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Stress upregulates 2-arachidonoylglycerol levels in the hypothalamus, midbrain, and hindbrain, and it is sustained by green nut oil supplementation in SAMP8 mice revealed by DESI-MSI

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ABSTRACT

The endocannabinoid 2-arachidonoylglycerol (2AG) is an important modulator of stress responses. Its level in the brain increases in response to stress, but region-specific effects of stress on brain 2AG are not well known yet. Moreover, green nut oil (GNO), oil extracted from the seeds of *Plukenetia volubilis* has several health benefits, but its effects on brain 2AG levels are unknown. Therefore, we conducted this study to explore the effects of stress and GNO supplementation on 2AG levels in specific brain regions of senescence-accelerated mouse prone 8 (SAMP8). In this study, desorption electrospray ionization-mass spectrometry imaging (DESI-MSI) revealed that water-immersion stress for three days significantly increased 2AG levels in several brain regions of SAMP8 mice, including the hypothalamus, midbrain, and hindbrain. No significant change was observed in the relative abundance of brain 2AG in stress given SAMP8 mice after eighteen days of removing stress load compared to control SAMP8 mice. GNO supplementation also increased brain 2AG in SAMP8 mice without stress load. Additionally, GNO supplementation sustained the increased brain 2AG levels in stress given SAMP8 mice after eighteen days of removing stress load. Among all brain regions, a relatively higher accumulation of 2AG was noted in the hypothalamus, midbrain, and hindbrain of GNO-fed SAMP8. Our data explored the potentiality of GNO supplementation to improve brain 2AG levels which might be used to treat anxiety and depressive behaviors.

1. Introduction

The endocannabinoid signaling system in the brain modulates different biological processes. It consists of two G protein-coupled receptors (CB1 and CB2) and two major endogenous ligands, 2-arachidonoylglycerol (2AG) and *N*-arachidonoylethanolamine (anandamide, AEA). Among these two ligands, 2AG is the most studied and primary endogenous agonist for CB1 and CB2 receptors, and its concentration in the brain is about 50–200 times higher than that of AEA [1]. It is crucially significant for the development of the central nervous system (CNS) [2]. It is synthesized on-demand from phospholipid precursors at the

Abbreviations: 2AG, 2-arachidonoylglycerol; CB1, Cannabinoid receptor type 1; CB2, Cannabinoid receptor type 2; AEA, Anandamide; CNS, Central nervous system; PLC, Phospholipase C; DAGL, Diacylglycerol lipase; FAAH, Fatty acid amide hydrolase; MAGL, Monoacylglycerol lipase; HPA, Hypothalamic-pituitary-adrenal; GNO, Green nut oil; FA, Fatty acid; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; SAMP8, Senescence-accelerated mouse prone 8; DESI-MSI, Desorption electrospray ionization-mass spectrometry imaging; CO, Corn oil; CRH, Corticotropin-releasing hormone.

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postsynaptic neurons due to phospholipase C (PLC) and diacylglycerol lipase (DAGL) activation [3]. After that, following the release of 2AG in the synaptic cleft, it is taken back to the cell and degraded by hydrolytic enzymes found in the presynaptic neurons, such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) [4,5].

Brain 2AG is involved in several physiological and pathophysiological mechanisms associated with stress responses, including increased anxiety, decreased feeding behavior, suppressed responsiveness to stimuli, impaired memory function [6], hypervigilance, and arousal [4]. It regulates synaptic function, neurophysiology, and behavior in response to internal and external stimuli, thus allowing organisms to adapt with changing environments [7]. It works at presynaptic neurons to inhibit the secretion of neurotransmitters by activating CD1 receptors by modulating the hypothalamic-pituitary-adrenal (HPA) axis [8]. Recent studies suggest bidirectional effects of physical and physiological stressors on 2AG levels in mice brains. Chronic and repetitive stresses can increase the 2AG levels in the brain of mice, but they return to the baseline gradually after removing the stressors [9]. This increased brain 2AG plays a vital role in inhibiting stress responses, including anxiety, depressive behavior, and declined memory function via modulating the HPA axis [4]. According to the previous reports, 2AG also has neuroprotective effects in Alzheimer's disease and Parkinson's disease [1,10]. However, little is known about the effects of stress on the 2AG levels in specific brain regions. Additionally, therapeutic options for increasing brain 2AG levels through dietary intervention which might be used to prevent anxiety-like behaviors and neurodegenerative diseases associated with stressors yet to be explored.

