Negative air ion exposure ameliorates depression-like behaviors induced by chronic mild stress in mice

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Abstract

The presence of negative air ions (NAI) is suggested to be a good factor in improving psychological status and used in treating depression as an alternative approach. However, the biological explanation for effects of NAI on alleviating depression symptoms has less been explored. In this study, the chronic mild stress (CMS) protocol was used to induce transcriptional depressive-like behaviors in mice, and the effects of NAI exposure on CMS-induced depression-like behaviors were examined. Thirty-day NAI exposure prevented the CMS-induced depression-like behaviors as shown by the restoration of sucrose preference and reduced immobility time in the suspension test. In addition, the elevation of serous corticosterone was present in CMS-treated mice but not existed in those with the NAI exposure. Furthermore, we observed a shifted balance between the cytokines secreted by type 1 T helper (Th1) cells and type 2 T helper (Th2) cells. In conclusion, NAI intervention is able to ameliorate CMS-induced depression-like behaviors in mice, and this effect is associated with the alteration of corticosterone and functional rebalance between Th1 and Th2 cells.

Introduction

Air ions are molecules of ionized particles present in the atmosphere, either losing or gaining an electric charge. They are generated in a variety of natural or artificial ways, such as rain, wind, snow, lightning, and ion generators, and currently are available for either domestic or industrial uses (Jiang, Ma, & Ramachandran, 2018). It has been speculated that exposure to positive air ions in the environment is harmful to human health, while exposure to negative air ions (NAI) has beneficial health effects. Several explorations have focused on the biological effects of air ions on mood and behaviors (Bachman, McDonald, & Lorenz, 1966; Della Vecchia, Mucci, & Marazziti, 2020; Olivereau, Lambert, & Truongngoc, 1981). A meta-analysis reported that the exposure to the air ions show no consistent results on the performances of wheel running, spontaneous locomotion, brain electrical activity and sleep patterns in animals (Bailey, Williams, & Leonhard, 2018), while other studies measuring the effects of air ionization on various psychological parameters related to mood or emotional state have demonstrated that higher concentrations of NAI exposure is positively associated with mental health (Chu et al., 2019; Jiang et al., 2018; Perez, Alexander, & Bailey, 2013).

The etiology of depression is complex and diverse (Duman, Aghajanian, Sanacora, & Krystal, 2016; Krishnan & Nestler, 2008). Etiological hypotheses of this disorder include the dysfunctions of monoaminergic system, hyperactivity of hypothalamic-pituitary-adrenal (HPA) axis, inflammatory alterations and neurotrophic abnormalities (Villas Boas et al., 2019). Hyperactivity of HPA axis shown by high serous level of cortisol is present in a large population of depressed patients (Hinkelmann et al., 2009; Leonard, 2018), and also observed in animal models (X. Q. Wang et al., 2021; Zaletel, Filipovic, & Puskas, 2016). Cortisol is released in response to stress and regulates immune and inflammatory processes, energy metabolism and neuronal survival (Nikkheslat, Pariante, & Zunszain, 2018; Zunszain, Anacker, Cattaneo, Carvalho, & Pariante, 2011). Increasing data have evidenced that inflammation and

HPA axis hyperactivity often coexist in the episodes of depression (Cernackova, Durackova, Trebaticka, & Mravec, 2020; Gold, 2015).

Inflammation has been shown to interact with almost all pathophysiological domains known to be related to depression (Kim, Na, Myint, & Leonard, 2016; A. H. Miller, V. Maletic, & C. L. Raison, 2009). Cytokines are polypeptides or glycoproteins synthesized and secreted by peripheral monocytes, macrophages, lymphocytes, and as well as brain cells such as neurons, astrocytes and microglia. They play important roles in the bidirectional immune communication between the brain and the periphery (Leonard, 2018). Specifically, cytokines produced in the periphery can access and together with those generated within the brain influence the function state of the brain through humoral and neural pathways (Capuron & Miller, 2011). They are critical in the immune response to help to mark and eliminate the pathogens, and also affect many aspects of brain functions such as neurotransmitter metabolism and its activity, neuroendocrine, neurogenesis, and neurocircuits relevant to mood, alarm, anxiety and motor activity (Himmerich, Patsalos, Lichtblau, Ibrahim, & Dalton, 2019). Previous studies have found that the administration of interferon-γ (IFN-γ) and inflammation inducers, lipopolysaccharides in rodents results in altered behaviors similar to those of depressed patients (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Kentner et al., 2008). Whereas, cytokine antagonists, such as interleukin-1 (IL-1) receptor antagonist and prebiotics attenuate social and/or anxiety behavior in rodents (Arakawa, Blandino, & Deak, 2009; Dantzer, 2004; Savignac et al., 2016).

Cytokines can be divided into several categories including IL, colony stimulating factor (CSF), IFN, tumor necrosis factor (TNF) and chemokine family. Circulating level of cytokins ranges according to the condition of the body. Importantly, cytokines respond to stress-triggered neuroinflammation, and modulate the function of neurons in coordination with astrocytes in the development of depression (Jia, Gao, & Hu, 2020; C. Zhang et al., 2019). Various alterations of cytokines have been reported in patients with depression (Lotrich, 2015), including IL-1, IL-6, IL-2, IL-4, IL-10, IL-21, IL-22, TNF-α, TGF-β and IFN-γ (Kohler et al., 2018; Mosiolek et al., 2021; Nobis, Zalewski, & Waszkiewicz, 2020). Besides, it was suggested that antidepressant treatment could decrease peripheral levels of IL-6, IL-10, TNF-α and IFN-γ (C. Y. Chen et al., 2018; Kohler et al., 2018).

Chronic mild stress (CMS) is a crucial trigger of depression (Paul Willner, 2017), and CMS-treated animals exhibit depression-like behaviors, such as helplessness and anhedonia (L. Chen et al., 2021; Garcia et al., 2009; X. Q. Wang et al., 2021). The objective of this study is to investigate the roles of NAI exposure in behavioral alterations induced by CMS treatment in mice. Contents of cortisol and cytokines in the serum were examined to explore possible contribution of immune responses and HPA axis in this process. We found that the NAI exposure could ameliorate CMS-induced depression-like behaviors shown by sucrose preference test and tail suspension test. In addition, the NAI exposure also interfered the alterations of cortisol levels and multiple cytokines in the serum, which may contribute to the behavioral changes in CMS-treated mice.

