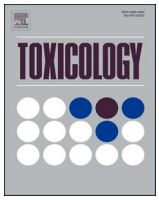
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Role of brain monoamines in acetamiprid-induced anxiety-like behavior

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ABSTRACT

Neonicotinoid (NN) pesticides have been linked to increased brain dysfunction in mammals, such as anxiety-like behavior; this is thought to involve monoamines (MA), neurotransmitters that control behavior, memory, and learning. However, the mechanism by which NNs affect the central nervous system is not fully understood. In this study, we aimed to investigate whether MAs affect NNs-induced anxiety-like behavior. Mice were orally administered acetamiprid (ACE), an NN, at the no observed adverse effect level (NOAEL) of mouse (20 mg/kg body mass) set by the Food Safety Commission of Japan, and the elevated zero-maze (EZM) test was performed 30 min after administration. After behavioral analysis, levels of four MA (dopamine, 3-MT, serotonin, and histamine) in selected brain regions were determined by liquid chromatography mass spectrometry (LC/MS/MS). In the exposed group, a trend toward increased anxiety-like behavior was observed, and at least one MA concentration was significantly increased in each region. Further, significant correlations were found between behavioral test results and hippocampal serotonin and striatal dopamine concentrations, as well as between dopamine and serotonin concentrations, in the exposed group. As anxiety can influence activity in the behavioral tests, the activity of neurons in the raphe nuclei (RN), a brain region greatly involved in anxiety via the serotonergic system, was examined by staining with anti-serotonin antibodies, and increased serotonergic activity was observed. Taken together, these results suggest that ACE regulates MA levels, notably serotonin levels in the hippocampus and that RN plays an important role in ACE-induced anxiety-like behavior.

1. Introduction

Neonicotinoids (NNs) are pesticides with a nicotine-like structure that act as nicotinic acetylcholine receptor (nAChR) agonists. Due to the varying properties of nAChRs, NNs have a higher affinity for insect nAChRs than for mammalian nAChRs (Tomizawa and Casida, 2003); therefore, NNs has been considered to be highly selective, with low toxicity to mammals and non-target species. These circumstances, coupled with their flexible application in sprays and seed treatments, have led to the widespread use of NNs, accounting for more than 25 % of the global market (Bass et al., 2015; Klingelhöfer et al., 2022; Stehle et al., 2023; Thompson et al., 2020). Owing to their increased usage,

Abbreviations: ACE, acetamiprid; CE, collision energy; DRN, dorsal raphe nuclei; ESI, electrospray ionization; EZM, elevated zero maze; LC/MS/MS, liquid chromatography mass spectrometry; MA, monoamines; MRM, multiple reaction monitoring; NAChRs, nicotinic acetylcholine receptors; NNs, neonicotinoid pesticides; NOAEL, no observed adverse effect level; RN, raphe nuclei; RT, retention time; SSRI, selective serotonin reuptake inhibitors; 3-MT, 3-methoxytyramine HCl.

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NNs have been detected in agricultural surface waters in several regions (Stehle et al., 2023), in commercial teas and juices (Li et al., 2020; Ly et al., 2022; Suzuki et al., 2021), and in human urine, including that of newborns (Ichikawa et al., 2019; Taira et al., 2021), indicating that humans are routinely exposed to NNs, albeit at low doses.

However, recent reports have shown that non-observed adverse effect level (NOAEL) of NNs induce behavioral changes such as increased anxiety-like behavior in mice (Hirai et al., 2022a; Hirano et al., 2018) and elevated dopamine release in the striatum (de Oliveira et al., 2010; Faro et al., 2019; Hirai et al., 2022b; Yoneda et al., 2018). As abnormalities in the monoamine (MA) neurotransmitter system can lead to the development of psychiatric disorders such as depression, anxiety disorders, and Parkinson's disease (Hamon and Blier, 2013; Morilak and Frazer, 2004; Ohama and Ikuta, 1976), MAs have been suggested to be involved in mechanism of NN-induced behavioral changes. However, the effects of NNs on the mammalian central nervous system remain unclear, and reports on the effects of NNs on MA are limited.

Dopamine is reportedly involved in nicotine-induced anxiety-like behavior (Zarrindast et al., 2012), and various types of NNs increases striatal dopamine levels (de Oliveira et al., 2010; Faro et al., 2019; Hirai et al., 2022b; Yoneda et al., 2018). However, few studies have examined the effects of acetamiprid (ACE) on MA. Although the hippocampus is associated with memory, it is also important for anxiety (Bannerman et al., 2004): it has been reported that injection of dopamine receptor antagonists into the dorsal or ventral hippocampus reduces nicotine-induced anxiety-like behavior (Nasehi et al., 2011; Zarrindast et al., 2010), and that the cholinergic system in the hippocampus can modulate anxiety (Degroot and Treit, 2002; Mineur et al., 2013).

Several studies have reported that NNs cause a decrease in activity as well as an increase in anxiety-like behavior in the elevated plus maze (EPM) (Hirano et al., 2021; Kubo et al., 2022). Additionally, the index of activity in the EPM is also affected by anxiety (Shepherd et al., 1994); for example, freezing, which refers to immobility in mice and rats, is known to be a defensive response (Blanchard et al., 2003) that may affect activity in the EPM. The serotonergic system is involved in freezing (da Silva Soares et al., 2019), and the major dopaminergic pathways, including the nigrostriatal pathway, and dopaminergic neurons in the hippocampus are innervated by serotonergic nerves originating in the raphe nuclei (RN) (Alex and Pehek, 2007; Moore and Halaris, 1975). The RN is another brain region that is critically involved in anxiety, and stimulation of serotonergic neurons in the RN increases anxiety-like behavior via the release of hippocampal serotonin (Abela et al., 2020; Ohmura et al., 2014). In addition, nAChRs are also expressed in the RN, and nicotine increases the firing frequency of serotonergic neurons in the RN (Garduño et al., 2012). These studies suggest that MAs in the striatum, hippocampus, and RN may play an important role in NNs-induced anxiety-like behavior.

Therefore, in this study, we sought to clarify the involvement of MAs in NNs-induced behavioral changes by examining the correlation between the behavioral effects of ACE, and MAs in the brain, as well as serotonin activity in the RN, which plays an important role in anxiety.