Green nut oil (GNO) is also known as Sacha inchi oil. It is extracted from the seeds of the *Plukenetia volubilis*. It is a perennial plant of tropical South America. Oil extracted from its seeds is rich in omega-3 fatty acids (FAs) and processes several health benefits [11]. Its supplementation improves memory function and omega-3 FAs levels, including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in the brain of dementia model mice [12,13]. This increased brain omega-3 FA levels after GNO supplementation is expected to have protective effects against stress-mediated deleterious physiological and pathophysiological changes [14]. Omega-3 FAs also inhibit inflammatory responses, sustain increased corticosterone levels in stress-given mice and increase brain 2AG levels [4,14]. As dietary GNO improves omega-3 FAs levels in the brain, it might also increase brain 2AG levels. However, still, there is a lack of evidence about it. Therefore, we conducted this study to explore the effects of water-immersion stress and GNO supplementation on 2AG levels in the different brain regions of senescence-accelerated prone mouse 8 (SAMP8), model mice of dementia, accelerated brain aging, and oxidative stress [15].

To reveal the effects of stress and GNO supplementation on 2AG levels in the different brain regions of SAMP8 mice for the first time, we applied desorption electrospray ionization-mass spectrometry imaging (DESI-MSI). DESI-MSI is a recently developed tool of MSI widely used for the detection and visualization of drugs [16], small metabolites [17], neurotransmitters [18], lipids [19], and proteins [20] under ambient conditions [21]. Recently, Nielsen et al. also reported the capability of DESI-MSI to detect 2AG with higher sensitivity [22].

2. Materials and methods

2.1. Animals

We purchased twenty-weeks-old male SAMP8 mice from Sankyo Labo Service Corporation (Tokyo, Japan). Each mouse was reared in individual cages at 22 ± 2 °C with a light/dark cycle of 12 h. The diet consisted of CRF-1 pellets from Charles River International Laboratories, Inc. (Kanagawa, Japan) that were given throughout the first week of the rearing period. After preparatory rearing periods, all mice were divided into four groups as follows; group I: corn oil-fed (CO-fed), group II: CO-fed with stress, group III: GNO-fed, and group IV: GNO-fed with stress (Fig. S1). SAMP8 mice of groups I and II were fed on a diet consisting of AIN-93G for four weeks, in which soybean oil was replaced with 7% CO. On the other hand, SAMP8 mice of groups III and IV were fed on a diet consisting of AIN-93G for four weeks, in which soybean oil was replaced with 7% GNO. After one week, SAMP8 mice of groups II and IV received a stress load for three days (72 h) at the age of twenty four weeks. For water-immersion stress, tap water (temperature: 22–25 °C) was poured into the mice case (water depth: 7 mm). During the stress period, mice cases were changed every day, and all mice were washed from the neck to the bottom with tap water and wiped with a Kim towel. After finishing three days of stress load, three mice from group I and three mice from group II were euthanized by decapitation and dissected after 14 h of fasting (Fig. S1). After removing mice brains, all samples were stored at −80 °C until further analysis. The remaining mice were kept in the same condition as groups I and III, and reared for eighteen days. Mice brain samples were collected and stored at −80 °C following the method mentioned above on the last day of the rearing period. The Animal Research Committee of Showa Women's University (ethical permission numbers: 19-09 and 20-11) and the Animal Care and Use Committee of the Hamamatsu School of Medicine (ethical permission number: 2020027) approved this study.