Material And Methods

Animals

Six-week-old male C57BL/6 mice were obtained from Shanghai Lingchang Biotechnology Co. Ltd, China. Animals were provided regular rodent chow and water *ad libitum* and were maintained on a 12 h light/dark cycle (lights on at 7:00 a.m.), with a temperature of 25 ± 1°C and a humidity level of 50 ± 10%. Prior to the experimental procedure, mice were accommodated in the experiment room for two weeks. All procedures were carried out in compliance with the Animal Experimental Ethics Committee of Shanghai Medical School, Fudan University.

Apparatus

Filtered air (FA) box and NAI box (50 cm length× 40 cm width × 100 cm height) were equipped with a fan (ERF500D1N, Honeywell). The NAI box contained a negative ion generator purchased from Shanghai Sailumei Environmental Protection Technology Co., Ltd, China, and provided approximately 4×10^4 small NAIs per cubic centimeter (high-density exposure) at 1 meter to the feeding cages. Automatic observation system of atmospheric negative ions (Wide Creative Science & Technology, Beijing, China) was used to monitor the concentration of NAIs whose ion mobility is not less than 0.4 cm^2 /(V·s).

Experimental procedure

Mice were housed either in the FA box or NAI box. In each box, animals were randomly assigned into two groups: one suffered from CMS for 30 days and the other stayed as is. Therefore, the research was consisted of 4 groups: 1) Control mice in FA box, 2) CMS-treated mice in FA box, 3) Control mice in NAI box and 4) CMS-treated mice in NAI box. Mouse body weight between groups was evaluated before and after 30-day exposure. After the 30-day exposure, behavioral tests were performed and mice were sacrificed after completing these tests for examination of contents of cortisol and cytokines in the serum.

CMS paradigms

The CMS protocol was conducted according to the well-established methods with minor modifications (L. Chen et al., 2021; X. Q. Wang et al., 2021; Zhao et al., 2021). The mouse was restained in a 50-ml tube with no space to turn over for 6 hours every day, along with unpredictable 3-minute shaking for 5-7 times during this period.

Behavioral testing

Behavioral experiments were performed in a sound-proof room with a neutral environment. All behavioral tests were conducted during the light phase of the light/dark cycle. All mice were given a 30-minute habituation in the behavioral room before the start of each test. After each animal completed the behavioral test, the equipment was thoroughly cleaned to eliminate olfactory effects. The experimenter was blind to the group identity of the tested mice. Some behavioral tests were recorded by a video camera, then the footages were analyzed by a trained researcher.

Open field test (OFT)

A black square arena (45 × 45 × 30 cm) was used to examine locomotor activity. Mice were placed in the center of the arena and allowed to explore the apparatus freely for 5 min (Y. L. Wang et al., 2018). Total distance moving in the field was analyzed by the EthoVision XT video tracking software (Ver. 12).

Sucrose preference test (SPT)

All stages of the test were carried out at the same time of the day (C. Zhang et al., 2019). Each cage was provided with two drinking tubes containing sucrose water (2% w/v) during the first 24-hour training phase. Then the next day, one bottle with 2% sugar solution and another bottle with regular water were provided to mice. After training, mice were deprived of water and food for 24 hours, then the mice were given the free choice to drink from two bottles for 24 hours: one was filled with a sucrose solution, and the other was filled with water. The positions of the bottles in the cage were switched after the first 12 hours. Sucrose and water consumptions were recorded separately before and after the test. Sucrose preference % = (sucrose intake/total intake) × 100%. The total intake value is the sume of water intake value and sucrose intake value.

Forced swimming test (FST)

Animals were individually placed in a transparent acrylic cylinder (height 30 cm, diameter 15 cm) for 6 minutes (Zhao et al., 2021), which was filled with tap water to a depth of 20 cm. The first 2 minutes were spent for adaptation, and the last 4 minutes were analyzed. Immobility time was evaluated as floating or no active movements except those necessary for the mouse to keep its head above water.

Tail suspension test (TST)

In the TST, mice were suspended 30 cm above the floor with an adhesive tape applied approximately 1 cm from the end of the tail on a metal hook (Xu et al., 2021). At the beginning of the test, nearly all the mice attempted to escape from hanging, but after a period of struggling, it showed intermittent immobility, displaying a state of "behavioral despair". The duration of this state was considered as the immobility time. The activities of the mice were recorded by a video camera, then the immobility time during the last 4 minutes of a 6-minute testing period was evaluated.

Rotarod test (RTT)

Mice were habituated to the rod for 2 minutes while it slowly rotated (10 rpm-rotations per minute) and they were replaced on the rod if they fell off during the 2 min. Testing consisted of three-trial sessions; each session had a progressively increasing speed from 4 rpm to 40 rpm within 5 minutes. On each of the three trials, the mouse was placed on the rod and left there until either 300 seconds elapsed or until the mouse fell off. There was a 1-hour break between trials. The average performance of total time on the rod for the three-trial session was analyzed.

Y maze (YMT)

Working memory was evaluated by spontaneous alternation Y-maze test (Pontifex et al., 2021). The apparatus comprised of three identical arms (30cm× 5cm x 10 cm), spaced 120° apart. The mouse was

placed in one arm of the maze and allowed to explore freely for 8 minutes. At the same time, zone transitionings were recorded by tracking software (EthoVision XT video tracking software, Ver. 12). Spontaneous alternation was calculated using the following formula: Spontaneous alternation % = (Number of alternations/Total Arm entries − 2) × 100.