2. Material and methods

2.1. Chemicals

ACE was purchased from Cosmo Bio Co., Ltd. (100 % purity, Tokyo, Japan). Dopamine, 3-methoxytyramine HCl (3-MT), and 3-MT-d4 HCl were purchased from Cerilliant Corporation (Round Rock, TX, USA). Serotonin was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA); histamine and serotonin-d4 hydrochloride from Toronto Research Chemicals, Inc. (Toronto, ON, Canada); and dopamine-d4 and histamine-d4 from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Py-Tag for histamine was purchased from TAIYO NIPPON SANSO CORPORATION (Tokyo, Japan); formic acid and sucrose from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan); L-ascorbic acid and Triton™ X-100 from Sigma-Aldrich Co. LLC (St. Louis, MO, USA); 4 % paraformaldehyde phosphate buffer solution (PFA/PB) from Bioenno Lifesciences (Santa Ana, CA, USA); goat anti-serotonin IgG antibodies from Abcam plc. (Cambridge, UK); Alexa Fluor™ 594 donkey anti-goat IgG (H+L) from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). All other chemicals and solvents were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.2. Animals

All animal experiments were approved by the Experimental Committee of the Faculty of Veterinary Medicine, Hokkaido University, and were conducted in accordance with the ARRIVE guidelines, as well as the Guide for the Care and Use of Laboratory Animals and with the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC; approval date: 17th March 2022, approval number: 22–0024).

Male C57BL/6 J mice (11-weeks-old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). They were maintained at 22±1 °C (room temperature), 70±5 % humidity, and a 12-h light/dark cycle (8:00–20:00; light, 20:00–8:00; dark), with food (breeding solid food for mice, rats, and hamsters: CE-2, CLEA Japan, Inc., Tokyo, Japan) and tap water available *ad libitum*. Food and water were changed twice a week, and cages were changed once a week. Experiments were performed after at least one week of acclimation.

2.3. Elevated zero maze (EZM) tests

Mice ($n = 41$) were randomly divided into two groups: control ($n = 20$) and ACE ($n = 21$) groups. These number of mice in each group was calculated based on a previous study using similar behavioral test (Biala and Budzynska, 2006). The ACE group received oral ACE dissolved in normal saline at 20 mg/kg body mass, which was estimated to be the NOAEL of ACE in mice (Food Safety Commission, 2014), while the control group received saline at 10 mL/kg body mass. These doses were administered under light anesthesia with isoflurane using sonde (FUCHIGAMI, Kyoto, Japan). After administration, mice were placed individually in a dark room for 30 min, and their behavior was observed in the EZM for 5 min.

The EZM apparatus has an 0-shaped corridor, placed at 50 cm from the floor, with an outer diameter of 46 cm and an inner diameter of 35 cm, and this corridor is divided into four areas. Alternating areas are surrounded by a 17-cm high wall (closed arm), while the others are without walls (open arm). This apparatus was placed in a quiet room with no human access during test, surrounded on all sides by a white enclosure, and the light intensity inside the enclosure was kept at 20 lx. Behavior was automatically recorded and analyzed by SMART 3.0 (Panlab, S.L., Barcelona, Spain), and mice were defined as having entered the zone when their center of gravity entered each area. All administrations and behavioral tests were performed during the light cycle (12:00–18:00), ensuring that there was no group bias. The EZM tests measured the percentage of time and distance in open arms and the number of entries into open arms as an index of anxiety-like behavior, and the total distance traveled as an index of activity. Mice that fell out of the EZM apparatus during testing were excluded from this and subsequent LC/MS/MS analyses.

2.4. MA concentration measurement using liquid chromatography mass spectrometry

Immediately after the behavioral tests were performed, mice were euthanized by cervical dislocation under deep isoflurane anesthesia. Brains were sampled and visually dissected on an ice-cold tray using tweezers and spatulas into 6 brain regions, primarily containing the following areas: hippocampus (including both dorsal and ventral parts), striatum, brainstem, cerebellum, cerebral cortex, and thalamus

(including hypothalamus). The regions sampled are shown in Figure S1, and we refer to them in this study as the above six brain regions that each primarily contains. Samples were flash frozen in liquid nitrogen and stored at −20 °C until further use.

Approximately 10 mg of the sample was weighed into a 1.5 mL tube to which 200 μL of 0.05 % formic acid in acetonitrile and 0.5 mM ascorbic acid solution and 20 μL of an internal standard master mix (100 ppb) containing dopamine-d4, 3-MT-d4, histamine-d4, and serotonin-d4 were added. This tube was homogenized with two zirconia beads (2.0 mm; Toray Industries, Inc., Tokyo, Japan) in a Tissue Lyser (30/s, 1.5 min; QIAGEN K.K., Tokyo, Japan), the tissue homogenate was centrifuged at 10,000 xg for 10 min, and the supernatant was carefully transferred to a new tube. Then, to derivatize the monoamine, 20 μL of Py-Tag for histamine, a derivatization reagent, was added to 30 μL of this supernatant, vortexed, and incubated at 50 °C for 15 min using a thermal cycler. Finally, 70 μL of this derivatized sample was diluted with 50 μL of distilled water (DW) and analyzed by liquid chromatography mass spectrometry (LC/MS/MS).

2.5. LC/MS/MS measurement conditions

The LC and MS apparatus used to measure MA concentrations were an Agilent 1290 Infinity ultra-high performance liquid chromatography system and an Agilent 6495 triple quadrupole mass spectrometer, respectively (Agilent Technologies, Inc., Santa Clara, CA, USA). The column used was a 150 × 2.1 mm Kinetex Biphenyl column with 2.6 μm particle size (Phenomenex Inc., Torrance, CA, USA), and the temperature was set at 60 °C.

Using DW containing 0.1 % formic acid as mobile phase A and methanol containing 0.1 % formic acid as mobile phase B, the analytes were separated at a flow rate of 0.2 mL/min until 0.8 min and 0.4 mL/min after 0.8 min at an injection volume of 5 μL. The gradient was started at 5 % mobile phase B. After a linear increase of B to 75 % from 0.8 min to 17 min, the mobile phase B was switched to 100 % and maintained until 18.5 min. It was then returned to 5 % B and equilibrated for 1.5 min until the next injection. Table 1 shows the retention time (RT), multiple reaction monitoring (MRM) transition, and collision energy (CE) for each analyte. Electrospray ionization (ESI) was performed in positive mode.

The product ion listed in the top row for each compound was used as the target ion, and the other product ions were used as qualifiers.

Table 1
Parameters in MA analysis by LC/MS/MS.