2.2. Chemicals and reagents

We received GNO from NPO Arcoiris Naturaleza (Matsudo, Chiba, Japan) and purchased CO from Oriental Yeast Co., Ltd. (Tokyo, Japan). LC/MS grade ultrapure water, acetonitrile, methanol, and 2-propanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Standard 2AG and sodium formate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Tissue preparation and DESI-MSI data acquisition

Sagittal sections of SAMP8 mice brains were prepared according to the previously described method [12]. Before acquiring DESI-MSI data, brain sections containing glass slides were kept at room temperature for 2–3 min to remove extra water. Thereafter, DESI-MSI data from SAMP8 mice brain samples were acquired using Omni Spray-2D DESI ion source (Prosolia Inc, Indianapolis, IN, USA) equipped with a quadrupole time-of-flight mass spectrometer (Xevo G_2-XS Q-TOF; Waters, USA) in positive ion mode. Before DESI-MSI data acquisition, mass spectra were calibrated using 0.5 mM sodium formate solution prepared in 90% 2-propanol. ACQUITY UPLC Binary Solvent Manager (Waters, Milford, MA, USA) was used to deliver spray solvent during data acquisition. Other parameters used to optimize DESI-MSI signals were listed in Table S1. Standard 2AG was dissolved in acetonitrile (3 μg/mL), and 0.3 μL of that solution was applied on the normal glass slide and mice brain section to confirm the detection of 2AG by DESI-MSI and optimize DESI parameters for better ionization of 2AG.

2.4. DESI-MSI data analysis

MassLynx 4.1 and HDImaging 1.4 (Waters, Milford, MA, USA) software were used to acquire DESI-MSI data. After that, MSI raw data were converted into .imzML by HDImaging software and then further converted to .imdx using IMDX converter (Shimadzu,

version 1.20.0.10960). Then .imdx data was used to analyze the distribution of candidate ions by IMAGE REVEAL (Shimadzu, version 1.20.0.10960) software. Top 1000 *m/z* peaks with a mass window of 0.02 Da were extracted from total ion current (TIC) normalized mass spectra, and 2D ion images were constructed to visualize and analyze the spatial distribution of candidate ions. We used MS Excel (version 2019) and IBM SPSS (version 22) software for statistical analyses. All values were expressed as mean ± SD (standard deviation). Differences were considered significant with p values less than 0.05 (two-tail t-test).

3. Results

3.1. Detection of 2AG by DESI-MSI

Before DESI-MSI data acquisition from SAMP8 mice brain sections, DESI-MSI signals were optimized for better ionization and detection of 2AG with higher sensitivity. A solution of standard 2AG (3 μg/mL in acetonitrile) was applied on the glass slide and mice brain section (0.3 μL/spot). Thereafter, DESI-MSI data were acquired in positive ion mode. Standard 2AG was detected as two different adducts, sodium adduct $[M+Na]^+$ and potassium adduct $[M+K]^+$ (Fig. S2 and Table S2). After optimizing DESI-MSI parameters, we acquired DESI-MSI data in positive ion mode over 300–900 *m/z* range from the sagittal brain sections of SAMP8 mice to analyze the effects of water-immersion stress and dietary GNO on brain 2AG distribution/levels (qualitative analysis) in SAMP8 mice. Similar to standard 2AG, two *m/z* peaks corresponding to two different adducts of 2AG were detected in DESI-MSI data acquired from SAMP8 mice brain sections (Fig. 1A–B).

3.2. Effects of water-immersion stress on 2AG distribution in the brain of SAMP8 mice

To elucidate the effect of water-immersion stress on the distribution of 2AG in the different brain regions of SAMP8 mice, we acquired DESI-MSI data from the sagittal brain sections of stress given (CO-fed with stress) and control (CO-fed) SAMP8 mice. We found a significantly increased accumulation of 2AG in the brain of stress given SAMP8 mice compared to control mice (Fig. 2A–B). Although accumulation of 2AG was noted throughout the brain of stress given SAMP8 mice (1.4–1.5 folds), a relatively higher accumulation of 2AG was observed in the hypothalamus, hindbrain, and midbrain compared to other brain regions (Fig. S3).