Cytokine and cortisol measurement

Blood was collecting from obital sinus in anesthetized mice. The serum from blood was isolated by centrifugation for 20 min at 1000×g at 4℃, then the aliquots of the samples were stored at -80°C before they were measured. Cytokines, including granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-21, IL-23, IFN-γ and TNF-α in the serum were determined using commercial Interleukin Antibody Arrays (QAM-INT-1, RayBiotech Inc). Serum concentration of cortisol was determined by using an enzyme linked immunosorbent assay (ELISA) kit (Enzo, ADI-900-071). Experiments were performed in accordance with the manufacturer's protocols.

Statistical analysis

All figures were performed using Graph Pad Prism 6 and IBM SPSS statistics 20.0. Data were expressed as the mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used for statistical analysis of data, followed by Bonferroni (test of homogeneity of variance α is more than 0.05) or Tamhane's T2 (test of homogeneity of variance α is less than 0.05) post-hoc multiple comparison test. Statistical significance was defined as $*P< 0.05$, $**P< 0.01$, $***P< 0.001$.

Results

NAI exposure ameliorates CMS-induced depression-like behaviors

During the 30-day experiment, the concentration of negative oxygen ions was monitored (Fig. 1), showing a consistent supply of NAIs.

The CMS design and timeline of behavioral observations are shown in Fig. 2a. Depression-like behaviors were examined by the sucrose preference, force swimming and tail suspension tests. The reduction of preference to sucrose reflects the core depression-like behaviors in CMS-induced depression animal model (Czeh, Fuchs, Wiborg, & Simon, 2016). As expected, the CMS treatment reduced the consumption of sucrose solution in FA group (Fig. 2b). Importantly, the negative ion intervention restored the sucrose preference in CMS-treated mice in NAI group compared with those in FA group (Fig. 2b). To further evaluate the effect of NAI on depression-like behaviors, TST and FST tests were performed. In the TST test, the CMS treatment led to significantly-increased immobility time in FA group, but did not do so in NAI group as shown by similar immobility time compared with control mice in NAI group (Fig. 2c). However, in the FST test, there weren't any differences revealed between control and CMS-treated mice in either FA or NAI group (Fig. 2d). In addition, short-term memory, a type of working memory responsibe for the

temporary storage of information, (Czaczkes, 2018), was examined using Y maze, and there were no differences found among the four groups (Fig. 2e).

It is well known that the CMS treatment prevents the increase of body weight during the one-month CMS period (Lu, Yang, Geng, Ding, & Hu, 2014). The body weight before the experiment was not significantly different among the four groups (Fig. 2f). After 30-day experiment, in FA group, there was no weight gain in the CMS-treated mice, whereas control mice gained more weight than before (Fig. 2f, g). However, it should be noted that the unchanged body weight during the research in CMS-treated mice was also present in NAI group (Fig. 2f, g), showing that the NAI exposure has no effects on the alteration of body weight in CMS-treated mice, although it ameliorates CMS-induced depression-like behaviors as mentioned above.

To exclude the possibility that the behavioral alterations were associated with inability of locomotion activity, we carried out the rotarod test and OFT test, which are used to examine motor coordination and spontaneous locomotion (Moniruzzaman, Mannan, Hossen Khan, Abir, & Afroze, 2018; Ramshini et al., 2018). It was found that there was no significant difference among the groups in the two tests in terms of time stayed on the rod and traveled distance in the open field (Fig. 2h, i). Taken together, we demonstrate that NAI exposure prevents the occurrence of “anhedonia” behavior and some aspects of “despair” behaviors induced by the CMS treatment.

Effects of NAI exposure on cortisol levels of CMS-treated mice

Hyperactivity of the HPA axis is one of well-documented factors in the etiology of depression, which can be reflected by the increased level of cortisol in serum (Dean & Keshavan, 2017; Kim et al., 2016). Consistently, the CMS treatment did induce an increase of the concentrations of serous cortisol in FA group, but not in CMS-treated mice in NAI group (Fig. 3), showing that one-month NAI exposure relieves the HPA axis hyperactivity induced by CMS in mice.

Effects of NAI exposure on cytokine levels of CMS-treated mice

The CMS treatment increases the levels of corticosterone, which is known to negatively regulate immune responses (Villas Boas et al., 2019; Zaletel et al., 2016). To explore possible mechanism underlying the effect of NAI exposure on CMS-induced depression-like behaviors, we then measured the contents of a panel of cytokines in the serum, based on the concept that cytokines play important roles in immune responses as well as HPA axis activation (Kim et al., 2016). Comparing the data from control and CMS-treated mice in FA group would be useful to evaluate if CMS treatment itself interferes the serous levels of cytokines. The results showed that the levels of IL-15 were up-regulated in CMS-treated mice, while those of IL-7 were down-regulated in the serum (Fig. 4a, b). Comparing the data from control mice in NAI and FA groups would provide the information if the NAI exposure itself has any contribution to alterations of cytokines, and it showed that the levels of IL-15 and IL-21 were increased, and those of IL-7 and TNF-α were decreased (Fig. 4a-d). In this study, a total of 20 cytokines were examined, and only a small number

of them displays the changes in the serum, showing that specific cytokines are affected by the CMS or NAI exposure. The data showing unchanged levels of cytokines are included in Fig. S1.

Next, we asked if the NAI exposure contributes to the alteration of cytokines in CMS-treated mice. The increased levels of IL-15 were no longer existed, while the deceased levels of IL-7 were still present in CMS-treated mice with the NAI exposure, as compared with those without NAI exposure (Fig. 4a, b). There were increasing tendency of IL-13 and TNF-α levels in CMS-treated mice although the p value was not statistically significant relative to control mice in FA group; while in NAI group, their levels of CMS-treated mice were reduced to the control levels (Fig. 4d, e). These results showed that the NAI exposure interferes the changes of serous cytokines in CMS-treated mice.

