intake, two-month-old ethanol-naïve UChB animals received continuous free-choice access to 10% ethanol solution and water for 58 days. On that day, animals were divided into three groups (n = 6 per experimental group) and treated by oral gavage with either (1) a combination of SCFAs (300 mg/kg each sodium acetate, sodium propionate, and sodium butyrate); (2) 554 mg/kg NaCl solution; or (3) distilled water (vehicle). Treatments were administered twice-daily for five days. Free-choice ethanol and water access was maintained during SCFAs and vehicle administrations. Ethanol and

water intake were recorded daily. After the last measuring of ethanol consumption animals were anesthetized with a cocktail of 60 mg/kg ketamine, 10 mg/kg xylazine, and 4 mg/kg acepromazine, and blood samples were obtained by cardiac puncture, and were intracardially perfused with 100 mL of 0.1 M PBS (euthanasia). After perfusion, samples of proximal colon, liver, and brain were obtained and frozen or fixed in 4% paraformaldehyde (Merck). One brain hemisphere was fixed for the analysis of astrocyte and microglia immunoreactivity in the stratum radiatum of the CA1 region of the hippocampus. From the contralateral frozen hemisphere, the nucleus accumbens was used to assess transcriptional dynamics, while the striatum and prefrontal cortex were used to assess histone H3 acetylation levels.

Histopathological analysis of intestinal architecture

Samples of proximal colon (n=6 per experimental group) were paraffin-embedded, and 4 µm-histological sections were stained with hematoxylin/eosin, and evaluated based on intestinal damage criteria³³ by an experienced pathologist. Crypt length and inflammatory infiltrate were analyzed using QuPath software (v.0.5.1). Crypt length was determined by measuring a straight line from the base of the crypt to the apical surface. Inflammatory infiltrate was determined by counting cell numbers per area in the lamina propria compartment of each sample³³.

Immunohistochemistry staining and analysis of proximal colon samples

Paraffin-embedded histological sections (4 µm thick) derived from proximal colon samples obtained from animals of the different experimental groups (n=6 per experimental group), were stained with rabbit polyclonal anti-PCNA antibody (1:300, Novus Biologicals, Cat. NB600-1331) to evaluate the proportion of proliferating cells in colon tissue. Briefly, the sections were subjected to deparaffinization with NeoClear, (Merck) and rehydrated with a battery of alcohols from absolute ethanol to 70% ethanol. Antigen retrieval was performed with sodium citrate buffer (pH 6). Then, IHC was performed with VECTASTAIN® Elite® ABC-HRP Kit, Peroxidase, R.T.U. (Universal) (PK-7200) and DAB substrate from VectorLabs, following the manufacturer instructions.

Positivity Index (pixels per area) was calculated with QuPath software (v.0.5.1), creating a classifier with the following parameters: Moderate resolution (2.01 mm/pixel), DAB channel, smoothing sigma 1, 0.29 threshold. Staining above this threshold index was considered positive and below was identified as negative. We selected lamina propria areas for each sample and mean positivity index from DAB stain was compared among different experimental groups.

Quantification of mRNA levels of pro-inflammatory factors in the proximal colon and liver

Total RNA was purified from proximal colon and liver samples (n=6 per experimental group) using Trizol (Invitrogen) and used to perform reverse transcription with MMLV reverse transcriptase (Invitrogen). qPCR reactions were conducted using a QuantStudio 12 K-Flex equipment (Thermo-Fisher) to amplify the mRNA levels of interleukins IL1β, IL6, IL8, TNF-α and lipopolysaccharide binding protein (LBP) using specific primers (Supplementary Table 1). The mRNA level of each target gene was normalized against the mRNA level of β-actin in the same sample.

Determination of LPS levels in blood samples

LPS levels were determined in plasma samples (n=6 per experimental group) using the LPS enzyme-linked immunosorbent assay (ELISA) kit (MyBiosource) as previously reported⁹.

Determination of astrocyte and microglia immunoreactivity

Double-labeling immunofluorescence against the astrocyte marker glial fibrillary acidic protein (GFAP) (Sigma-Aldrich G3893; 1:500 dilution) and the microglial marker ionized binding protein1 (Iba1) (Wako 019-19741, 1:400 dilution) was performed on 30 µm-coronal cryosections of one brain hemisphere (n=6 per experimental group). Six microphotographs of the stratum radiatum of the CA1 region in the hippocampus per hemisphere were captured using an Olympus FV10i confocal microscope with a 60× objective lens (NA 1.30) and FV10-ASW-2b software. Astrocyte surface area, total process length, and the ramification index (ratio of total process length to the number of branch points) were quantified using FIJI software with the Simple Neurite Tracer plugin, according to previous reports⁸⁴. Microglial reactivity, typically involving a shift from a highly ramified to a more compact, amoeboid shape characterized by a larger soma and retracted processes was quantified using the Solidity metric. Solidity is defined as the ratio of total cell area (including soma and processes) to convex cell area (the smallest convex polygon encompassing the entire cell). Higher solidity values indicate a more compact morphology typical of reactive microglia^{58,85}.

