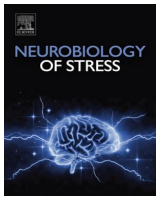
Neurobiology of Stress 27 (2023) 100572

Contents lists available at ScienceDirect

Neurobiology of Stress

journal homepage: www.elsevier.com/locate/ynstr



# Effect of acetate supplementation on traumatic stress-induced behavioral impairments in male rats

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ARTICLE INFO

*Keywords:*
SPS
Anxiety
Acetate
ß-hydroxybutyrate
Neuroinflammation

ABSTRACT

Gut microbiota and their metabolites have emerged as key players in the pathogenesis of neuropsychiatric disorders. Recently, we demonstrated that animals susceptible to Single Prolonged Stress (SPS) have an overall pro-inflammatory gut microbiota and significantly lower cecal acetate levels than SPS-resilient rats, which correlated inversely with the anxiety index. Here, we investigated whether the microbial metabolite, acetate, could ameliorate SPS-triggered impairments. Male rats were randomly divided into unstressed controls or groups exposed to SPS. The groups received continued oral supplementation of either 150 mM of sodium acetate or 150 mM of sodium chloride-matched water. Two weeks after SPS, a battery of behavioral tests was performed, and the animals were euthanized the following day. While not affecting the unstressed controls, acetate supplementation reduced the impact of SPS on body weight gain and ameliorated SPS-induced anxiety-like behavior and the impairments in social interaction, but not depressive-like behavior. These changes were accompanied by several beneficial effects of acetate supplementation. Acetate alleviated the stress response by reducing urinary epinephrine levels, induced epigenetic modification by decreasing histone deacetylase (HDAC2) gene expression, inhibited neuroinflammation by reducing the density of Iba1+ cells and the gene expression of IL-1ß in the hippocampus, and increased serum β-hydroxybutyrate levels. The findings reveal a causal relationship between oral acetate treatment and mitigation of several SPS-induced behavioral impairments. Mechanistically, it impacted neuronal and metabolic pathways including changes in stress response, epigenetic modifications, neuroinflammation and showed novel link to ketone body production. The study demonstrates the preventive-therapeutic potential of acetate supplementation to alleviate adverse responses to traumatic stress.

## 1. Introduction

Stress-associated anxiety, depression, and post-traumatic stress disorder (PTSD) are among the most common mental health disorders, affecting more than 60 million adults in the United States in any given year (Health (2021); Affairs, 2022; Association (2013); Greenberg et al., 2015; Kessler et al., 2005). Although current treatments can help manage symptoms, they do not always address the root cause of these disorders. Moreover, not all individuals can afford or respond to existing treatments, and some experience significant side effects (McHugh and Barlow, 2010). Thus, there is a need for new, affordable, and effective treatments that can address these underlying factors and provide more complete relief for those who suffer from stress triggered disorders.

The recognition of critical bidirectional signaling along the microbiota-gut-brain axis and the involvement of the gut microbiota in the pathogenesis of many psychiatric disorders has led to a paradigm shift in the understanding of many neuropsychiatric disorders and their potential treatments (Dinan et al., 2015; Kelly et al., 2015; Mayer et al., 2014; Sarkar et al., 2016). Clinical and preclinical studies have indicated that the composition of the gut microbiota is associated with stress resilience or susceptibility to traumatic stress (Bassett et al., 2019; Tanelian et al., 2022, 2023); and microbiota-modifying interventions, such as pro/prebiotics, have been demonstrated to be effective in modulating stress reactivity, cognitive processes, and behavior in both animal models and humans (Ait-Belgnaoui et al., 2014, 2018; Bravo et al., 2011; Desbonnet et al., 2008; Gacias et al., 2016; Schmidt et al.,

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https://doi.org/10.1016/j.ynstr.2023.100572
Received 28 June 2023; Received in revised form 6 September 2023; Accepted 16 September 2023
Available online 19 September 2023

2015; Steenbergen et al., 2015). However, the specific mechanisms and biological mediators involved in this communication remain largely unknown.

Short-chain fatty acids (SCFAs) are microbial metabolites produced mainly in the gut through fermentation of complex polysaccharides and dietary fibers (Miller and Wolin, 1996). Owing to their neuroactive properties and ability to affect different gut-brain signaling pathways, SCFAs are often considered key candidate mediators along the microbiota-gut-brain axis (Silva et al., 2020). In fact, changes in SCFA levels have been documented in several neurological and psychiatric disorders, including Alzheimer's, Parkinson's, anorexia nervosa, autism, and depression, with specific SCFAs exhibiting varying patterns of elevation or reduction depending on the particular disease (Mirzaei et al., 2021).

Among the SCFAs, acetate (C2) is the smallest and structurally simplest SCFA in both human and animal bodies (Dalile et al., 2019; Kaji et al., 2015). In the colon, acetate is typically present in an approximate molar ratio of 60:20:20 relative to propionate, and butyrate, respectively. In plasma, acetate also exhibits the highest concentration, ranging from 25 to 250 μM (Dalile et al., 2019). However, the specific amounts and relative proportions of each SCFA can vary based on dietary substrates, the composition of gut microbiota, and gut transit time (Dalile et al., 2019). Acetate also represents the basic building block of all long-chain fatty acids and sterols through its association with coenzyme A (Barko et al., 2018; Moffett et al., 2020; Ríos-Covián et al., 2016). Although initially considered solely as metabolic fuel, several clinical and preclinical studies have recently reported its neuroprotective and anti-inflammatory properties. For instance, in an Alzheimer's disease model, acetate supplementation ameliorated working and behavioral memory deficits, improved cognitive impairment, and suppressed neuroinflammation (Kobayashi et al., 2017; Liu et al., 2020). In patients with multiple sclerosis and depression, serum acetate levels were lower and inversely correlated with pro-inflammatory cytokines (Olsson et al., 2021; Skonieczna-Żydecka et al., 2018). Acetate has also been reported to be a crucial regulator of epigenetics in both pre-clinical and clinical studies. By being converted to acetyl-CoA, acetate participates in histone acetylation which can have long-lasting effects on depression and anxiety (Huang et al., 2021; Misztak et al., 2018; Soliman and Rosenberger, 2011; Soliman et al., 2012).

While the documented benefits of acetate in treating various neurological diseases are well-established, its effect within the context of stress-related conditions remain in the early stages of exploration (Arielle Kasindi et al., 2022; Dalile et al., 2019). Currently, only a limited number of studies have delved into the impact of acetate in stress models. For instance, in the social defeat stress model, researchers have observed that oral supplementation of a mixture of SCFAs, which includes acetate, can alleviate stress-induced anhedonia and enhance stress responsiveness (van de Wouw et al., 2018). Similarly, in chronic social defeat stress model, the administration of Glyceryl triacetate via oral gavage has shown promise in ameliorating depressive-like behaviors, reducing the transcription levels of various histone deacetylases, and increasing histone acetyltransferase activity (Huang et al., 2021). Furthermore, using chronic mild stress model, researchers have reported that subcutaneous injection of glatiramer acetate leads to improvements in learning performance among stressed animals, restoration of normal adult neurogenesis levels, rebalancing of the immune system, normalization of reactive oxygen species (ROS) levels, and the restoration of neuronal nitric oxide synthase (nNOS) activity (Palumbo et al., 2012; Pascuan et al., 2015). It's noteworthy that most of these studies employed invasive methods for acetate administration.

Recently, using single prolonged stress (SPS), an animal model for PTSD, we found differences in the fecal microbial composition and cecal metabolites of male rats that were susceptible or resilient to the development of SPS-induced behavioral impairments (Tanelian et al., 2022). The levels of cecal acetate were about 25% lower in susceptible rats compared to resilient rats and were strongly and inversely correlated with the anxiety index ($r = -0.89$, $p = 0.005$) (Tanelian et al., 2022). Here, we aimed to further test the cause-and-effect relationship by (1) assessing whether non-invasive acetate supplementation protects against SPS-triggered behavioral impairments and (2) determining some of the potential mechanisms by which acetate can improve behavioral deficits triggered by SPS.

## 2. Materials & methods

### 2.1. Animals

All animal experiments complied with the ARRIVE guidelines and NIH Guide for the Care and Use of Laboratory Animals. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of New York Medical College. Male Sprague–Dawley rats 6–7 weeks of age (150–160 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Throughout the experiment, the animals were housed two per cage to avoid isolation stress and were maintained under a 12-h light/dark cycle at 23 ± 1 °C. Food and water were provided *ad libitum*.