Leukocytes, especially T cell population plays a key role in immune responses, in which cytokines mediate intercellular communication. Th1 cells and Th2 cells are the two major subgroups of T cells that are characterized primarily on the basis of cytokines they secreted (Varade, Magadan, & Gonzalez-Fernandez, 2021). Th1 cells secret types I cytokines (e.g. IL-2 and IFN-γ) which are mainly pro-inflammatory, while Th2 cells secret type II cytokines (e.g. IL-4, IL-5, IL-6, IL-10 and IL-13) which are mainly anti-inflammatory (Gharagozloo et al., 2013; Maher, Griffith, Lau, Reeves, & Higgins, 2014). Accumulated evidence supports the idea that stress leads to an increase of pro-inflammatory cytokines (N. Li et al., 2016; S. Li et al., 2020). However, the data from animal models and clinical research revealed significant variabilities in type I and type II cytokine profiles (Cuervo, Sordillo, & Abuelo, 2021; Koivisto et al., 2019; Razali et al., 2020). It seems to be clear that the examination of a single cytokine, or small groups of cytokines are not sufficient to evaluate the alterations of cytokines in depression-related animal models. Instead, one way to gain a better insight might be achieved by examining the ratios between the two types of cytokines, which reflects the balance between them and the tilt of immune response (Rostaing et al., 1999; Yoon, Kim, Lee, Kwon, & Kim, 2012).

To this end, the ratios of type II (i.e., IL-4, IL-5, IL-6, IL-10 and IL-13) and type I cytokines (i.e., IL-2 and IFN-γ) were calculated. We found that these ratios of IL-4/IL-2, IL-5/IL-2 and IL-13/IL-2 were elevated in CMS-treated mice, and remarkably the elevations of IL-4/IL-2 and IL-13/IL-2 were prevented by NAI exposure (Fig. 5a, c). There is also a decrease tendency of the ratio of IL-5/IL-2 after NAI exposure, although the p value was not statistically significant (Fig. 5b). The others were not significantly different (Fig. S2). It can be concluded that the NAI exposure is likely having the ability of preventing the shift from Th1 to Th2 cytokine profiles in CMS-treated mice.

Discussion

In this study, we showed that NAI intervention can improve the depression-like behaviors in CMS-treated mice. We also demonstrated that the NAI intervention reduces the increase of serous cortisol levels and reestablish the balance between type I and type II cytokines in CMS-treated mice.

The core depressive symptom of CMS model is decreased sucrose preference in rodents (Czeh et al., 2016; P. Willner, 2005), and it was confirmed in our CMS-treated mice. The role of NAI exposure in

alleviating depression-like behaviors are supported by the data of restoration of sucrose preference and reduction of immobility time in the TST in CMS-treated mice. Clinical data have shown that high-density negative air ionization is effective in treating seasonal affective disorders including major depression (Michael Terman, 2006; M. Terman, Terman, & Ross, 1998). Based on these findings, it is recommended that NAI intervention is a useful tool or providing as an alternative way in treating depression. In addition to therapeutic application, it is also recommended that NAI exposure either in natural environment or by artificial way can be used in preventing the onset of mental illness for people who had experienced chronic/acute stress and/or displayed a trend of developing the disorders.

Glucocorticoids are the final products of the HPA axis, and regulates stress-triggered responses through a negative feedback with the hypothalamus and pituitary gland (van Bodegom, Homberg, & Henckens, 2017). A wealth of evidence documented that chronic stress exposure leads to an impairment of the negative feedback of the HPA axis with increased levels of cortisol (Barfield & Gourley, 2018; Kvarta, Bradbrook, Dantrassy, Bailey, & Thompson, 2015). The hyperactivity of HPA axis is observed in the majority of depressed patients (Nemeroff & Vale, 2005; Pruessner, Hellhammer, Pruessner, & Lupien, 2003). In this study, we showed that the elevation of cortisol in the CMS-treated mice could be prevented by the NAI intervention, and this may be one of possible mechanisms underlying its role in ameliorating CMS-induced depression-like behaviors in mice.

Patients with depression often show signs of inflammation, which was illustrated by the increased concentrations of cytokines such as IL-1, IL-6, and TNF-α in the peripheral blood and cerebrospinal fluid (Leonard, 2007; Paudel, Shaikh, Shah, Kumari, & Othman, 2018). It has been reported that administration of IFN-α, used to treat infective diseases, is a significant risk factor in inducing major depressive episode (Andrew H. Miller, Vladimir Maletic, & Charles L. Raison, 2009; Su et al., 2019). Among 20 cytokines examined, two (IL-15 and IL-7) were altered in CMS-treated mice, and more (IL-7, IL-15, IL-21 and TNF-α) were changed by NAI exposure either in control mice or CMS-treated mice. To evaluate possible contribution of cytokines in the ameliorated depression-like behaviors by NAI intervention, we analyzed the alterations of ratios of proinflammatory and anti-inflammatory cytokines, which may provide a whole view concerning the net effects of altered cytokines in modulating brain functions of CMS-treated mice. The ratios of IL-4/IL-2, IL-5/IL-2 and IL-13/IL-2 were elevated in CMS-treated mice. After NAI treatment, two of them (IL-4/IL-2 and IL-13/IL-2) were reduced significantly similar to the level of control mice. The ratio of IL-5/IL-2 was also showing reduced tendency with no statistical difference. IL-2 functions as a type I cytokine, the alterations of IL-2-related ratios support the idea that CMS treatment may lead to the shift of Th1 inflammatory pathway with deterious effects on mood-related brain functions, whereas NAI intervention may prevent or reduce the activation whereby it displays beneficial effect in mood regulation.

Finally, preliminary data from patients with inflammatory disorders, as well as depressed patients without appearing any other medical problems, suggest that inhibiting inflammatory signaling pathways may improve depressed mood and increase effective treatment response to conventional antidepressant medication (Malemud & Miller, 2008; J.-c. Zhang, Yao, & Hashimoto, 2016). Translational implications of these findings include the unique opportunity to identify relevant patient populations, apply immune-

targeted therapies, and monitor therapeutic efficacy at the level of the immune system in addition to behaviors.

Conclusions

In conclusion, chronic stress results in depressive-like behaviors and has a significant effect on HPA axis and immune system parameters, which are the risk factors in development of depression. We demonstrated that NAI exposure is capable of preventing depression-like behaviors induced by chronic stress, and this positive effect is likely achieved by rebalancing activity of HPA axis and immune-response under the condition of chronic stress.