3.3. Effects of stress and GNO supplementation on the distribution of 2AG in SAMP8 mice brain after eighteen days of removing stress load

After eighteen days of removing stress load, almost no difference was observed in the distribution of brain 2AG in SAMP8 mice compared to CO-fed SAMP8 mice without stress load (Fig. 3). However, dietary GNO increased the accumulation of 2AG in the brain of SAMP8 mice (GNO-fed) without water-immersion stress (Fig. 3A–B). Interestingly, GNO supplementation also sustained the increased distribution of 2AG in the brain of SAMP8 even after eighteen days of removing stress load compared to CO-fed SAMP8 mice without stress load. About 2-fold increased accumulation of 2AG was recorded in GNO-fed SAMP8 mice brains, and about 1.6-fold increased accumulation of 2AG was found in GNO-fed SAMP8 mice which received stress load compared to CO-fed SAMP8 mice without stress load. Although increased accumulation of 2AG was noted in almost all brain regions after GNO supplementation, we observed a relatively higher accumulation of 2AG in the hypothalamus, midbrain, and hindbrain of SAMP8 mice. Among all brain regions, maximum accumulation of 2AG (1.7–2.3 folds) was observed in the hypothalamus of GNO-fed and GNO-fed with stress given SAMP8 mice compared to that of CO-fed SAMP8 mice (Fig. 3C). The distribution of 2AG was also significantly increased in the midbrain (1.3–1.6 folds), and hindbrain (1.4–1.7 folds) of SAMP8 mice supplemented with GNO (GNO-fed and GNO-fed with stress) compared to CO-fed SAMP8 mice (Fig. 3D–E). No significant change in the distribution of 2AG was found in the hypothalamus, midbrain, and hindbrain of CO-fed SAMP8 mice after eighteen days of removing stress load compared to CO-fed SAMP8 mice without stress.

4. Discussion

In this study, we have investigated the effects of water-immersion stress and GNO supplementation on the distribution of brain 2AG in SAMP8 mice, a model mouse of accelerated brain aging and dementia. Water-immersion stress significantly increased the accumulation of 2AG in the brain of SAMP8 mice. However, after eighteen days of removing stress load, no change in the spatial distribution of brain 2AG in stress given SAMP8 mice compared to CO-fed SAMP8 mice was noted in our current study. Interestingly, we found significantly increased accumulation of brain 2AG in GNO supplemented SAMP8 mice with or without

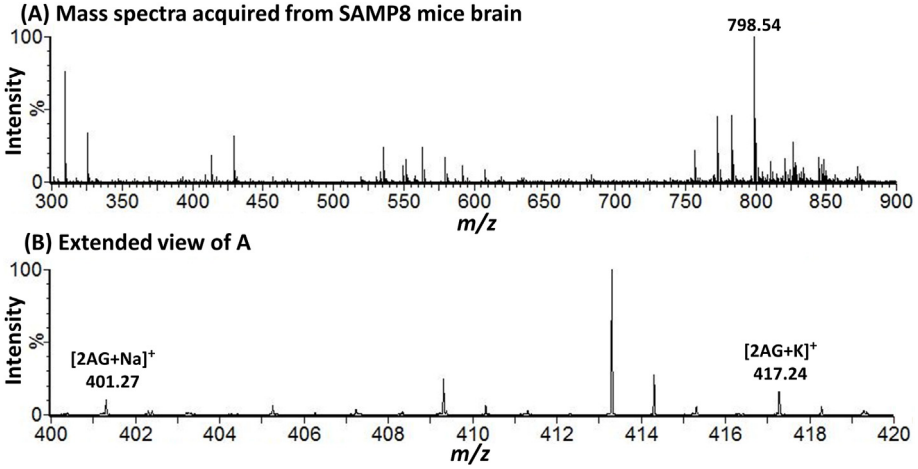

Fig. 1. DESI-MSI mass spectra acquired from SAMP8 mice brain in positive ion mode. 2AG: 2-arachidonoylglycerol; *m/z*: mass to charge ratio.