Compound	RT [min]	MRM Transition [*m/z*]		CE [V]
		Precursor Ion	Product Ion	
3-MT	14.5	342.31	192	28
			151.1	28
			91	60
3-MT-d4	14.5	346.41	192	16
			155	28
			95.1	48
Dopamine	14	328.31	192.3	24
			91.2	56
Dopamine-d4	14	332.41	192.2	20
			95.1	56
Histamine	7.7	286.21	192.3	24
			95.1	28
Histamine-d4	7.7	290.31	192.2	32
			99.1	32
Serotonin	14.3	351.41	192.1	20
			160	28
Serotonin-d4	14.3	355.41	192.1	20
			164	20

2.6. Anti-serotonin staining

Two groups of mice were randomized: control ($n = 6$) and ACE ($n = 7$) groups. Solutions, methods of administration, and concentrations administered were the same as those used in the behavioral test. In brief, 30 min after administration, mice were cervically dislocated under deep anesthesia with isoflurane and immediately perfusion fixed with 0.9 % NaCl with 10 mg/L heparin, and whole brains were sampled. The sampled brains were shaken at 4 °C in 4 % PFA/PB overnight, then replaced with 30 % sucrose in 0.1 M phosphate buffer (PB) and shaken again at 4 °C overnight. After fixation, frozen sections were prepared by embedding in O.C.T. Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and freezing in liquid nitrogen. Frozen sections were stored at −20 °C until sectioning; then, after cryostat thinning to 50 μm thickness, they were stored at 4 °C in 0.1 M PB until staining. Sections were then blocked with 4 % Triton™ X-100 and incubated with goat anti-serotonin IgG antibody (1:1000) overnight at room temperature with protection from light. After incubation with Alexa Fluor™ 594 donkey anti-goat IgG (H+L) (1:500) as a secondary antibody for 2 h at room temperature, the slides were mounted with DAPI Fluoromount-G® (Southern Biotechnology Associates Inc., Birmingham, AL, USA).

The prepared slides were observed using an all-in-one fluorescence microscope, BZ-9000 (KEYENCE CORPORATION, Osaka, Japan), and slides containing the middle part of the dorsal RN (DRN) (Bregma −4.54 mm to −4.90 mm (Abrams et al., 2004)), were visually identified for all mice. These slides were observed using a confocal laser-scanning microscope, Zeiss LSM 700 (Carl Zeiss Co., Ltd., Tokyo, Japan; objective lens: 20×), and fluorescence images of 8 z-stacks (3.05 μm interval) of the DRN were captured. The maximum intensity projections of these z-stacks were used for image analysis. All image data were mixed so that groups were not recognized, and cell counts were performed in a blinded manner using Fiji Image J (1.53e; NIH, Java 1.8.0_172; 64 bit) (Schindelin et al., 2012).

2.7. Statistical analysis

Microsoft Excel (2016) and JMP (SAS Institute Inc., Cary, NC, USA) were used for statistical analysis. Non-parametric tests were used for experiments in which there were data sets that did not show either normal distribution or homogeneity of variance; the Wilcoxon test was used to compare behavioral test results, the Steel test to compare MA concentrations, and the Spearman's rank correlation coefficient test to analyse the correlation between behavioral test results and MA concentrations or body mass. The number of cells in the RN showed a Gaussian distribution and homogeneity of variance in both groups and was assessed by one-tailed t-test. All data are presented as mean ± standard error (SEM), and the significance level was set at $P < 0.05$.

3. Results

3.1. EZM tests

The results of EZM tests are shown in Fig. 1. During the test, the control group spent 17.94 ± 1.37 % of the total time in the open arms and the ACE group spent 14.15 ± 1.69 % of the total time, showing a decreasing trend (Fig. 1a, $P = 0.0569$). The control group traveled 19.30 ± 1.30 % of the total distance in the open arms, while the ACE group traveled 16.04 ± 1.85 %, showing no significant difference (Fig. 1b, $P = 0.0951$). The entries into the open arms were 22.45 ± 1.47 times in the control group and 13.76 ± 1.53 times in the ACE group (Fig. 1c, $P = 0.0001$), and the total distance traveled was 1431 ± 43.66 cm in the control group and 1120 ± 66.19 cm in the ACE group (Fig. 1d, $P = 0.0026$), wherein a significant decrease was observed in the ACE group. The correlation coefficients between mouse body mass and time in the open arms and total distance were −0.06 and 0.26, respectively (Figure S2). Neither of these coefficients was significant ($P = 0.7182$,