Declarations

Availability of data

All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Contributions

YQD and JZ design this work. YQH and TTN performed the animal model and histological examination. The first draft of the manuscript was written by YQH and all authors commented on previous versions of the manuscript. JMX, LP, QHS analyzed and interpreted the research data. YH finally review and edit the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval

All procedures were carried out in compliance with the Animal Experimental Ethics Committee of Shanghai Medical School, Fudan University.

Consent for publication

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Competing interests

The authors declare no competing interests.

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Figures

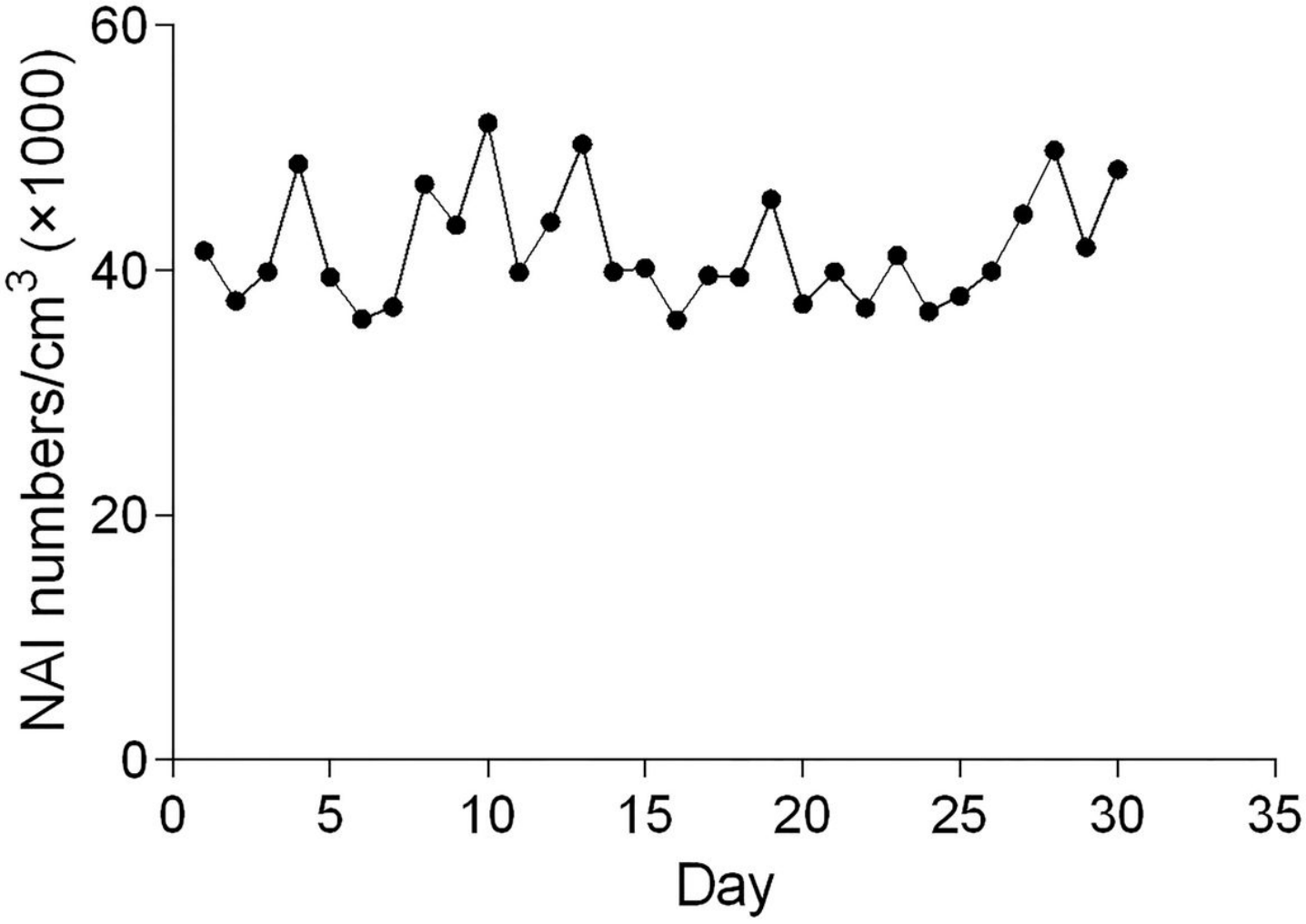

Figure 1

The concentration of negative air ions detected daily

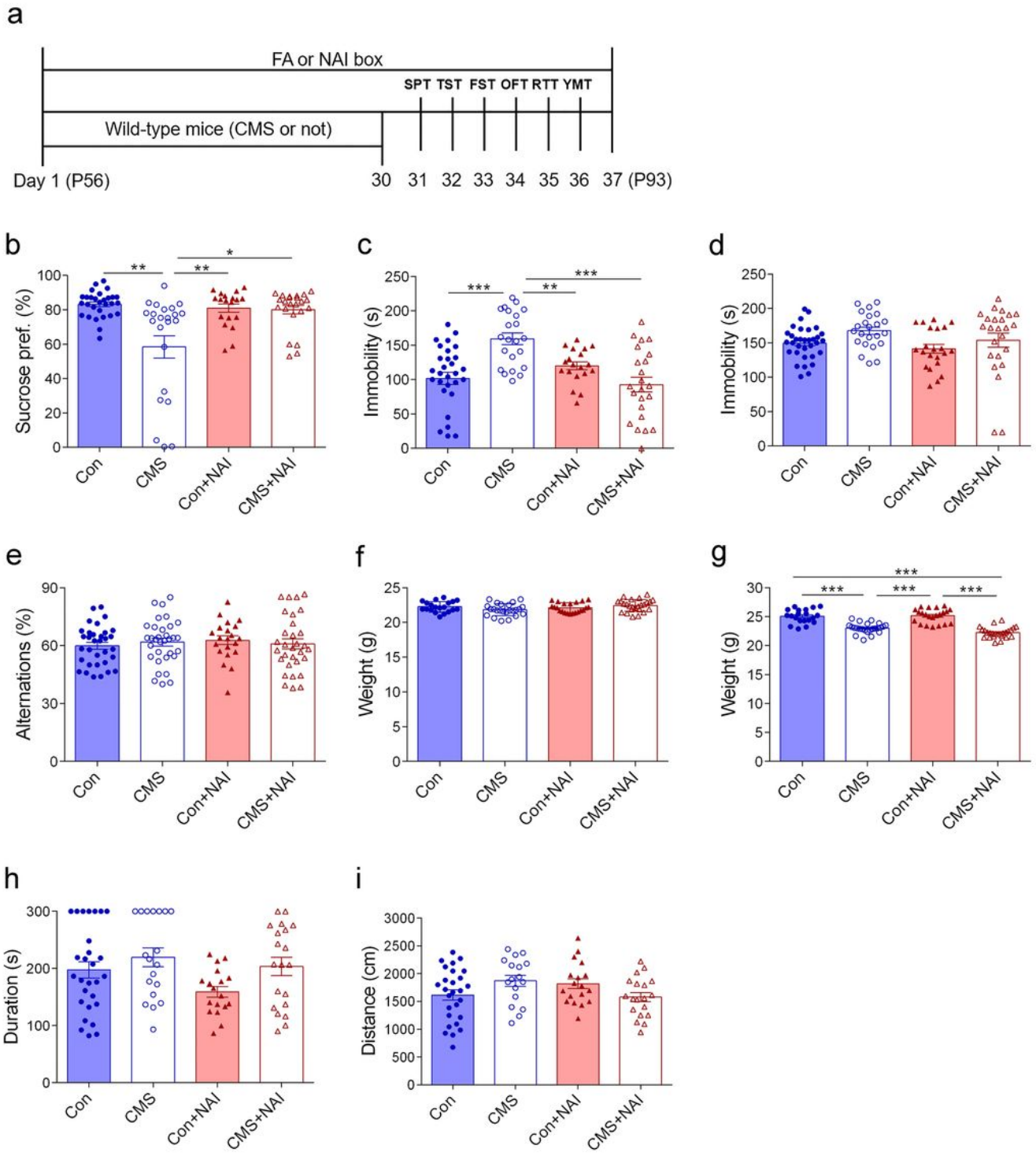

Figure 2

NAI intervention alleviates depression-like behavior in mice after stress exposure (a) Diagram of experiment design and timeline. (b) Sucrose preference in the SPT. The one-way ANOVA test measured the significant differences between these groups {F[3,92] = 11.247, ***P < 0.0001; Tamhane's T2 multiple comparisons test showed **P = 0.003 (Con vs. CMS); *P = 0.012 (CMS vs. CMS+NAI); **P = 0.009 (CMS vs. Con+NAI)}. (c) Time spent immobile in the TST. The one-way ANOVA test measured the significant