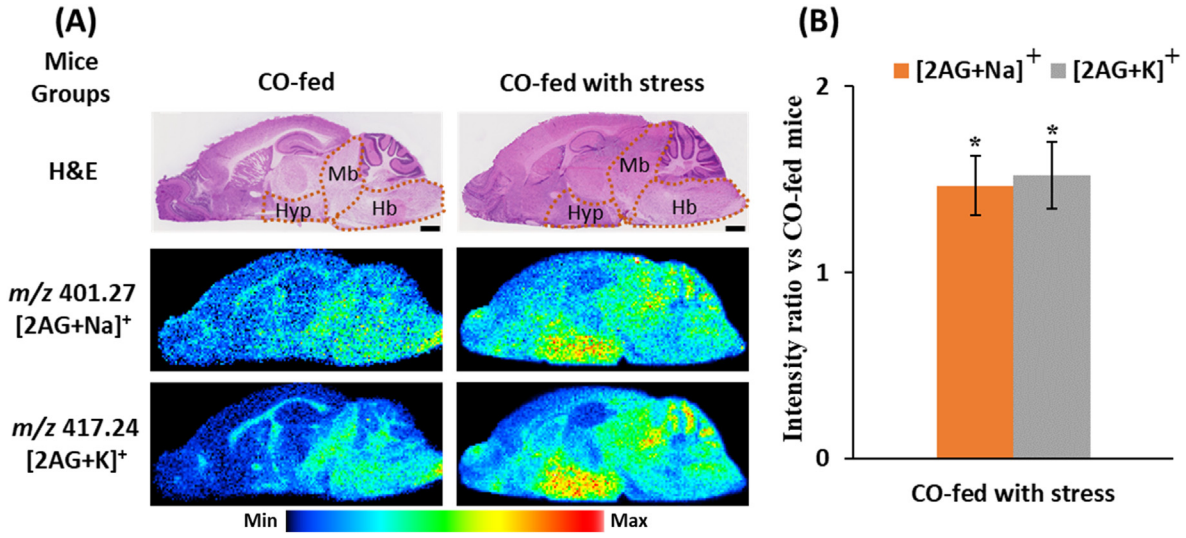

Fig. 2. Effects of stress on the distribution of brain 2AG in SAMP8 mice. (A) Spatial distribution of 2AG in the SAMP8 mice brain. (B) Fold changes in the average intensity of 2AG in stress given SAMP8 mice compared to CO-fed mice. *$P < 0.05$ vs CO-fed SAMP8 mice (two-tail *t*-test; n = 3). Hyp: hypothalamus, Mb: midbrain, Hb: hindbrain, Scale bar: 1 mm.

stress load. Although GNO supplementation increased the accumulation of 2AG throughout the brain of SAMP8 mice, a relatively higher accumulation of 2AG was found in the hypothalamus, midbrain, and hindbrain compared to other brain regions. To the best of our knowledge, this is the first study investigating the effects of stress and GNO supplementation on the spatial distribution of brain 2AG in mice.