### 2.2. Drugs

Anhydrous sodium acetate (Sigma-Aldrich, cat# S2889-1 KG) and sodium chloride (Sigma-Aldrich, cat# 7647-14-5) were dissolved in distilled water (pH7.0). The dose of sodium acetate (150 mM) was based on previous studies which demonstrated its effectiveness in rodents (Xie et al., 2021).

### 2.3. Experimental design

Animals were allowed to accommodate to the animal facility for 14 days (Fig. 1A). Three days after arrival, the animals were randomly divided into four groups and received either freshly prepared 150 mM sodium acetate or 150 mM sodium chloride in their drinking water every three days, until the end of the experiment. The groups were as follows: control + NaAcetate (n = 8), SPS + NaAcetate (n = 14), control + NaCl (n = 8), and SPS + NaCl (n = 14). Two weeks after the start of the treatment (day 17), the rats in the SPS groups were subjected to SPS stressors, whereas the control rats were briefly handled. The SPS-exposed groups were left undisturbed without bedding changes for seven days to consolidate the experience of traumatic stress, after which they were kept with normal bedding changes for the remainder of the experiment. Two weeks after SPS (day 31), all animals were subjected to a battery of behavioral tests in the following order: Open Field (OF), Social Interaction (SI), Elevated Plus Maze (EPM), and Forced Swim Test (FST). One day after the last behavioral test, the animals were sacrificed by decapitation and brain and blood were collected. Urine samples were collected before and 30 min into the SPS immobilization step. The animals were weighed on the day of SPS and two weeks after SPS (on the day of the OFT).

### 2.4. Water intake measurements

The amount of water consumed per cage was measured throughout the experiment. The sterile water bottles were changed every three days, and the remaining amount of water was measured. The average water consumption was similar among the groups (Fig. 1B).

### 2.5. Single prolonged Stress (SPS)

SPS was performed as previously described (Serova et al., 2013, 2019; Tanelian et al., 2022). Briefly, the animals were restrained by taping their limbs with surgical tape onto a custom-made metal board that restricted the motion of their heads. Immediately after 2 h of immobilization, the animals were subjected to a 20 min forced swim in a

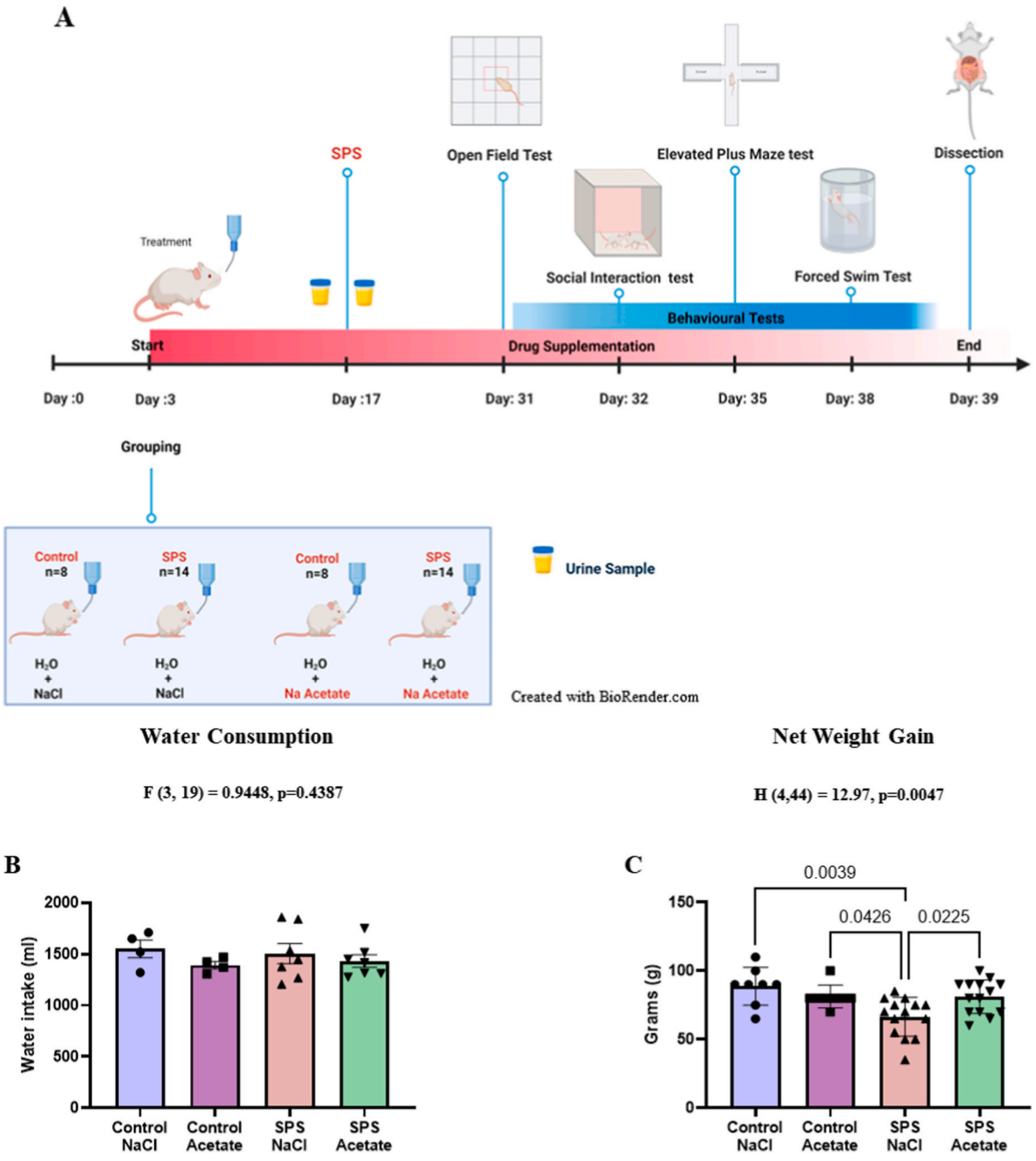


**Fig. 1.** *Experimental design, water consumption, and weight gain.*
**A**: **Timeline**: Three days after arrival, the animals were randomly divided into four groups and received either sodium acetate or sodium chloride in their drinking water. The groups were as follows: **control** + **NaAcetate**, **SPS** + **NaAcetate, SPS** + **NaCl**, **and control** + **NaCl**. On day 17, the rats in the SPS group were subjected to SPS stressors. Two weeks after SPS (days 31–38), all animals were exposed to a battery of behavioral tests and sacrificed one day later.
**B**: **Water consumption**: The amount of water consumed per cage was measured every three days, throughout the experiment, starting on day three. The total amount of water consumed per cage during the experiment was calculated. Data passed the normality test and were analyzed using one-way ANOVA. Each dot represents the value for two animals within a single cage (**Control** + **NaCl**: n = 4, **Control** + **NaAcetate**: n = 4 **SPS** + **NaCl**: n = 7, SPS + **NaAcetate**: n = 7)
**C**: **Net weight gain**. All animals were weighed on the day of SPS (day 17) and two weeks later (day 31), and the net weight gain was calculated. Data did not pass the normality test and were analyzed using the Kruskal-Wallis test. Each dot represents the value for an individual animal (**Control** + **NaCl**: n = 8, **Control** + **NaAcetate**: n = 8, **SPS** + **NaCl**: n = 14, SPS + **NaAcetate**: n = 14).

plexiglass cylinder filled two-thirds with fresh water at 24 °C. They were then dried and allowed to recuperate for 15 min, after which they were exposed to ether in a glass desiccator chamber until loss of consciousness.

### 2.6. Behavioral tests

All behavioral tests were performed between 10 a.m. and 3 p.m., and were administered in the order of least to most stressful to reduce possible carryover effects from prior behavioral tests (Tanelian et al., 2022). All tests, except FST, were performed in a room with dim lights, videotaped, and analyzed by trained individuals blinded to the groups.

#### 2.6.1. Open field (OF)

The animals were accommodated to the room 30 min before the start of the test. Individual rats were placed in an open arena (40 × 32 × 24 cm, L × W × H) and were allowed to explore the arena for 5 min, after which they were removed and placed in their respective home cages. Duration and number of entries into the virtual center zone (defined as 50% away from the edges), as well as number of rears were analyzed.