differences between these groups {F[3,90] = 10.431, ***P < 0.0001; Tamhane's T2 multiple comparisons test showed ***P < 0.0001 (Con vs. CMS); **P = 0.003 (CMS vs. Con+NAI); ***P < 0.0001 (CMS vs. CMS+NAI) }. (d) Time spent immobile in the FST. The one-way ANOVA test with Tamhane's T2 multiple comparisons test measured the significant differences between these groups {F[3,98] = 2.475, P = 0.066 }. (e) Spontaneous alternation in Y maze. The one-way ANOVA test with Bonferroni multiple comparisons test measured the significant differences between these groups {F[3,122] = 0.366, P = 0.778 }. (f) Body weight evaluated before the experiment. The one-way ANOVA test with Bonferroni multiple comparisons test measured the significant differences between these groups {F[3,87] = 2.509, P = 0.064 }. (g) Body weight evaluated after 30-day CMS exposure. The one-way ANOVA test measured the significant differences between these groups {F[3,87] = 45.286, ***P < 0.0001; Bonferroni multiple comparisons test showed ***P < 0.0001 (Con vs. CMS); ***P < 0.0001 (Con vs. CMS+NAI); ***P < 0.0001 (CMS vs. Con+NAI); ***P < 0.0001 (CMS+NAI vs. Con+NAI) }. (h) Time spent on the rod. The one-way ANOVA test with Tamhane's T2 multiple comparisons test measured the significant differences between these groups {F[3,81] = 2.681, P = 0.052 }. (i) Distance traveled in the open field. The one-way ANOVA test with Bonferroni multiple comparisons test measured the significant differences between these groups {F[3,77] = 2.338, P = 0.08 }. *P < 0.05, **P < 0.01, ***P < 0.001. Data are presented as mean ± S.E.M.