Evaluation of transcriptomic dynamics in the nucleus accumbens

The nucleus accumbens of one brain hemisphere was used for RNA purification with the total RNA purification kit (Qiagen). mRNA samples (n=4 per experimental group) were sequenced using TruSeq stranded mRNA library + NovaSeq6000-150PE platform (Macrogen) with 6 million reads per sample. The raw data from RNA-seq libraries were processed, and reads of low quality were filtered out with Trimmomatic software (v0.39)⁸⁶. The clean reads were mapped to the rat reference genome (Rattus_norvegicus.mRatBN7.2) with HISAT2 software (v2.2.1)⁸⁷, and quantified with featureCounts software (V2.0.1)⁸⁸. The differential expression analysis was performed using pyDESeq2 software (v0.4.4)⁸⁹. Differentially expressed genes (DEGs) were determined based on the criteria of p.adj < 0.05 and log₂ fold change (FC) > 0.58, selecting genes with a change in expression of at least 50%. The gene ontology analysis (GO) was performed with the DAVID (v2024q1) database⁹⁰. The RNA sequencing data have been deposited in the Genome Sequence Archive (GSA) under the accession number PRJCA032120.

Comparison of the potency of the three SCFAs to inhibit ethanol consumption

Two-month-old UChB ethanol-naïve animals received continuous free-choice access to 10% ethanol solution and water for 44 days. On that day, animals were divided into five groups ($n = 6$ per experimental group) and treated by oral gavage with either (1) a combination of SCFAs (300 mg/kg each sodium acetate, sodium propionate, and sodium butyrate); (2) 300 mg/kg sodium acetate; (3) 300 mg/kg sodium propionate; (4) 300 mg/kg sodium butyrate; or (5) distilled water. Treatments were administered twice-daily for five days. Free-choice ethanol and water access was maintained during treatment administration. Ethanol and water intake were recorded daily.

Dose–response determination of sodium butyrate in reducing alcohol intake in chronically ethanol-consuming animals

Two-month-old UChB ethanol-naïve animals received continuous free-choice access to 10% ethanol solution and water for 64 days. On that day, animals were divided into groups ($n = 6$ per experimental group) and treated by oral gavage with either (1) 300 mg/kg sodium butyrate; (2) 30 mg/kg sodium butyrate; (3) 3 mg/kg sodium butyrate; or (4) distilled water. Treatments were administered twice-daily for five days. Free-choice ethanol and water access was maintained during treatment administration and then continued for six additional days after treatments discontinuation to assess the duration of the effects. Ethanol and water intake were recorded daily.

Evaluation of H3 histone acetylation levels in the striatum and prefrontal cortex

Twenty-four hours after the last administration of 300 mg/kg sodium butyrate or vehicle, animals were anesthetized with a cocktail of 60 mg/kg ketamine, 10 mg/kg xylazine, and 4 mg/kg acepromazine, and were intracardially perfused with 100 mL of 0.1 M PBS (euthanasia). Then, striatum and prefrontal cortex of one brain hemisphere were dissected and frozen ($n = 6$ per experimental group). Total proteins were extracted using a T-per lysis buffer (Thermo-Fisher). For Western Blots, 25 μ g of proteins was utilized to assess striatal and prefrontal cortex levels of H3 histone acetylation (AcH3) with an anti-AcH3 primary antibody (Thermo, MA5-11,195, 1:500 dilution). Histone H4 and β -actin were used as loading controls. Histone H4 was detected with an anti-H4 primary antibody (Cell Signaling, 13,919, 1:500 dilution), while β -actin was evaluated with an anti- β -actin primary antibody (Santa Cruz Biotechnology, sc-47778, 1:2000 dilution). Detection of reactive bands was performed using the Odyssey Imagen System (Li-COR) as previously reported⁹¹.

Statistical analyses

Data are expressed as mean \pm SEM. Statistical analyses were performed using GraphPad Prism v.9.2.0 software. The normal distribution of data for all experiments was evaluated using the Shapiro–Wilk test. For normally distributed data, one-way ANOVA or two-way ANOVA was used followed by Bonferroni or Tukey *post-hoc* test. A level of $p < 0.05$ was considered for statistical significance.

Data availability

Data generated during this study are included in this published article and its supplementary information files. Additionally, the RNA sequencing data have been deposited in the Genome Sequence Archive (GSA) under the accession number PRJCA032120.

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MEQ: Conceptualization, methodology, data obtention and analysis, data curation, final approval of the manuscript; DS: Methodology, data obtention and analysis, data curation, final approval of the manuscript; ED: Data obtention and analysis, data curation, final approval of the manuscript; IVM: Methodology, data obtention and analysis, data curation, final approval of the manuscript; NM: Methodology, data obtention and analysis, data curation, final approval of the manuscript; GL: Methodology, data obtention and analysis, final approval of the manuscript; AD: Methodology, data obtention and analysis, final approval of the manuscript; PM: Methodology, data obtention and analysis, founding acquisition, final approval of the manuscript; DR: Data obtention and analysis, founding acquisition, final approval of the manuscript; MH: Data obtention and analysis, founding acquisition, final approval of the manuscript; BO: Data obtention and analysis, final approval of the manuscript; PBC: Data obtention and analysis, final approval of the manuscript; ME: Data obtention and analysis, final approval of the manuscript; MHM: Data obtention and analysis, founding acquisition, final approval of the manuscript; YI: Conceptualization, writing original draft, final approval of the manuscript; FE: Conceptualization, methodology, data obtention and analysis, founding acquisition, writing original draft, final approval of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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