Endogenous 2AG is an important component of the endocannabinoid system. Chronic and repetitive stresses can increase brain 2AG levels [4,23], but region-specific effects of stress on 2AG levels are yet to be explored. In this study, we attempted to investigate the effect of water-immersion stress on the 2AG levels in the specific brain regions of SAMP8 mice applying DESI-MSI. DESI-MSI revealed increased accumulation of 2AG throughout the brain of SAMP8 mice after stress load. Among all brain regions of SAMP8 mice, a higher accumulation of 2AG was noted in the hypothalamus, midbrain, and hindbrain after giving stress compared to control SAMP8 mice. However, no change was observed in the distribution of brain 2AG after eighteen days of removing stress load. According to previous studies, brain 2AG levels can be increased by upregulated corticosterone levels and decreased expression of MAGL in response to stress [24,25]. After removing the stress load, 2AG is degraded by the activity of the FAAH and MAGL, and goes back to normal levels [5,26]. Therefore, the findings of our study are in line with the previous reports.

GNO is gradually being recognized to have health benefits for humans due to its higher content of omega-3 FAs [27]. Its supplementation can increase brain omega-3 FAs levels in mice [13], which has the potentiality to improve brain 2AG levels by increasing corticosterone levels [14]. In our current study, we supplemented GNO to SAMP8 mice without stress load and with stress load to explore its effects on the distribution of brain 2AG even after eighteen days of removing stress load. We found a significantly increased accumulation of brain 2AG in the GNO-fed SAMP8 mice without stress load. We also observed that GNO supplementation could sustain the stress-mediated increased accumulation of 2AG in the brain of SAMP8 mice even after eighteen days of removing stress load. Among all brain regions of GNO-fed SAMP8 mice with and without stress load, a higher accumulation of 2AG was noted in the hypothalamus, hindbrain, and midbrain, respectively. All these brain regions are associated with several physiological functions which are affected by stress. The hypothalamus regulates the fundamental aspects of physiological homeostasis and behaviors of vertebrate animals. It plays a major role in the endocrine system by modulating the functions of the pituitary gland [28]. In response to stressors, the HPA axis of the hypothalamus is activated. Thereafter, it releases corticotropin-releasing hormone (CRH), which enhances the production of glucocorticoid hormones from the pituitary gland and corticosterone from the adrenal gland. These hormones can increase the 2AG levels in the hypothalamus [29,30]. This increased 2AG is essential for stress adaptation and habituation by modulating HPA responses. The hindbrain is another major part of the brain which connects the brain to the spinal cord so that messages can pass from and to the brain from other areas of the body. It controls motor reflexes, sleeping activity, blood pressure, and heart rate. According to recent reports, glucocorticoid receptors present in the hindbrain have important roles in developing anxiety and depression [31]. Midbrain also plays a vital role in processing information related to pain and sleep. All these functions are affected by different types of stressors [4]. Failure of the adaptative response to stress can cause cell death in the midbrain and can be a disease risk factor [32]. Accumulated 2AG in these regions after GNO supplementation might be beneficial for the adaptation of stress-mediated physiological and behavioral changes. However, additional studies are required to clarify the molecular mechanisms of increasing brain 2AG after GNO supplementation and their mode of action associated with the adaptation of stress responses.

In summary, we revealed that water-immersion stress could increase brain 2AG in the hypothalamus, hindbrain, and midbrain of SAMP8 mice, and it goes back to the basal levels after removing the stress load. However, GNO supplementation could sustain this increased accumulation of brain 2AG in those brain regions of SAMP8 even after removing the stress load. This increased brain 2AG might have the potentiality to treat disorders associated with stress by modulating the HPA axis of the brain.