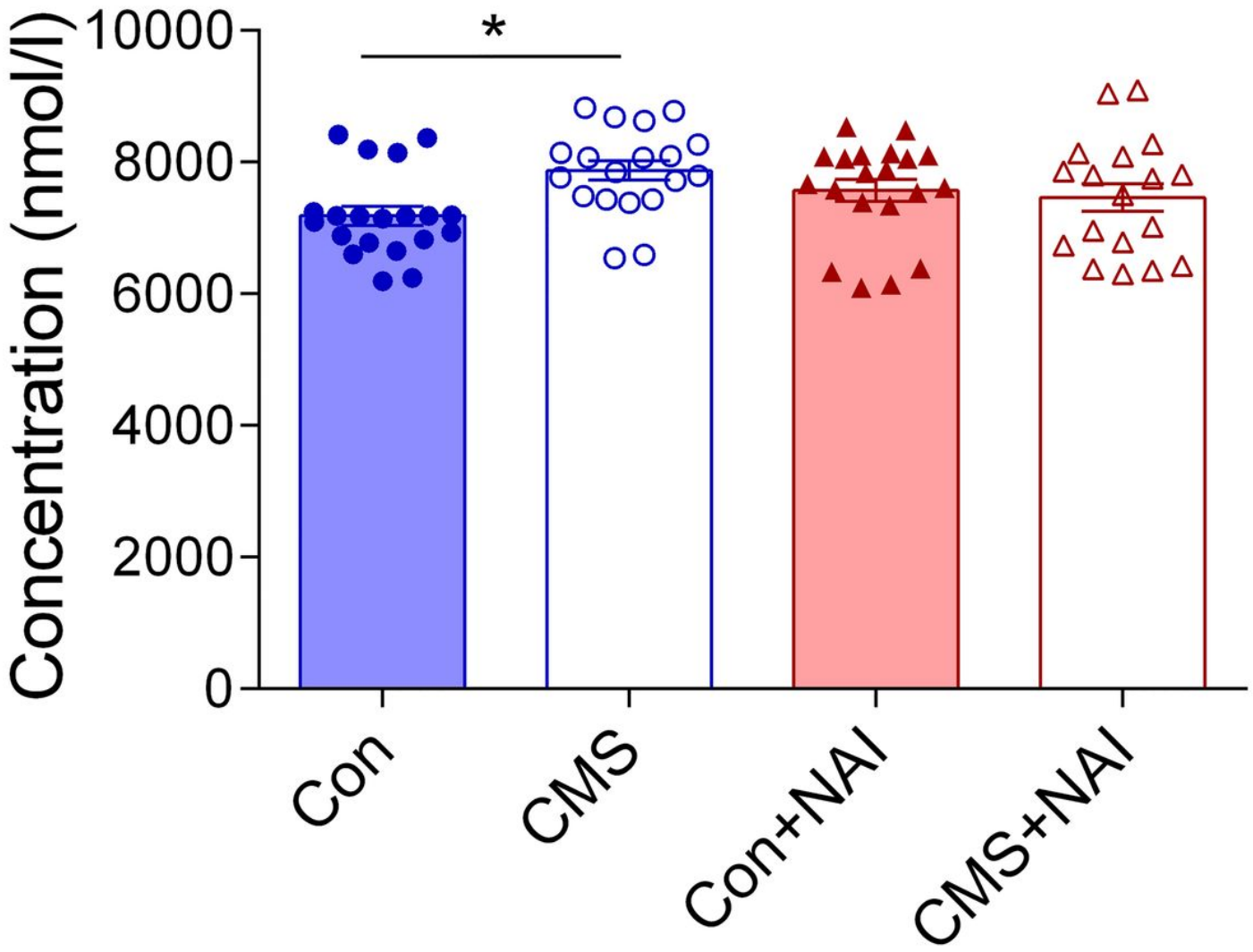

Figure 3

Effects of NAI intervention on cortisol levels in serum of mice with CMS. A significant difference is observed between control (Con) and CMS-treated (CMS) mice in FA group but not NAI group. The one-way ANOVA test measured the significant differences between these groups {F[3,73] = 2.924, *P = 0.039; Bonferroni multiple comparisons test showed *P = 0.027 (Con vs. CMS) }.