#### 2.6.2. Social interaction (SI)

One day prior to the test, the animals were allowed to explore the open field arena for 5 min to reduce the anxiety component in a novel environment. On the testing day, the animals were acclimated to the room for 30 min and then allowed to explore the field for 2 min after which a juvenile rat (50–75 g) of the same sex was introduced into the center of the arena. The animals were allowed to interact for 5 min, and their behavior was recorded. The time spent interacting, the number of approaches initiated, and the latency to first approach by the test rats were scored and analyzed. The time spent in nose-to-nose sniffing, nose-to-anogenital sniffing, following, crawling over and under each other with physical contact, chasing, mounting, and wrestling initiated by the test rat was considered as the time spent engaged in social interaction (Varlinskaya and Spear, 2008).

#### 2.6.3. Elevated Plus Maze (EPM)

Anxiety/avoidance-like behavior was tested on the EPM. The apparatus (Stoelting, Wood Dale, IL, USA), 50 cm above ground level, has four cross-shaped platforms; two platforms with a 2-cm-high plexiglass fence wall are open, while the other two platforms with 40-cm-high opaque walls on the sides are closed. Arms of the same type are located opposite to each other. Each rat was placed on the central platform with its head towards an open arm and allowed 5 min to explore the maze. The following measurements were evaluated: Duration and number of entries into open arms (OA) and closed arms (CA); number of head dips; risk assessment duration; and anxiety index (AI). Arm entry was defined as the entry of an arm with all four paws. The percentage of entries was calculated as the percentage of total open or closed arm entries to the total number of arm entries, and the time in the arms was calculated as a percentage of the total time of the test. The anxiety index was calculated as 1 − [(time spent in open arm/total time on the maze)/2 + (number of entries into the open arms/total exploration on the maze)/2] (Cohen et al., 2012). Head dips were defined as the frequency at which the animal lowered its head towards the floor over the sides of the open arms. Risk assessment was assessed by the rat poking its head or trunk into an OA while its hind quarters were located in one of the CAs (Augustsson et al., 2005).

#### 2.6.4. Forced Swim Test (FST)

The FST was performed for 5 min in plexiglass cylinders filled to two-thirds with 24 °C fresh water and videotaped. Time spent swimming, defined as the movement of the forelimbs and hind limbs, and the time spent immobile when the animal showed no movement, or only movements needed to keep its head above the water were scored (Nahvi et al., 2021; Serova et al., 2013).

### 2.7. Tissue collection

Ventral hippocampus (vHipp) sections −4.80 mm to −5.20 mm to bregma were dissected using a brain matrix. The right vHipp was flash-frozen in liquid nitrogen for subsequent gene expression analysis, whereas the left vHipp was post-fixed in 4% paraformaldehyde for immunohistochemical analysis. Both sides were stored at −80 °C until use.

### 2.8. Gene expression analysis

Total RNA was isolated using RNA STAT-60 (Tel-test, cat# CS-502). RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, cat# K1622). Gene expression analysis was performed by quantitative real-time polymerase chain reaction (qRT-PCR) using the FastStart Universal SYBR Green Master (ROX) kit (Roche Diagnostics cat# 04913850001) with the following primers designed by QIAGEN: GAPDH (PPR06557B), *HatI* (PPR52132A), *HdacII* (PPR42588A), *HdacVII* (PPR53048A), and IL-1β (PPR06480B-200). Amplifications were performed in duplicates in 96 well plates using a QuantStudioTM 5 system. Data were normalized to GAPDH as an endogenous control and transformed using the 2-ΔΔCT method. All procedures were performed according to the manufacturer's instructions.

### 2.9. Immunohistochemistry

Frozen coronal sections of the left vHipp from 3 to 4 animals/group were cut to a thickness of 16 μm. The sections were blocked with 10% normal goat serum containing 0.3% Triton. Goat polyclonal Iba-1 (Abcam, 1:500; cat# ab5076) followed by Alexa Fluor 488 anti-goat antibody (Jackson Immunoresearch, 1:300, cat# 705-545-147) was used for Iba-1 visualization. All sections were mounted using DAPI (Thermo Fisher, 1:5000, cat# D1306) and imaged using a Keyence-BZ-X810 microscope. Regions (CA1-4) in the hippocampus were captured using 20X images and the total number of Iba1+ cells was counted.

### 2.10. Quantification of serum β-hydroxybutyrate

Whole blood samples were collected from each animal via decapitation. The samples were stored at room temperature for 30 min and then centrifuged at 4 °C for 10 min at 2000 g. Serum was collected and stored at −80 °C for later analysis. Serum β-hydroxybutyric acid quantification was performed in duplicates using EnzyChrom Ketone Body Assay kit (BioAssay Systems, cat# EKBD-100), according to the manufacturer's protocol.

### 2.11. Urine collection for epinephrine and corticosterone quantification

Urine samples were collected from each rat before and 30 min into the immobilization step of SPS as previously described (Tanelian et al., 2022). Urine epinephrine (Rocky Mountain Diagnostics cat# BAE-5100R) and corticosterone (Invitrogen, cat# EIACORT) levels were quantified in duplicates using a commercially available competitive enzyme immunoassay kit and normalized to urinary creatinine concentrations (Arbor Assays, cat# K002-H1) in the same samples.

### 2.12. Data analysis

Statistical analyses were performed using the GraphPad Prism 9 software. Data were assessed for normality using the Shapiro-Wilk test and for equality of variances using Brown-Forsythe and Bartlett's tests. Comparisons between two groups were performed using the Student's *t*-test. To compare more than two groups, one-way ANOVA for Gaussian distributions and Kruskal-Wallis test for non-Gaussian distributions were used. To compare group means from different time points, two-way repeated measures ANOVA was used. Pearson's correlation coefficient was used to assess correlations. To correct for multiple testing, the Benjamini-Krieger-Yekutieli post-hoc test was used with a q-value of 0.05 as a cut-off in all ANOVA and correlational tests. Statistical significance was set at an α level of less than 0.05 (two-tailed). Data are expressed as mean ± SEM.

## 3. Results

### 3.1. Acetate supplementation mitigated SPS-elicited reduction in weight gain

Body weight was determined as an initial measure of stress response. Animals in the SPS + NaCl group gained less weight. The net weight gain of the animals in the SPS + NaAcetate group was significantly higher than that of the animals in the SPS + NaCl group and was similar to that of the unstressed controls (Fig. 1C).

### 3.2. Acetate supplementation ameliorated SPS-triggered anxiety/avoidance-like behavior

To investigate whether acetate supplementation can ameliorate SPS-induced anxiety/avoidance-like behavior, OF and EPM tests were employed. As anticipated, exposure to SPS led to a decrease in the number of entries into the center of OF in the SPS + NaCl group. While acetate supplementation did show a trend toward preventing this effect, it did not reach statistical significance (Fig. 2A). Similarly, no significant differences were observed in the time spent in the center of the OF arena between SPS + NaCl and SPS + NaAcetate groups (Fig. 2B). Yet, acetate supplementation affected the number of rears. Animals in the SPS + NaAcetate group showed significantly more rears than those in the SPS + NaCl group. Although acetate treatment led to more rears in the SPS + NaAcetate group, it was still lower than that of the control + NaCl, indicating partial improvement in anxiety-like behavior in the OF test (Fig. 2C).

On the EPM, animals in the SPS + NaAcetate group spent significantly more time (Fig. 2D) and had more frequent entries (Fig. 2E) into the open arms (OA) of the maze compared to the SPS + NaCl group. Similarly, the SPS + NaAcetate group had a significantly lower anxiety index than that of the SPS + NaCl group (Fig. 2F), consistent with the strong correlation observed between cecal acetate levels and anxiety index in our previous study (Tanelian et al., 2022). Frequent head dips on the EPM are associated with decreased anxiety (Braun et al., 2011). Rats in the SPS + NaAcetate group showed significantly more frequent head dips than did those in the SPS + NaCl group (Fig. 2G). The number of head dips inversely correlated with the anxiety index (Fig. 2H). Another valuable measure of EPM that identifies anxiolytic-like actions of drugs that are not detected by conventional scoring methods is risk assessment (Rodgers et al., 1999). The risk assessment duration was significantly lower in the SPS + NaAcetate group than that in the SPS + NaCl group (Fig. 2I) and positively correlated with the anxiety index (Fig. 2J). Overall, the animals' behavior on the EPM indicated that acetate reduced the measures of anxiety/avoidance triggered by SPS without affecting the behavior of the controls, except for the number of head dips.