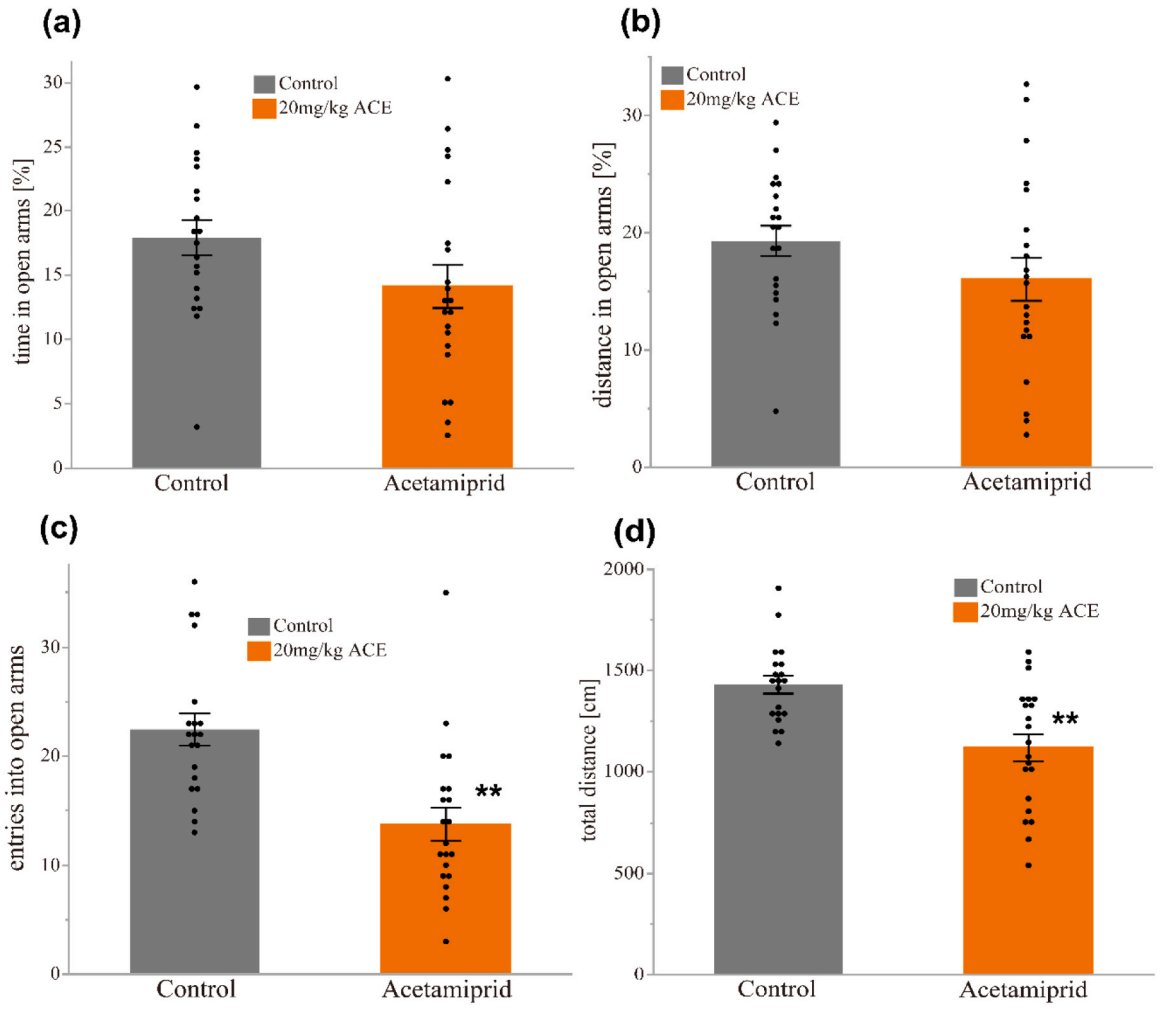


Fig. 1. ACE-induced behavioral changes in the EZM tests. (a) The percentage of time spent in the open arms tended to decrease in the ACE group. (b) The percentage of distance traveled in the open arms did not change significantly between the two groups. (c) The number of entries into the open arms and (d) the total distance traveled in the EZM were significantly lesser in the ACE group than the control. Bar graphs represent the mean ± SEM of each group (control: $n = 20$, ACE: $n = 21$). ** $P < 0.01$, Wilcoxon test.

0.0963), indicating that body mass did not significantly affect anxiety-like behavior and activity in the EZM.

3.2. MA concentrations in the hippocampus and striatum

Dopamine, 3-MT, histamine, and serotonin concentrations in the hippocampus, striatum, and brainstem were measured by LC/MS/MS analysis (Figs. 2–4). MA concentrations in the cerebellum, cerebral cortex, and thalamus are shown in the Supplementary Material, as the brain regions sampled were extensive and the accuracy of the analysis was reduced due to region-specific analysis.

The concentrations of dopamine, 3-MT, histamine, and serotonin in the hippocampus were 0.0049 ± 0.0008, 0.0019 ± 0.0002, 0.012 ± 0.001, and 0.13 ± 0.007 ng/mg in the control group, and 0.012 ± 0.002, 0.011 ± 0.002, 0.011 ± 0.002, and 0.68 ± 0.1 ng/mg in the ACE group, respectively (Fig. 2a–d).

In the striatum, the concentrations of dopamine, 3-MT, histamine, and serotonin were 1.7 ± 0.3, 0.27 ± 0.03, 0.12 ± 0.02, and 0.26 ± 0.01 ng/mg in the control group, and 3.1 ± 0.5, 0.66 ± 0.1, 0.11 ± 0.02, and 0.80 ± 0.1 ng/mg in the ACE group, respectively (Fig. 3a–d).

In contrast, in the brainstem, dopamine was detected in only three mice in the control group (0.0027 ± 0.002 ng/mg) and eight mice in the ACE group (0.0083 ± 0.005 ng/mg) (Fig. 4a); 3-MT was detected in only one mouse in the ACE group (0.00022 ± 0.001 ng/mg). Histamine and serotonin levels were 0.032 ± 0.006 and 0.014 ± 0.004 ng/mg in the control group, and 0.037 ± 0.007 and 0.15 ± 0.03 ng/mg in the ACE group, respectively (Fig. 4b, c).

Dopamine in the cerebellum of the ACE group, 3-MT in the cerebellum and cerebral cortex of both groups, and serotonin in the cerebellum of the control group were not detected (Table S1). In other brain regions, each MA was detected in more than two mice, and the mean concentrations are shown in Table S1.

In the hippocampus and striatum, dopamine ($P = 0.0049$ and 0.0460), 3-MT ($P < 0.0001$ and $P = 0.0005$), and serotonin ($P < 0.0001$ for both) levels were significantly increased in the ACE group. There was no significant difference in the concentration of histamine in the striatum ($P = 0.2154$) of the two groups, while it tended to decrease in the hippocampus ($P = 0.0535$). In the brainstem, cerebellum, and cerebral cortex, only serotonin concentration was significantly increased in the ACE group ($P < 0.0001$, $P = 0.0057$ and 0.0127, respectively). In the thalamus, levels of all four MAs were significantly increased (dopamine: $P = 0.0011$, 3-MT: $P = 0.0047$, histamine: $P = 0.0210$, and serotonin: $P < 0.0001$).

3.3. Correlation analysis of EZM test results and MA concentrations

Correlations were examined for two parameters of the behavioral test: time in open arms as a representative index of anxiety-like behavior