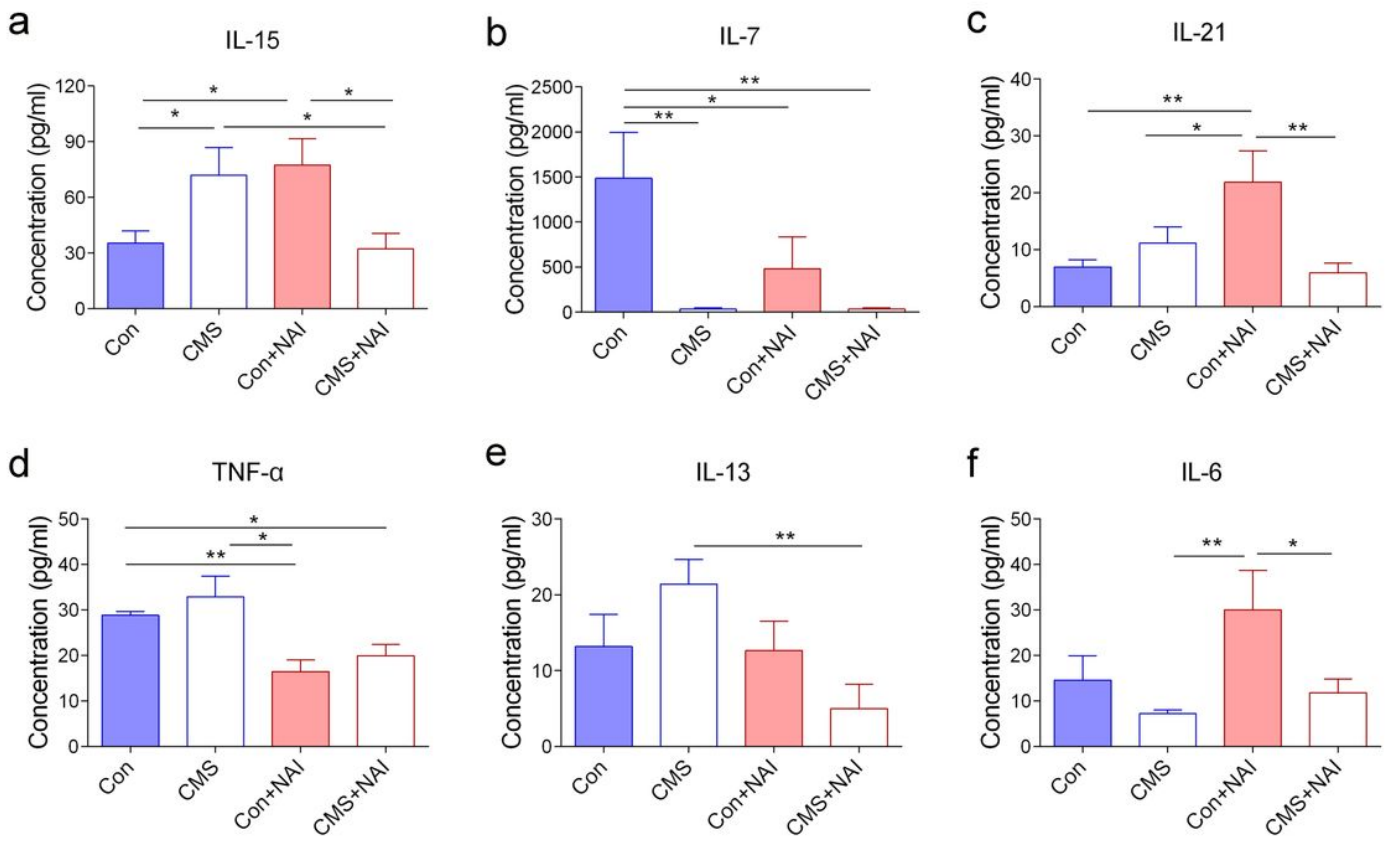

Figure 4

Effects of NAI innervation on inflammatory cytokine levels in serum of mice with CMS (a) The protein concentration of IL-15. The one-way ANOVA test measured the significant differences between these groups {F[3,35] = 4.055, *P = 0.014; Bonferroni multiple comparisons test showed *P = 0.033 (CMS vs. Con); *P = 0.015 (Con+NAI vs. Con); *P = 0.024 (CMS vs. CMS+NAI); *P = 0.011 (CMS+NAI vs. Con+NAI) }. (b) The protein concentration of IL-7. The one-way ANOVA test measured the significant differences between these groups {F[3,34] = 4.485, **P = 0.009; Bonferroni multiple comparisons test showed P = **0.003 (CMS vs. Con); **P = 0.003 (CMS+NAI vs. Con); *P = 0.032 (Con vs. Con+NAI) }. (c) The protein concentration of IL-21. The one-way ANOVA test measured the significant differences between these groups {F[3,35] = 4.703, P = **0.007; Bonferroni multiple comparisons test showed **P = 0.004 (Con vs. Con+NAI); *P = 0.029 (CMS vs. Con+NAI); **P = 0.002 (CMS+NAI vs. Con+NAI) }. (d) The protein concentration of TNF-α. The one-way ANOVA test measured the significant differences between these groups {F[3,35] = 6.485, **P = 0.001; Tamhane's T2 multiple comparisons test showed *P = 0.033 (CMS+NAI vs. Con); **P = 0.005 (Con vs. Con+NAI); *P = 0.044 (CMS vs. Con+NAI) }. (e) The protein concentration of IL-13. The one-way ANOVA test measured the significant differences between these groups {F[3,34] = 3.328, *P = 0.031; Bonferroni multiple comparisons test showed **P = 0.003 (CMS vs. CMS+NAI) }. (f) The protein concentration of IL-6. The one-way ANOVA test measured the significant differences between these groups {F[3,33] = 3.304, *P = 0.032; Bonferroni multiple comparisons test

showed **P = 0.006 (CMS vs. Con+NAI); *P = 0.025 (CMS+NAI vs. Con+NAI) }. (*P< 0.05, **P< 0.01). 9≤n≤11/group.

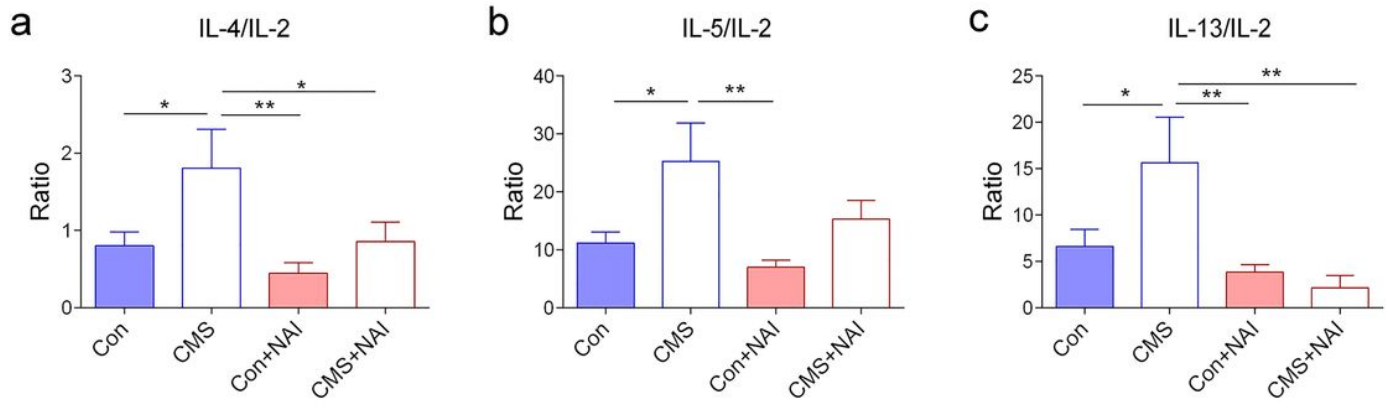

Figure 5

Comparison of ratios of IL-4/IL-2, IL-5/IL-2 and IL-13/IL-2 in CMS-treated mice with and without NAI exposure (a) The ratio of serous concentration of IL-4/IL-2. The one-way ANOVA test measured the significant differences between these groups {F[3,35] = 3.599, *P = 0.023; Bonferroni multiple comparisons test showed *P = 0.030 (CMS vs. Con); *P = 0.035 (CMS vs. CMS+NAI); **P = 0.003 (CMS vs. Con+NAI) }. (b) The ratio of serous concentration of IL-5/IL-2. The one-way ANOVA test measured the significant differences between these groups {F[3,35] = 3.981, *P = 0.015; Bonferroni multiple comparisons test showed *P = 0.018 (CMS vs. Con); P = **0.002 (Con+NAI vs. CMS) }. (c) The ratio of serous concentration of IL-13/IL-2. The one-way ANOVA test measured the significant differences between these groups {F[3,33] = 5.016, P = 0.006; Bonferroni multiple comparisons test showed *P = 0.024 (CMS vs. Con); **P = 0.001 (CMS+NAI vs. CMS); **P = 0.003 (CMS vs. Con+NAI) }. (*P< 0.05, **P< 0.01). 9≤n≤11/group.

Supplementary Files

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