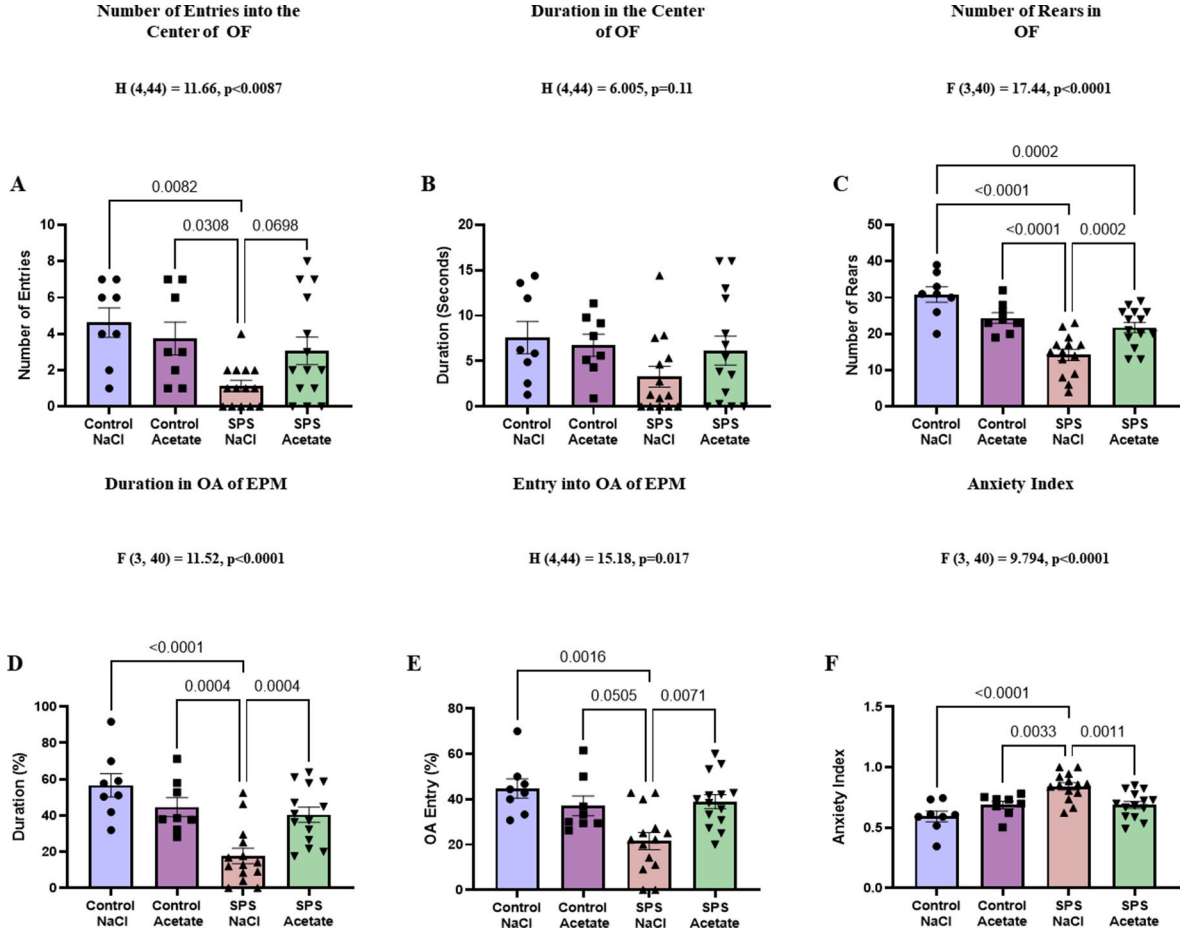


**Fig. 2.** *Effect of acetate supplementation on anxiety-like behavior*. The animals' anxiety-like behavior was assessed using the Open Field (OF) and Elevated Plus Maze (EPM) tests. *On OF* **A**: Number of entries into the center, **B**: Duration in the center, **C**: Number of rears. *On EPM* **D**: Duration in open arms (OA), **E**: % entry into OAs, **F**: Anxiety Index, **G**: Total number of head dips, **H**: Correlation between head dips and anxiety index, **I**: Risk assessment (RA) duration, **J**: correlation between risk assessment (RA) duration and anxiety index. Data from the number of entries and duration on OF test as well as % OA entries and risk assessment on EPM did not pass the normality test and were analyzed using the Kruskal-Wallis test. The rest of the behavioral data passed the normality test and were analyzed using one-way ANOVA. Correlation analysis was performed using Pearson's correlation coefficient. Each dot represents the value for an individual animal (**Control + NaCl**: n = 8, **Control + NaAcetate**: n = 8, **SPS + NaCl**: n = 14, SPS + **NaAcetate**: n = 14).

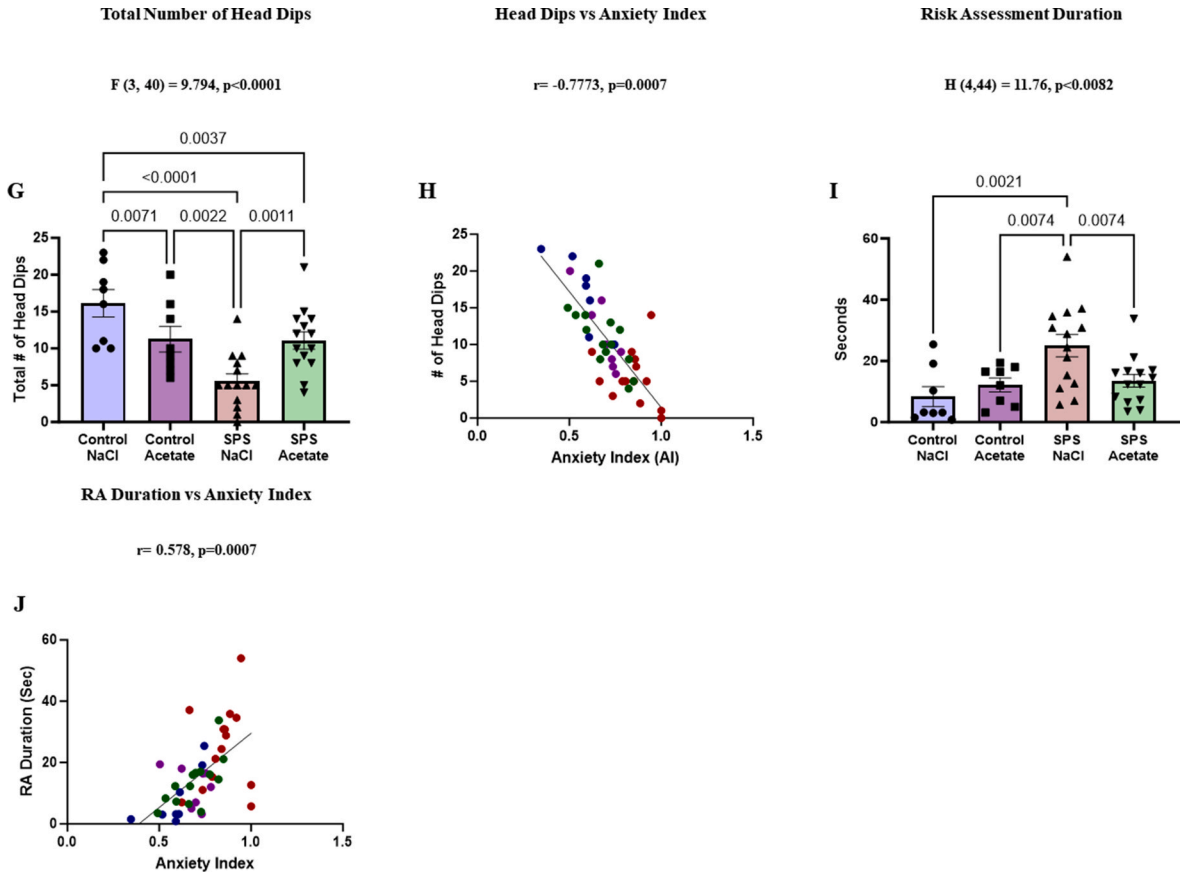


**Fig. 2.** (*continued*).

### 3.3. Acetate supplementation alleviated SPS-induced social impairments but not depressive-like behavior

To evaluate the effect of acetate on social behavior, we used the SI test, which assesses the active interaction of a test animal with a novel juvenile rat. The animals in the SPS + NaAcetate group spent more time (Fig. 3A) and initiated more interactions (Fig. 3B) towards the juvenile rat than did those in the SPS + NaCl group. Moreover, the latency to the first approach to the juvenile rat was significantly shorter in the SPS + NaAcetate group than that in the SPS + NaCl group (Fig. 3C). As in the previous tests, the social behavior of the SPS + NaAcetate group was comparable to that of the unstressed controls, and acetate supplementation did not alter the performance of the controls. Finally, we assessed despair depressive-like behavior using FST. No significant differences in the immobility time were observed among the groups (Fig. 3D).