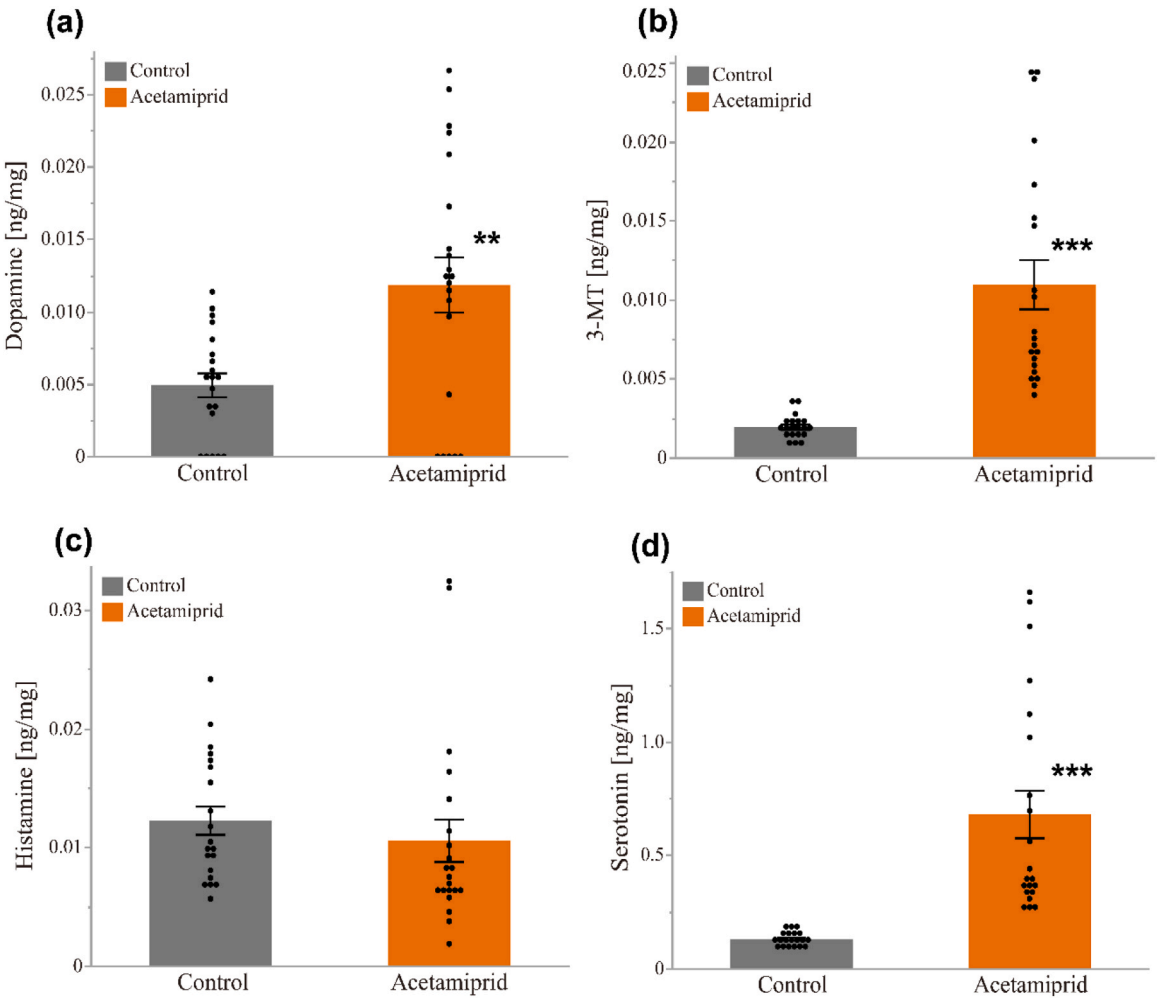


Fig. 2. MA concentrations in the hippocampus. The concentrations of (a) dopamine, (b) 3-MT, and (d) serotonin were significantly high in the ACE group. (c) The concentration of histamine tended to decrease in the ACE group. Bar graphs represent mean ± SEM of each group (control: $n = 20$, ACE: $n = 21$). ** $P < 0.01$, *** $P < 0.0001$, Steel test.

and total distance as a representative index of activity, and each of the four LC/MS/MS analysis results: concentrations of dopamine, 3-MT, histamine, and serotonin in the hippocampus, striatum, and brainstem (Figs. 5–7).

The correlations between 3-MT and dopamine and between 3-MT and serotonin were significant in the hippocampus and striatum of both the control (hippocampus: $P = 0.0305$ and 0.0027, striatum: $P = 0.0016$ and 0.0081, respectively) and ACE groups (hippocampus: $P = 0.0011$ and 0.0004, striatum: $P = 0.0444$ and 0.0006, respectively), and between dopamine and histamine only in the striatum of both groups (control: $P = 0.0435$, ACE: $P = 0.0343$), while histamine and serotonin levels were correlated only in the brainstem of both groups (control: $P = 0.0067$, ACE: $P = 0.0054$). The significant correlations observed only in the ACE group were between dopamine and serotonin in both the hippocampus ($P = 0.0019$) and striatum ($P = 0.0132$), between time spent in the open arms and serotonin in the hippocampus ($P = 0.0305$), and between time in open arms and dopamine in the striatum ($P = 0.0320$). In contrast, the correlations between dopamine and histamine ($P = 0.0082$), and between 3-MT and histamine ($P = 0.0184$) in the hippocampus, and between dopamine and serotonin in the brainstem ($P = 0.0369$) were significant only in the control group. Time spent in the open arms and total distance traveled were significantly correlated only in the control group ($P = 0.0064$). The other correlations were not significant.

3.4. Neuronal count of the DRN

In the DRN of the control group, 113 ± 10 neurons were stained with anti-serotonin antibodies (Fig. 8b). In contrast, 142 ± 9.3 neurons were stained in the ACE group (Fig. 8c). Thus, the number of neurons actively producing serotonin in the DRN was significantly increased in the ACE group ($P = 0.0307$).

4. Discussion

In this study, four MAs, dopamine, 3-MT, histamine, and serotonin, were measured in different brain regions using LC/MS/MS, and the correlation between these levels and behavioral test results was plotted for each mouse. Dopamine, 3-MT, and serotonin levels were significantly increased in the hippocampus and striatum, and striatal dopamine and hippocampal serotonin levels, in particular, were significantly correlated with time spent in the open arms, an index of anxiety-like behavior, in the ACE group.

Although NNs are known to affect dopamine transmission (Faro et al., 2019; Hirai et al., 2022b; Yoneda et al., 2018), reports on the involvement of the dopaminergic system in NNs-induced anxiety-like behavior are limited. Expression of dopamine transporters responsible for dopamine clearance in the striatum are reduced in Parkinson's disease patients, which correlates with the severity of anxiety symptoms (Erro et al., 2012). Dopamine signaling in the hippocampus and striatum