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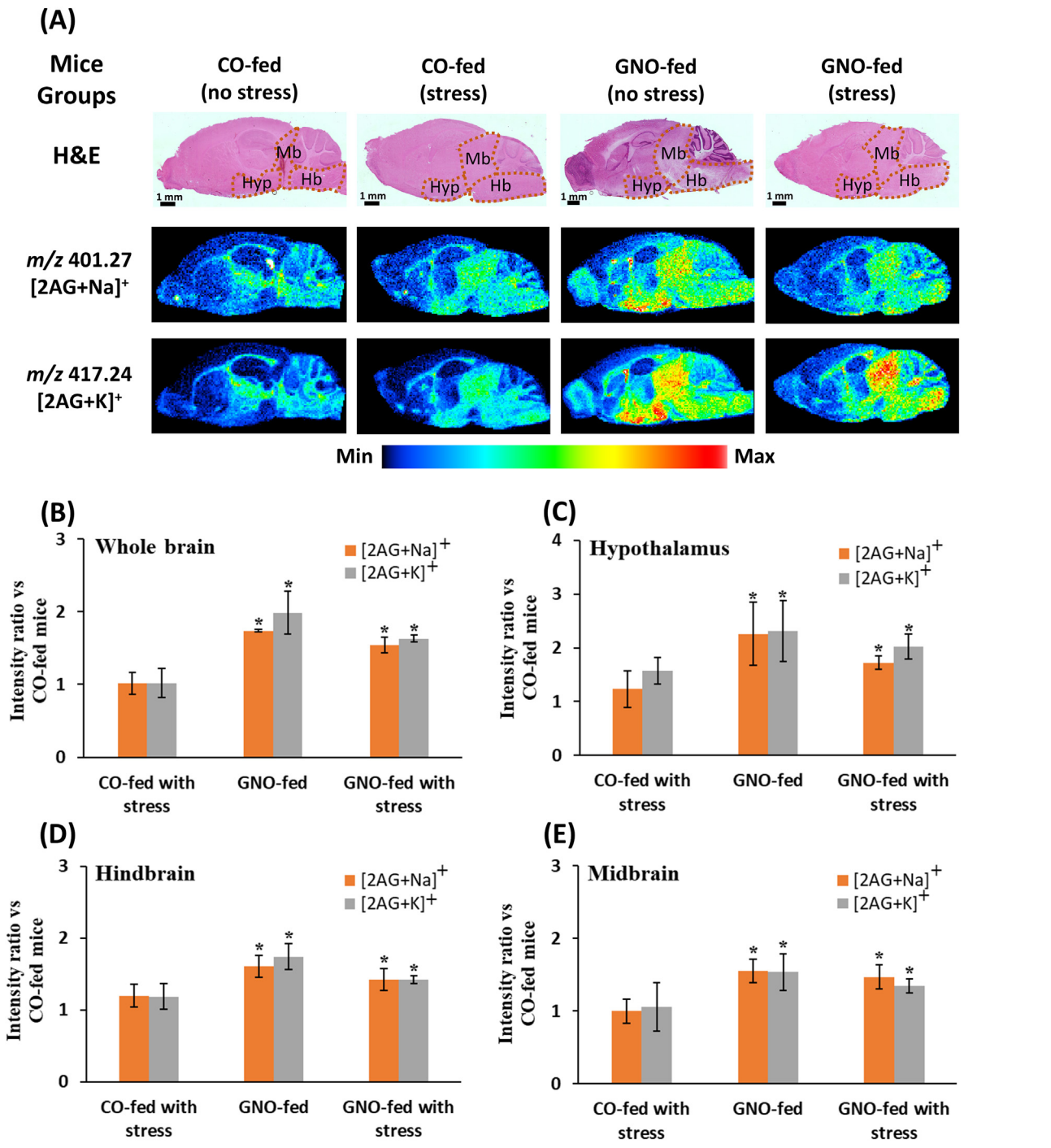

Fig. 3. Effects GNO supplementation on the distribution of 2AG after eighteen days of removing stress load in SAMP8 mice brain. (A) Spatial distribution of 2AG in SAMP8 mice brain. (B–E) Fold changes in the average intensity of 2AG in the whole brain section, hypothalamus, hindbrain, and midbrain of CO-fed with stress, GNO-fed, and GNO-fed with stress given SAMP8 mice compared to CO-fed SAMP8 mice. *$P < 0.05$ vs CO-fed SAMP8 mice (two-tail t-test, n = 3). Hyp: hypothalamus, Mb: midbrain, Hb: hindbrain, Scale bar: 1 mm.

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Declaration of competing interest

All authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.04.004.

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