To assess whether the animals maintained an overall consistent behavior in the different behavioral tests, we performed a set of correlational analyses. The number of rears in the OF test correlated positively with the number of interactions in SI (Fig. 3E), as well as with OA duration on the EPM (Fig. 3F). SI duration was also positively correlated with OA duration (Fig. 3G). No correlations were observed between immobility in the FST and any of the other behavioral tests.

### 3.4. Acetate supplementation regulated the stress response by inhibiting the SPS-induced increase in urinary epinephrine levels

To assess the effect of acetate supplementation on the stress response, we measured the levels of urinary corticosterone and epinephrine. The levels of urinary corticosterone immediately before immobilization were significantly lower in the acetate-supplemented group than in the SPS + NaCl group. However, 30 min into the immobilization step of SPS, the levels increased markedly to a similar level in both groups, and no group differences were observed (Fig. 4A). Whereas, the levels of urinary epinephrine were significantly lower in the SPS + NaAcetate group than in the SPS + NaCl group before immobilization and remained significantly lower even after 30 min into the immobilization step (Fig. 4B). Moreover, while the levels of epinephrine increased significantly in the SPS + NaCl group during immobilization, there was no increase in the SPS + NaAcetate group.

### 3.5. Acetate supplementation altered the mRNA gene expression of histone acetylase/deacetylase in ventral hippocampus

Acetate can participate in histone acetylation by altering the expression of key enzymes involved in this process, including histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Soliman and Rosenberger, 2011). As the ventral hippocampus is one of the main brain regions consistently shown to be altered by stress, we focused on assessing gene expression in this region (McEwen, 2000). HAT1 mRNA expression was significantly higher in the control + NaAcetate group than in the SPS + NaCl group (Fig. 5A). Acetate supplementation also appeared to increase, yet not significantly, the expression of HAT1 in the SPS + NaAcetate group. However, no differences were observed in HAT1 mRNA expression between NaCl treated controls and SPS group. As for HDACs, acetate significantly decreased the expression of HDAC2 gene (Fig. 5B) but not HDAC7 (Fig. 5C) in the SPS +NaAcetate group compared to SPS + NaCl group. The mRNA levels of HDAC2 also correlated positively, albeit weakly, with the anxiety index (Fig. 5D).

### 3.6. Acetate supplementation reduced SPS-triggered neuroinflammation in ventral hippocampus

Several studies have indicated the potential benefits of acetate in ameliorating neuroinflammation (Reisenauer et al., 2011; Soliman et al.,

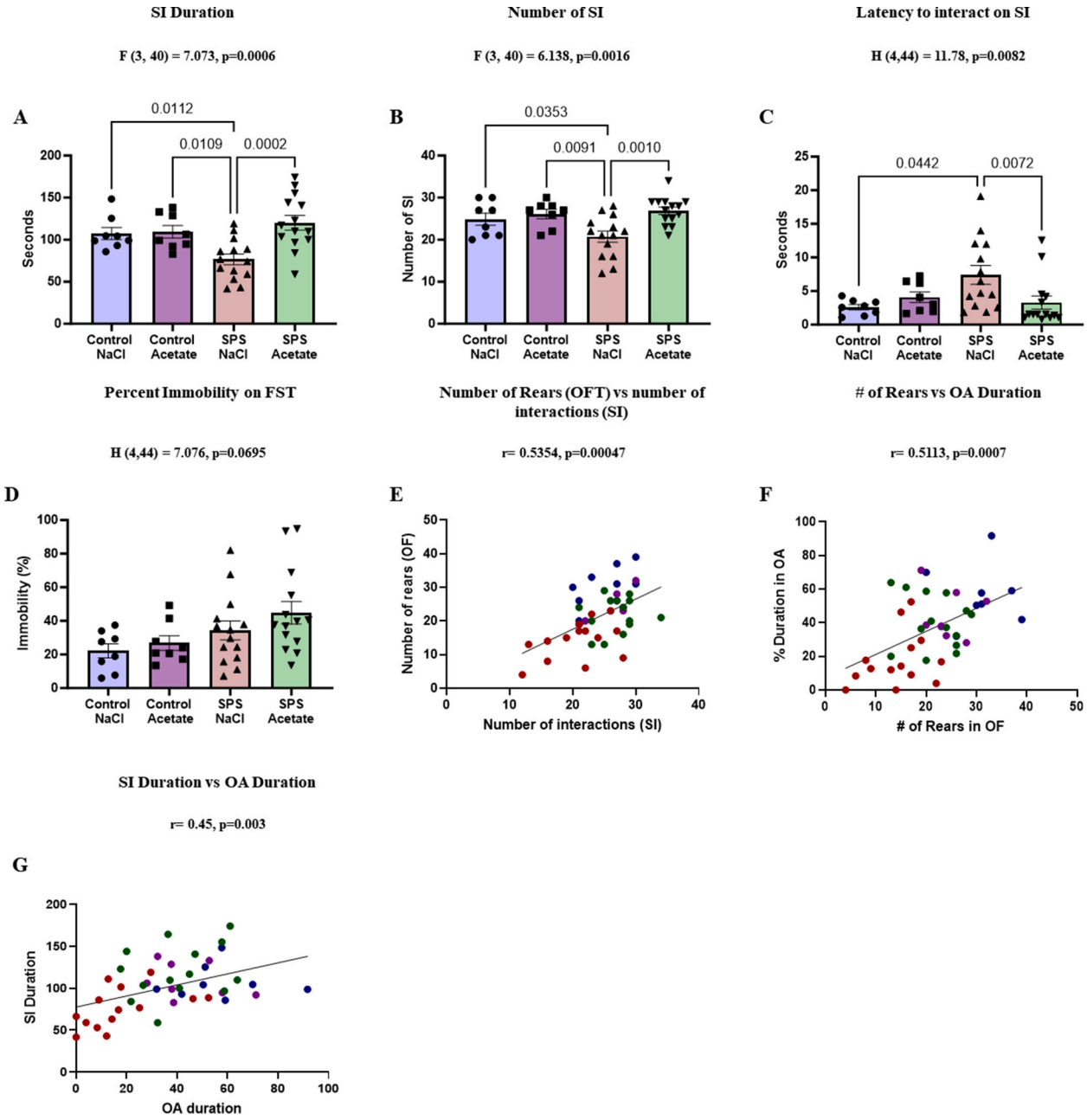


**Fig. 3.** *Effect of acetate supplementation on social impairment and depressive-like behavior*. The animals' social and depressive-like behaviors were assessed using Social Interaction (SI) and Forced Swim (FS) tests respectively. *On SI*: **A**: Duration of SI, **B**: Number of interactions initiated by the test rat towards the juvenile rat, **C**: Latency to initiate the first interaction. *On FST* **D**: % time spent immobile, **E**: Correlation between number of rears on OF and number of interactions on SI, **F**: Correlation between number of rears on OF and % time spent in the OAs of EPM, **G**: Correlation between time spent interacting on SI and time spent on the OAs of EPM. Data of duration and number of SI passed the normality test and were analyzed using one-way ANOVA. The remaining data did not pass the normality test and were analyzed using the Kruskal-Wallis test. Correlation analysis was performed using Pearson's correlation coefficient. Each dot represents the value for an individual animal (**Control + NaCl**: n = 8, **Control + NaAcetate**: n = 8, **SPS + NaCl**: n = 14, SPS + **NaAcetate**: n = 14).

2012). For this purpose, we assessed the role of oral acetate in reducing SPS-induced neuroinflammation in the ventral hippocampus. The mRNA levels of IL-1β in the vHipp were significantly higher in the SPS + NaCl group than in the SPS +NaAcetate and control groups (Fig. 5E) and correlated positively with HDAC2 mRNA levels (Fig. 5F). The acetate-treated SPS group also had fewer Iba1+ cells in various CA regions of vHipp compared to the untreated SPS group. Acetate supplementation did not affect the gene expression of IL-1ß or the number of Iba1+ cells in the controls (Fig. 5G).