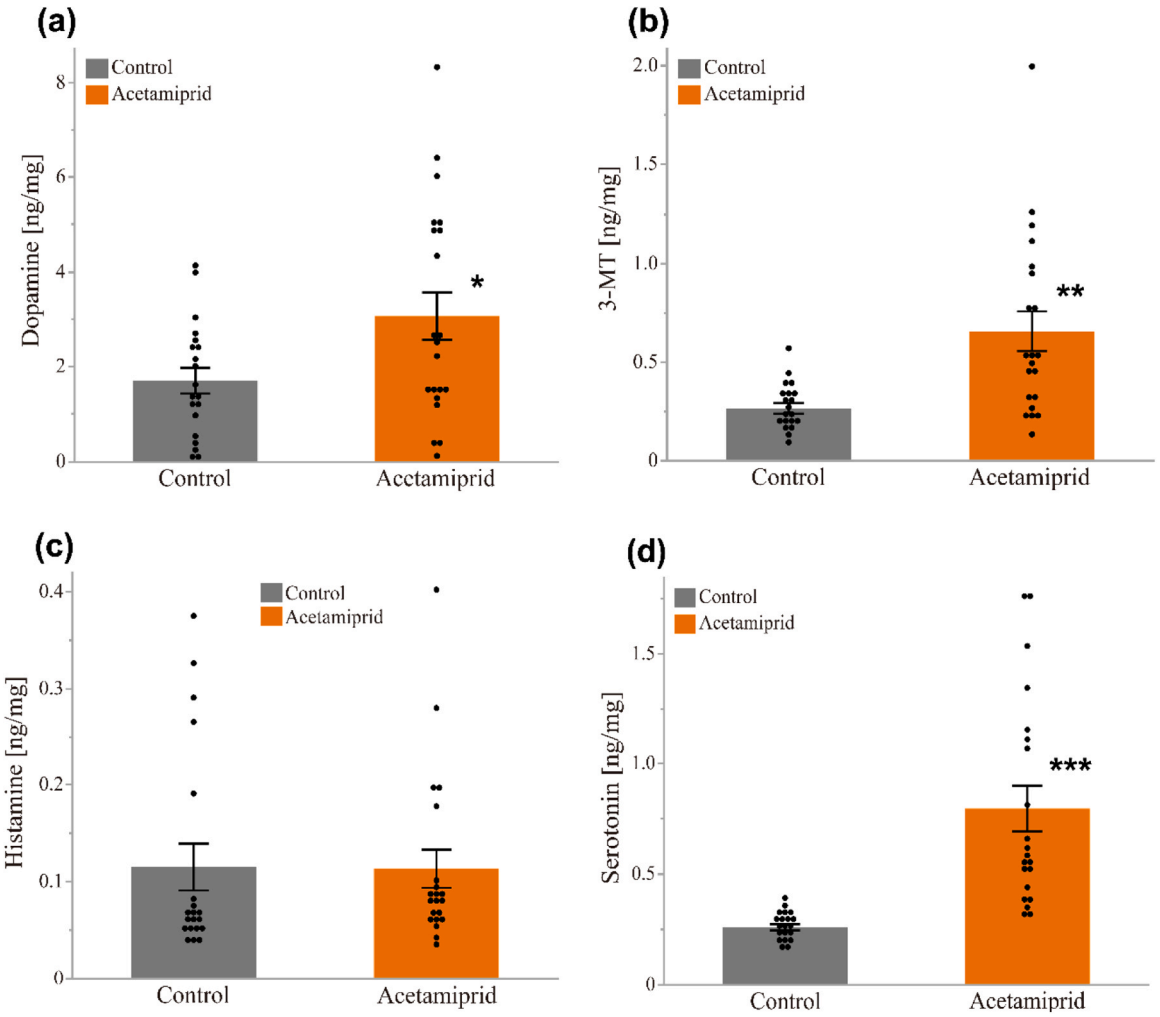


Fig. 3. MA concentrations in the striatum. The concentrations of (a) dopamine, (b) 3-MT, and (d) serotonin were significantly high in the ACE group. (c) The difference in histamine concentration was not significant. Bar graphs represent mean ± SEM of each group (control: $n = 20$, ACE: $n = 21$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, Steel test.

is involved in the anxiogenic-like effects of nicotine (Nasehi et al., 2011; Zarrindast and Khakpai, 2015; Zarrindast et al., 2012, 2010). We observed that increased striatal dopamine concentrations significantly increased the occurrence of anxiety-like behaviors, highlighting the importance of striatal dopamine in anxiety-like behaviors induced by NNs, which are structurally similar to nicotine.

The serotonergic system is also widely known to be involved in anxiety; for example, the selective serotonin reuptake inhibitors (SSRI) are typical anti-depressants (Żmudzka et al., 2018). Previous studies using social interaction tests have shown that microinjection of nicotine into the dorsal hippocampus induced anxiety-like behaviors, and this effect was reversed by serotonin receptor antagonists, but not by muscarinic receptor antagonists (Kenny et al., 2000). These studies suggest that hippocampal serotonin plays an important role in NNs-induced anxiety-like behavior; however, the present analysis was performed without separating the dorsal and ventral hippocampus, and further research is needed to determine which of these play a key role in NN-induced anxiety.

In this study, histamine levels did not show significant changes; however, a decreasing trend was observed in the hippocampus. The role of histamine in anxiety is thought to be complex (Haas et al., 2008); it has been reported that mice fed a diet low in L-histidine, which is necessary for histamine synthesis, show decreased histamine levels in the brain and increased anxiety-like behavior (Yoshikawa et al., 2014), while microinjection of histamine into the dorsal hippocampus induces anxiolytic effects (Zarrindast et al., 2006) and anxiogenic effects when injected in the ventral hippocampus (Rostami et al., 2006). Furthermore, as acute stress promotes histamine turnover in the striatum and other brain regions (Ito, 2000; Yoshitomi et al., 1986), measuring the concentrations of metabolites may further clarify the role of histamine in NNs-induced anxiety-like behavior.

Significant correlations were also found between several MA concentrations. The correlations between the levels of dopamine and histamine in the striatum and between dopamine and 3-MT, and 3-MT and serotonin in both the hippocampus and striatum were significant in both the control and ACE groups. As 3-MT is a metabolite of dopamine, its correlation with dopamine is likely due to metabolism of the increased dopamine. The correlation between dopamine or 3-MT and histamine in the hippocampus was significant only in the control group but not in the ACE group; the correlation disappeared with the administration of ACE. This may have been due to the decreasing trend of hippocampal histamine, although levels of the other MAs were significantly increased.

Conversely, the correlation between dopamine and serotonin in the hippocampus and striatum was significant in the ACE group, but not the control group, suggesting that it was caused by ACE administration. Three major dopaminergic pathways, including the nigrostriatal pathway, are innervated by serotonin (Alex and Pehek, 2007), and serotonergic stimulation of the striatum increases dopamine release (Dailly et al., 2004). Such innervations occur when the serotonergic tone is increased, rather than under physiological conditions (Alex and