### 3.7. Acetate supplementation induced increase in ketone body production

Ketone bodies are produced primarily by the liver and have been reported to have potential benefits for anxiety (Dilliraj et al., 2022). β-hydroxybutyrate is one of the main ketone bodies that may exert anxiolytic effects (Sleiman et al., 2016; Yamanashi et al., 2017; Youm et al., 2015). As acetate indirectly can be converted to ß-hydroxybutyrate through the ketogenic pathway, we assessed the serum levels of β-hydroxybutyrate in the acetate-treated and untreated groups. Acetate supplementation had no effect on serum β-hydroxybutyrate levels in the unstressed controls. However, animals in the SPS-acetate group

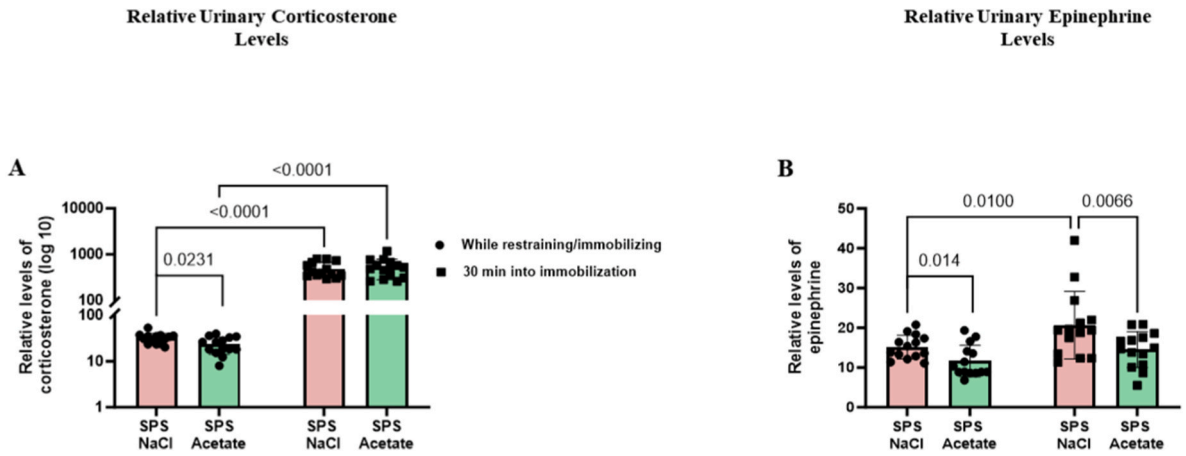


**Fig. 4.** *Effect of acetate supplementation on the HPA axis and sympathoadrenal system.* Urine samples from each rat were collected before SPS and 30 min into it. **A**: Relative urinary corticosterone levels, **B:** Relative urinary epinephrine levels. All data passed the normality test. Analysis was performed using repeated measures two-way ANOVA. Urinary corticosterone levels showed only significant group effect ($F(1,52) = 132.4$, $p < 0.0001$), whereas epinephrine levels showed both significant time ($F(1,52) = 11.07$, $p = 0.0016$) and group ($F(1,52) = 8.396$, $p = 0.0055$) effects, with no significant interaction between the two factors. Analysis of the data only while restraining was performed using student's t-test. Each dot represents the value for an individual animal (**Control + NaCl**: n = 8, **Control + NaAcetate**: n = 8, **SPS + NaCl**: n = 14, SPS + **NaAcetate**: n = 14).

showed, on average, 4-fold higher serum levels of β-hydroxybutyrate than the comparable group with NaCl (Fig. 6).

## 4. Discussion

The findings of this study demonstrate a causal relationship between oral acetate supplementation and the amelioration of various behavioral impairments induced by SPS. These results support the hypothesis that acetate, a microbial metabolite, can effectively mitigate some of the adverse effects of traumatic stress, particularly anxiety and social impairments. The observed improvements in behavior were accompanied by concomitant changes in the stress response, epigenetic modifications, neuroinflammation, and ketone body production. Overall, this study provides strong evidence for the preventive-therapeutic potential of acetate supplementation in ameliorating many negative effects of SPS on behavior and highlights its likely role in regulating various neurochemical and neuroendocrine pathways.

SPS, a severe and multifaceted traumatic stressor, elicits a robust stress response, resulting in a range of behavioral, neurobiological, and neuroimmune impairments (Lisieski et al., 2018). A key finding of this study is the efficacy of acetate supplementation in alleviating the effects of this potent stressor. While having no impact on the unstressed controls, acetate supplementation resulted in significant improvements in the SPS-exposed group. It mitigated the stress-induced decreased weight gain, reduced anxiety levels, as indicated by decreased number of risk assessments, frequent head dips, longer durations, and increased entries into the open arms of the EPM, and increased rearing behavior in the OF. Furthermore, acetate supplementation ameliorated social impairments by improving the duration, number, and latency of social interactions. Importantly, these behavioral improvements were consistent across different behavioral tests, as evidenced by various correlational analyses, suggesting that the beneficial effects of acetate treatment are not specific to any particular test, but rather have a broad impact, thus increasing confidence in the validity and reliability of the observed effects.

Our study is in line with recent report that demonstrated the efficacy of oral supplementation with glyceryl triacetate (GTA) in reducing several behavioral impairments induced by chronic social defeat stress (Huang et al., 2021). This study, like ours, did not find significant group differences in the time spent in the center of OF test, suggesting that this measure may not be sensitive enough to capture differences in anxiety-like behavior. Rearing behavior in the OF has emerged as an important component in evaluating an animal's behavioral state, with several studies reporting a strong correlation between rearing behavior and hippocampal morphology (Lever et al., 2006; Sturman et al., 2018). We observed significantly lower rearing behavior in the SPS +NaCl group than in the acetate-treated SPS group, despite the absence of differences in the duration and number of entries into the center of the OF arena. Rearing behavior in the OF test, as opposed to number of entries and duration, also correlated with several behavioral parameters in different tests, suggesting that it is useful behavioral measure.

In contrast to the findings of Huang et al. (2021), we did not observe any improvements in depression-like behavior using the FST. One possible explanation is the differences in treatment type/route. Another possibility is the fact that the FST was performed in the same tanks as a part of the SPS procedure; therefore, the observed results might reflect not only despair/depressive behavior but also an element of trauma memory in these animals. In the future, we plan to utilize other tests that are better suited to assess the effects of acetate on depression in this model.

The anxiolytic effect of acetate supplementation observed in this study may be related to its influence on the stress response. Animals treated with acetate exhibited reduced corticosterone levels at the start of the immobilization step of SPS, but it was markedly increased 30 min later. Alternatively, acetate may influence the stress response through the sympathoadrenal axis, which is evident by the significantly lower levels of urinary epinephrine, both at the beginning and 30 min into the immobilization step. Possible mechanisms for this modulation by acetate could be due to the ability of SCFAs to cross the blood-brain barrier via specific "leaky" brain regions involved in stress response (Ziegler and Herman, 2002), as well as through acetate's conversion to β-hydroxybutyrate, which was significantly higher in the SPS + NaAcetate group and was shown to inhibit the sympathetic nervous system (Kimura et al., 2011).

Histone modification, specifically histone acetylation and deacetylation, is an emerging area of research in the field of mood disorders (Bassett and Barnett, 2014; Howie et al., 2019; Plagg et al., 2015; Sen, 2015). Acetate has been shown to play an important role in regulating histone acetylation and subsequent gene expression (Huang et al., 2021; Soliman and Rosenberger, 2011; Soliman et al., 2012). Gut microbiota-derived acetate can increase the brain acetate levels (Perry et al., 2016). Studies have also demonstrated that sodium acetate (150 mM) administered through the drinking water can effectively penetrate the brain (Erny et al., 2021). We observed an increase in HAT1 mRNA