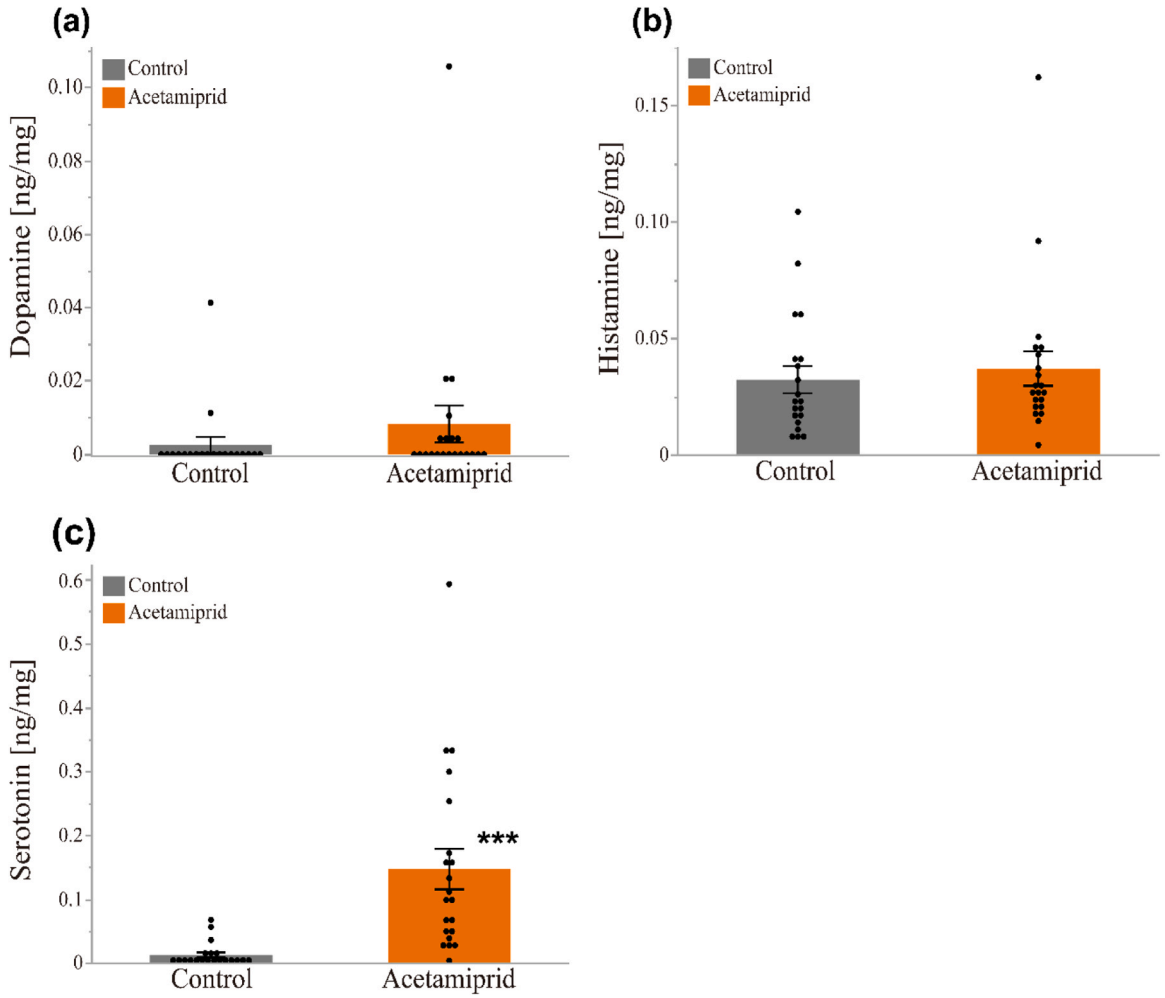


Fig. 4. MA concentrations in the brainstem. The concentration of (c) serotonin was significantly high in the ACE group. There were no significant difference in the concentration of (a) dopamine and (b) histamine between the two groups. Bar graphs represent mean ± SEM of each group (control: $n = 20$, ACE: $n = 21$). *** $P <$ 0.0001, Steel test.

Pehek, 2007). In light of these findings, the significant correlation between dopamine and serotonin observed only in the ACE group in this study may be the result of ACE increasing serotonin and modulating dopamine levels due to increased serotonergic tone.

The EZM, developed as an improvement on the EPM, is widely used to study anxiety-like behavior in rodents (Shepherd et al., 1994). This behavioral test uses a balance between curiosity about novel environments and fear of heights, and it is generally interpreted that the lesser time spent exploring in the open arm, the more fear avoidance behavior occurs, that is, a state of increased anxiety (Walf and Frye, 2007; Yamaguchi et al., 2005). In contrast to the EZM, the EPM has open and closed arms that cross each other in a crisscross pattern, and the stay in the central area is difficult to interpret; the EZM resolved this issue by using a circular design for the arms (Shepherd et al., 1994). In this study, the administration of ACE tended to decrease the time spent in open arms in the EZM, indicating increased anxiety-like behavior, our results support previous findings (Hirai et al., 2022b, 2022a; Kubo et al., 2022; Rodrigues et al., 2010; Sano et al., 2016). In addition, entries into the open arms and total distance traveled were significantly reduced. The rate of entries into the open arms is one of the parameters that is a typical indicator of anxiety-like behavior, especially in the EPM; however, due to the absence of a center area and the placement of open and close arms proximal to each other in the EZM, the number of entries into the open arms is almost the same as those into the closed arms. Therefore, in the EZM, this parameter is similar to that of total distance traveled rather than an index of pure anxiety-like behavior. Another parameter that was significantly reduced, total distance traveled, is often interpreted as an indicator of activity. However, this index is affected not only by activity but also by anxiety (Shepherd et al., 1994), and freezing can also occur as a defensive response (Blanchard et al., 2003). In the present results, total distance traveled was significantly correlated with time spent in the open arms in the control group, rendering it difficult to separate the effects of anxiety and activity on this parameter. One limitation of this study is the gender of mice; women have been reported to be at greater risk of anxiety and depression than men (Altemus et al., 2014), but only male mice were used in this study. Interestingly, in the EPM, a behavior test similar to the EZM, it has been reported that female mice and rats show less anxiety than males (An et al., 2011; Börchers et al., 2022). It is unclear whether female mice are better suited to investigate the effects of anxiety because of this species difference, but further studies of sex differences in monoaminergic systems may reveal not only sex differences in anxiety, but also the mechanism of this species difference.

As mentioned, the serotonergic system plays an important role in anxiety, and serotonergic neurons that project to the forebrain, including the serotonergic neurons that innervate the dopamine pathways, are largely derived from the RN (Abela et al., 2020; Alex and Pehek, 2007). The serotonergic system of the DRN, in particular, has been implicated in anxiety-related behaviors (Bouwknecht et al., 2007). In the DRN, nAChRs are expressed in both the serotonergic nerves and