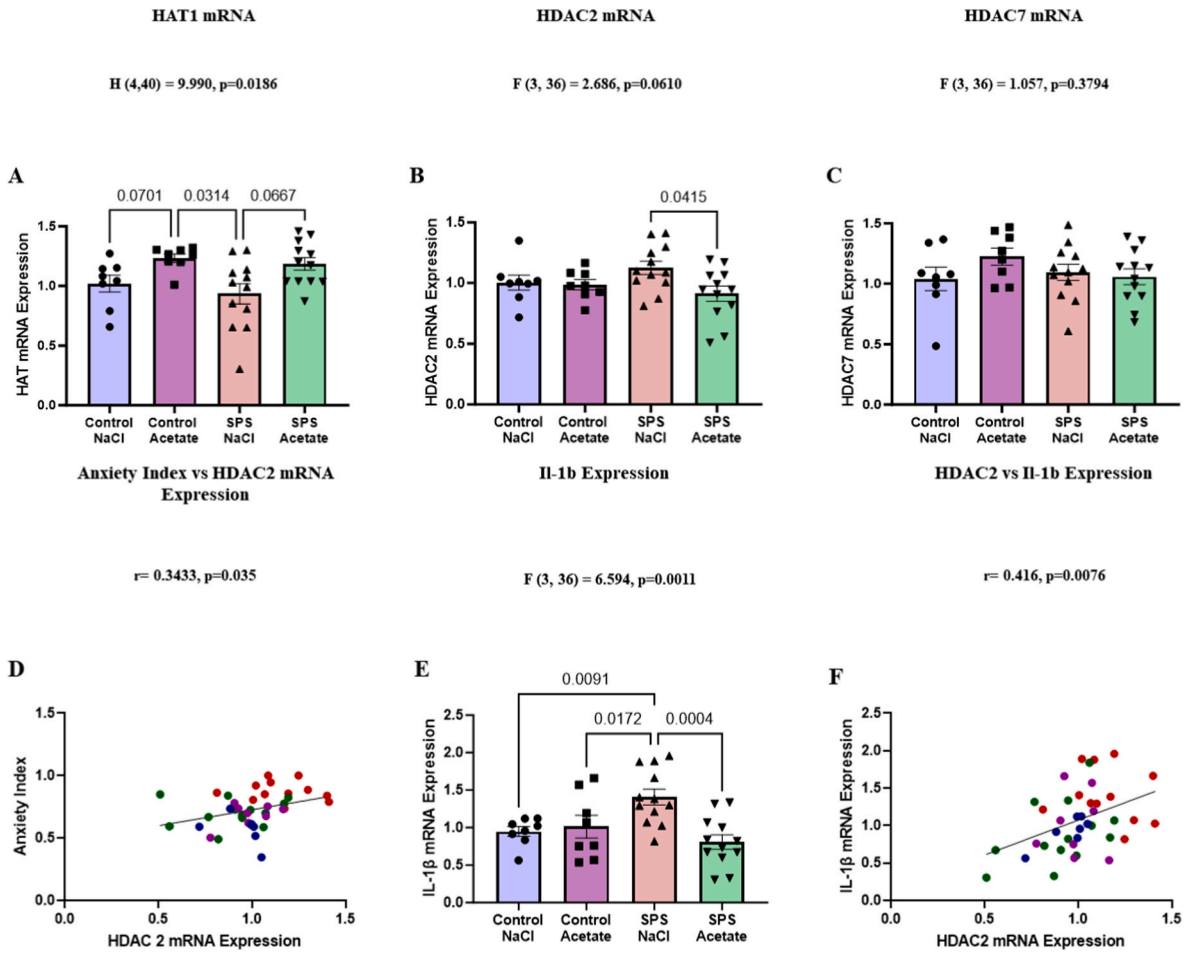


**Fig. 5.** *Effect of acetate supplementation on ventral hippocampal gene expression and microglial density.* The expression of several genes and the density of microglial cells were assessed. **A**: mRNA expression of HAT1 gene, **B**: mRNA expression of HDAC2 gene, **C**: mRNA expression of HDAC7 gene, **D**: Correlation between anxiety index and mRNA expression of HDAC2 gene, **E**: mRNA expression of IL-1ß gene, **F**: Correlation between mRNA expressions of HDAC2 and IL-1ß genes, **G**: Number of Iba1+ cells in ventral hippocampus. Data from HAT1 mRNA expression did not pass the normality test and were analyzed using the Kruskal-Wallis test. The remaining data passed the normality test and were analyzed using one-way ANOVA. For Iba1+ cells analysis, One way-ANOVA was followed by Tukey's multiple comparisons test (**Control + NaCl**: n = 3, **Control + NaAcetate**: n = 3, **SPS + NaCl**: n = 4, SPS + **NaAcetate**: n = 4). Representative IHC images are shown. Correlation analysis was performed using Pearson's correlation coefficient. Each dot represents the value for an individual animal (due to unavailability, 2 samples from SPS + NaAcetate and SPS + NaCl were not analyzed). (**Control + NaCl**: n = 8, **Control + NaAcetate**: n = 8, **SPS + NaCl**: n = 12, SPS + **NaAcetate**: n = 12

expression in the acetate-treated control, and to a lesser extent, in the SPS-acetate group compared to the untreated SPS group. However, no differences were observed in the HAT1 mRNA levels between the NaCl-controls and NaCl-SPS groups, suggesting that the differences observed in the acetate groups are a physiological response to acetate treatment rather than changes triggered by the SPS stressors. Regarding the HDACs, we found a significant decrease in the mRNA expression of HDAC2, a class one HDAC, primarily involved in gene expression regulation, but not in HDAC7, a class 2 HDAC associated with diverse cellular processes (Parra, 2015; Watson et al., 2016). These findings are in accordance with a study conducted by Soliman et al. (Soliman and Rosenberger, 2011) which showed that acetate treatment of male rats decreased the levels of HDAC2 of the total brain without affecting HDAC7 or HAT levels/activity. Interestingly, a chronic social defeat stress model altered the levels of both HDAC2 and HDAC7 (Huang et al., 2021). This suggests that alterations in HDAC levels might be stressor specific and thus modifications in the levels of class I, but not class II HDACs, might be required to withstand/overcome the effects of SPS, a traumatic stressor.

Although the mRNA levels of HDAC2 were not increased in SPS + NaCl group, we found a positive correlation between HDAC2 mRNA levels and anxiety index; suggesting that the enzymatic activity might be increased, and thus play a role in the development of anxiety symptoms. To date, only few studies have explored the involvement of acetate in histone acetylation (Huang et al., 2021; Soliman and Rosenberger, 2011; Soliman et al., 2012; Wolugbom et al., 2023). Notably, all these studies employed oral gavage as the delivery method for acetate. To our knowledge, this study is the first to investigate the effects of sodium acetate administered through drinking water on these aspects of epigenetics as well as on behavior. By using this non-stressful mode of delivery, we aimed to minimize the potential stress induced by oral gavage and provide acetate supplementation *ad libitum*.

Acetate supplementation also appeared to reduce SPS-induced neuroinflammation, as evidenced by lower mRNA expression of the proinflammatory cytokine IL-1ß, and fewer Iba1+ cells in the vHipp. Our findings support previous studies that have highlighted the anti-inflammatory effects of acetate. For example, acetate treatment decreased IL-1ß mRNA expression and neuroglial activation in a rat model of LPS-induced neuroinflammation (Soliman et al., 2012), and reduced colonic inflammation in an experimental colitis model (Maslowski et al., 2009). One potential mechanism underlying the anti-inflammatory action of acetate may involve epigenetic modulation,

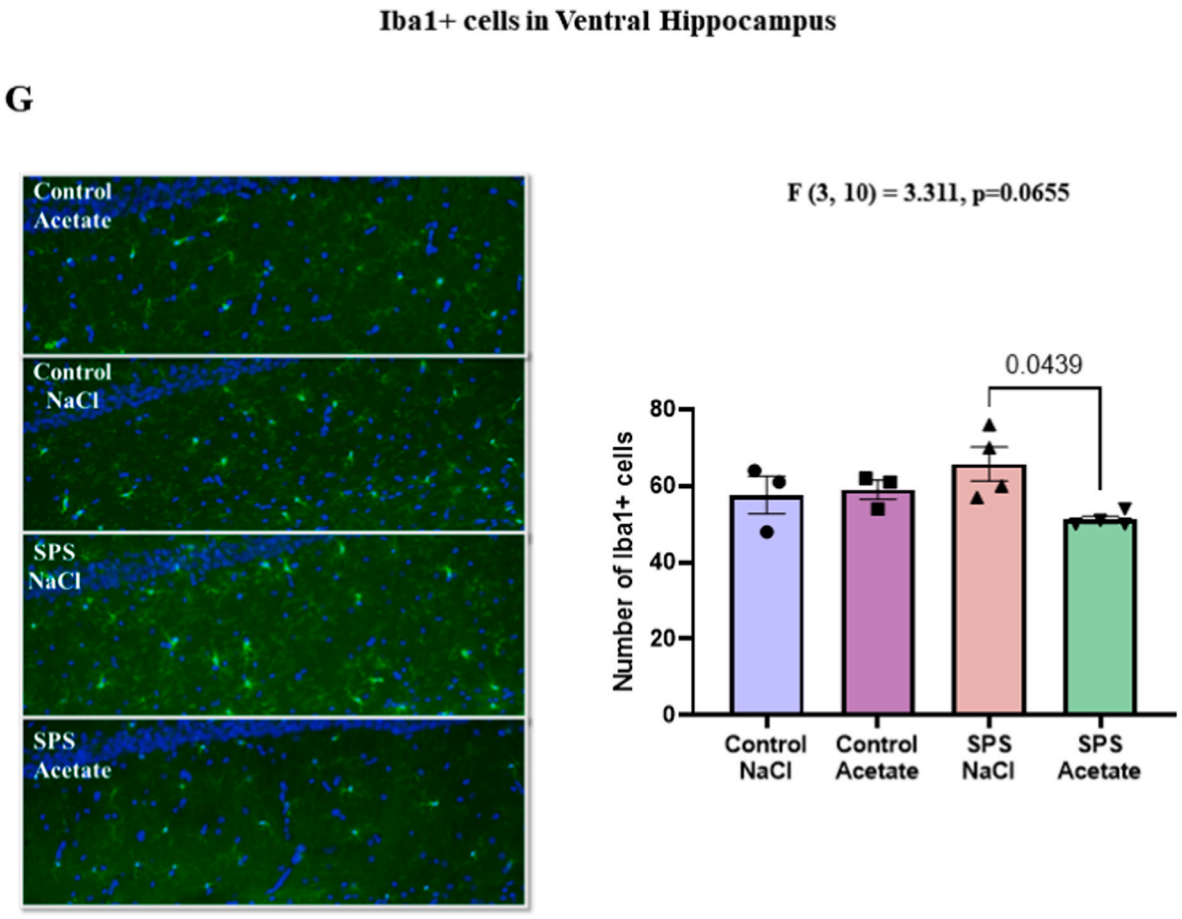


**Fig. 5.** (*continued*).

as histone hyperacetylation is associated with anti-inflammatory effects (Adcock, 2007; Kim et al., 2007; Langley et al., 2005; Zhang et al., 2008). We observed a significant reduction in HDAC2 mRNA levels in vHipp following acetate-treatment. The levels also showed positive correlation with IL-1ß expression. Although specific sites of histone acetylation were not assessed in our study, a separate study on LPS-induced neuroinflammation demonstrated a direct reduction in H3K9 acetylation, and increase in IL-1ß expression, which were reversed by acetate supplementation (Soliman et al., 2012). Additional studies have shown that the use of HDAC inhibitors can decrease microglial activation and TNF-α and IL-1ß gene expression while increasing histone H3 acetylation, particularly H3K9 (Li et al., 2009; Zhang et al., 2008). Considering HDAC2's role in deacetylating histone H3 lysine residues and its positive correlation with IL-1ß gene expression and anxiety index, our findings suggest that this mechanism may be relevant in the SPS model as well.

The benefits of acetate supplementation may also be mediated by its effects on ketosis. Upon production in the gut, any unmetabolized SCFA, particularly acetate, is transported to the liver, where it is converted to acetyl-CoA, leading to increased ketone body production. As the liver cannot utilize ketone bodies, their levels increase in the blood, providing energy to different organs. In this study, we observed higher levels of β-hydroxybutyrate specifically in the acetate-treated SPS group. This selective increase might be due to higher energy demand because of the stress response (indeed, the SPS + NaCl rats gained less weight), alterations in gut microbiota affecting acetate absorption and metabolism, or the potential underlying neuroprotective effects of acetate. β-Hydroxybutyrate, the major ketone body, can cross the blood-brain barrier and play a role in epigenetic regulation, specifically by decreasing the expression of HDAC2 and HDAC3 and increasing the transcription of BDNF, a trophic factor associated with cognitive improvement and alleviation of depression and anxiety (Sleiman et al., 2016). This might provide an additional mechanism by which acetate reduced the expression of HDAC2 in this study. Moreover, administration of β-hydroxybutyrate has been shown to attenuate SPS-induced anxiety and increase serum TNF-α levels (Yamanashi et al., 2020). In fact, a recent small study on adjuvant supplementation with β-hydroxybutyrate in patients with PTSD, showed significant decline in the PTSD checklist (PCL-5) in the treated group, although the differences between the groups were not significant (Youssef et al., 2022).

While this study demonstrated the potential benefits of oral acetate in mitigating certain behavioral measures induced by SPS, there are important limitations that warrant discussion. We did not directly measure the extent of acetate reaching the brain, as this has been established in previous research (Erny et al., 2021). However, future studies should incorporate labeled acetate to determine its uptake and levels in various organs, as well as the proportion of acetate incorporated into the histone markers. Additionally, we concentrated solely on the ventral hippocampus due to its consistent alterations observed by stress. Nevertheless, future research should explore the impact of acetate on gene expression in multiple brain regions, including the medial prefrontal cortex and amygdala. Furthermore, our focus on a limited number of histone acetylase and deacetylase markers was influenced by their known alterations in response to stress and acetate treatment (Huang et al., 2021; Soliman and Rosenberger, 2011; Soliman et al., 2012). However, future investigations, should expand the analysis to include several other markers, assess their enzymatic activities, and specific acetylation/deacetylation sites. The potential effect of acetate supplementation on gut microbial composition and cecal acetate levels was not assessed in this study. Existing literature suggests that

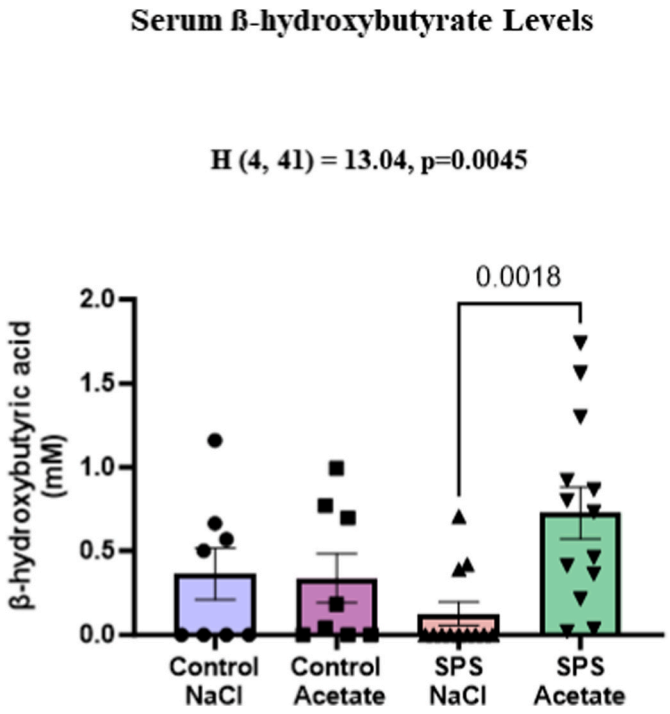


**Fig. 6.** *Effect of acetate supplementation on serum ß-hydroxybutyrate levels.* Serum was collected from each animal at the end of the experiment to assess the levels of β-hydroxybutyrate. Data did not pass the normality test and were analyzed using the Kruskal-Wallis test. Each dot represents the value for an individual animal (due to unavailability, serum samples from one animal in SPS + NaAcetate group and 2 animals from SPS + NaCl were not quantified). (**Control + NaCl**: n = 8, **Control + NaAcetate**: n = 8, **SPS + NaCl**: n = 12, SPS + **NaAcetate**: n = 13).

exogenous SCFA supplementation within the scope of our chosen intervention periods does not yield significant impacts on the microbiome (van de Wouw et al., 2018). Lastly, it is important to note that this study involved only male subjects, as previous findings indicated altered cecal acetate levels exclusively in male subjects susceptible to SPS (Tanelian et al., 2022, 2023). Nevertheless, future studies will expand the scope to include female subjects. Overall, addressing these limitations in future investigations will provide a more comprehensive understanding of the effects of acetate and its mechanisms of action in the context of stress responses and mood disorders.

In conclusion, this study established a causal relationship between oral acetate supplementation, a microbial metabolite, and the improvement of behavioral impairments induced by traumatic stress. Acetate supplementation through drinking water has significant effects in improving the negative consequences of stress, potentially through mechanisms involving stress response regulation, gene expression modulation, and reduction of neuroinflammation. These findings lay the groundwork for further investigation into the therapeutic potential and precise mechanisms of action underlying acetate supplementation in ameliorating adverse effects of the traumatic stressors.

## Funding

This study was partially funded by Student Touro Research Fellowship Grant Program Award 2022 (log # 017016).

## CRediT authorship contribution statement

Arax Tanelian: planning, experimentation, analysis, writing the manuscript: Bistra Nankova: planning, experimentation, analysis, writing the manuscript; Furung Hu, Jordan D. Sahawneh: experimentation; Esther L. Sabban: planning, supervision of experimentation, analysis and writing. All authors read and approved the submitted manuscript.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgements

We thank Dr. Edmund LaGamma for his insightful comments and support.

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