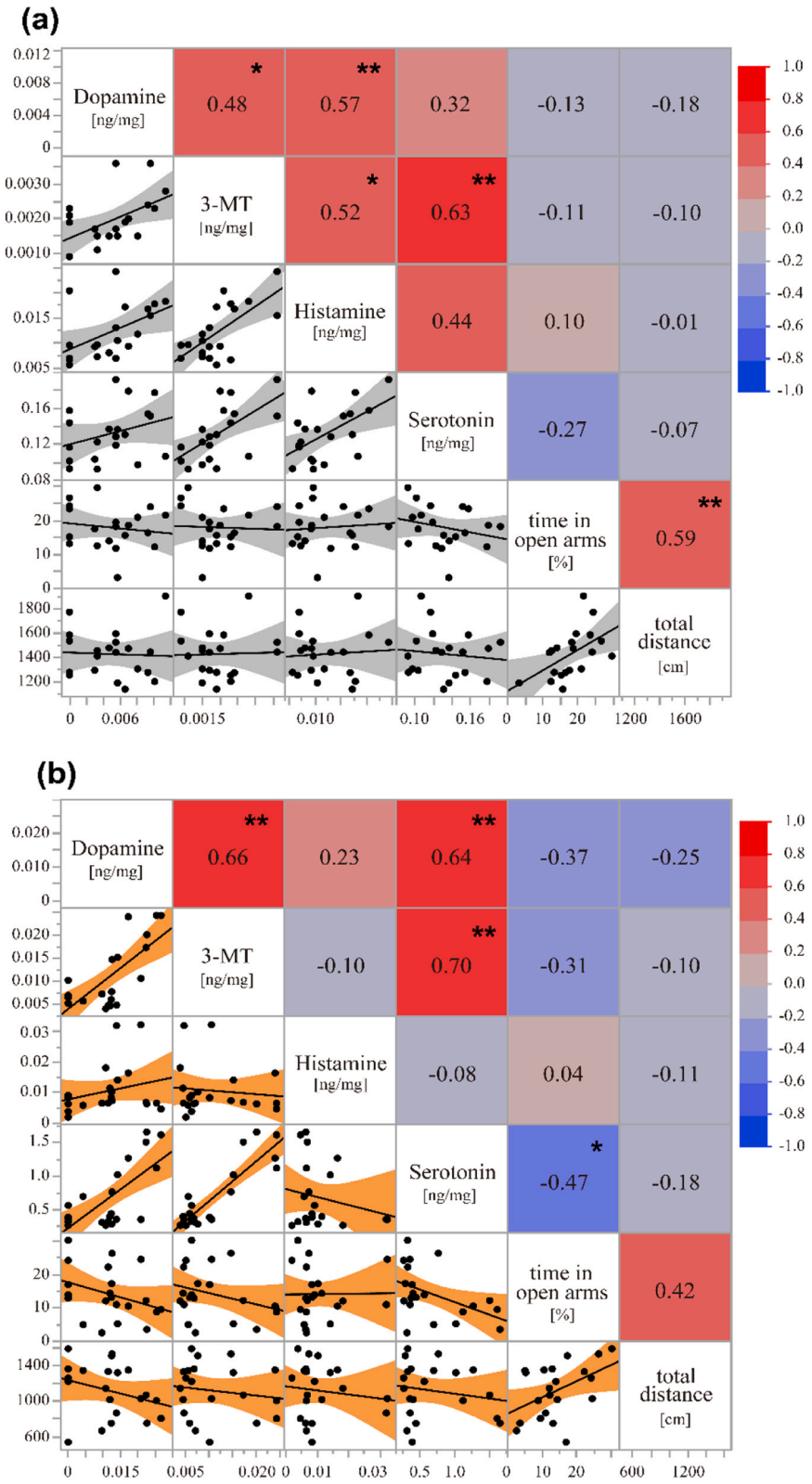


Fig. 5. Scatterplot matrix of correlation between the results of EZM test and MA concentrations in the hippocampus. The lower left half of the figures show the scatterplot matrix and the upper right half show a heat map of the correlation coefficients. (a) In the control group ($n = 20$ each), five correlations were significant. (b) In the ACE group ($n = 21$ each), four correlations were significant. * $P < 0.05$, ** $P < 0.01$, Spearman's rank correlation coefficient test.

presynaptic glutamatergic neurons, and nicotine increases the firing frequency of approximately 80 % of serotonergic neurons in the DRN (Mihailescu et al., 2002, 1998) and induces glutamate release via activation of the presynaptic α4β2 nAChRs subtypes (Garduño et al., 2012). It has also been reported that stimulation of serotonergic nerves in the RN increases anxiety-like behavior via release of hippocampal serotonin (Abela et al., 2020; Ohmura et al., 2014). In this study, serotonin concentration in the brainstem, the brain region containing the RN, was

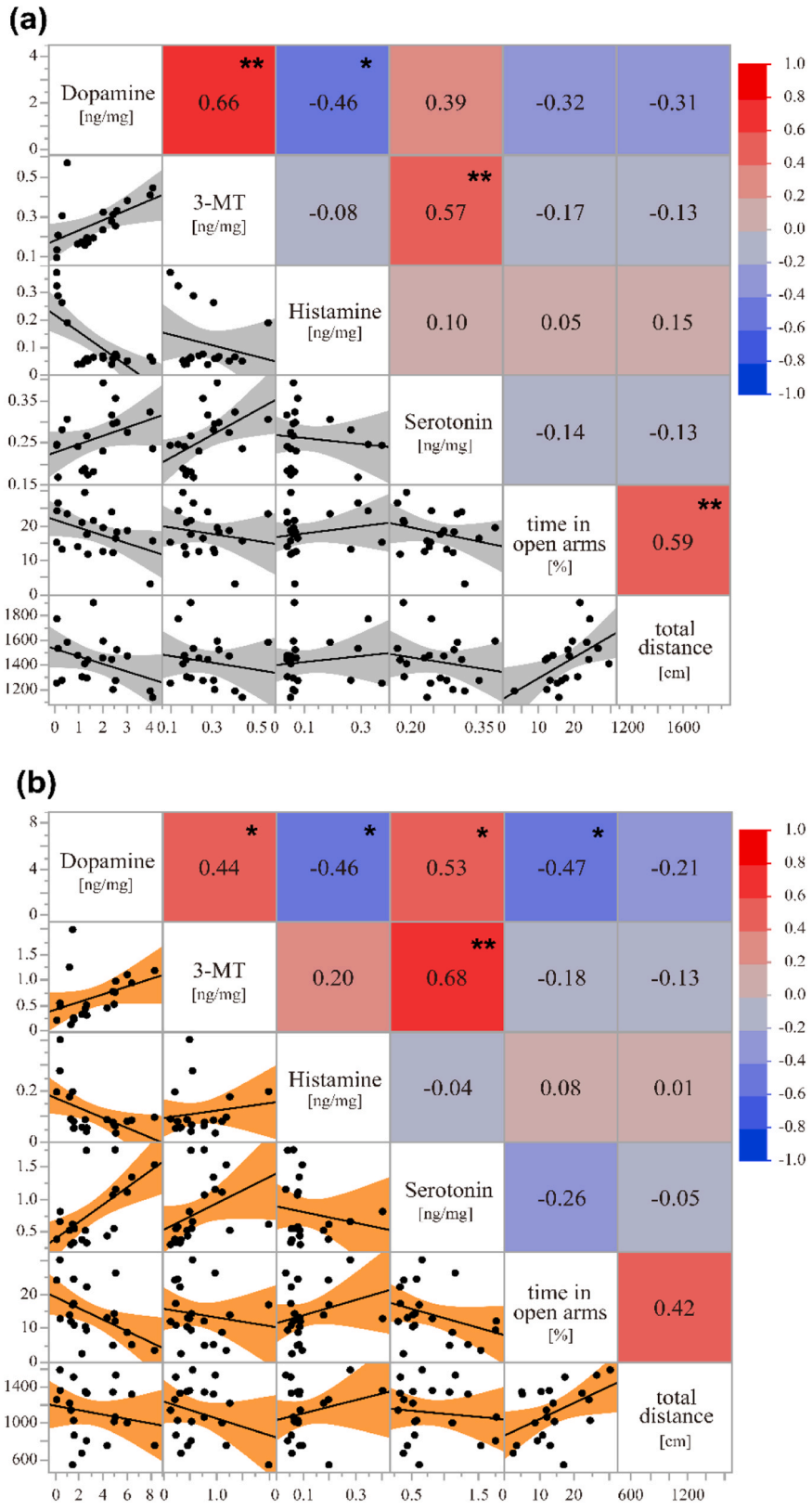


Fig. 6. Scatterplot matrix of correlation between the results of EZM test and MA concentrations in the striatum. The lower left half of the figures show the scatterplot matrix and the upper right half show a heat map of the correlation coefficients. (a) In the control group ($n = 20$ each), four correlations were significant. (b) In the ACE group ($n = 21$ each), five correlations were significant. * $P < 0.05$, ** $P < 0.01$, Spearman's rank correlation coefficient test.

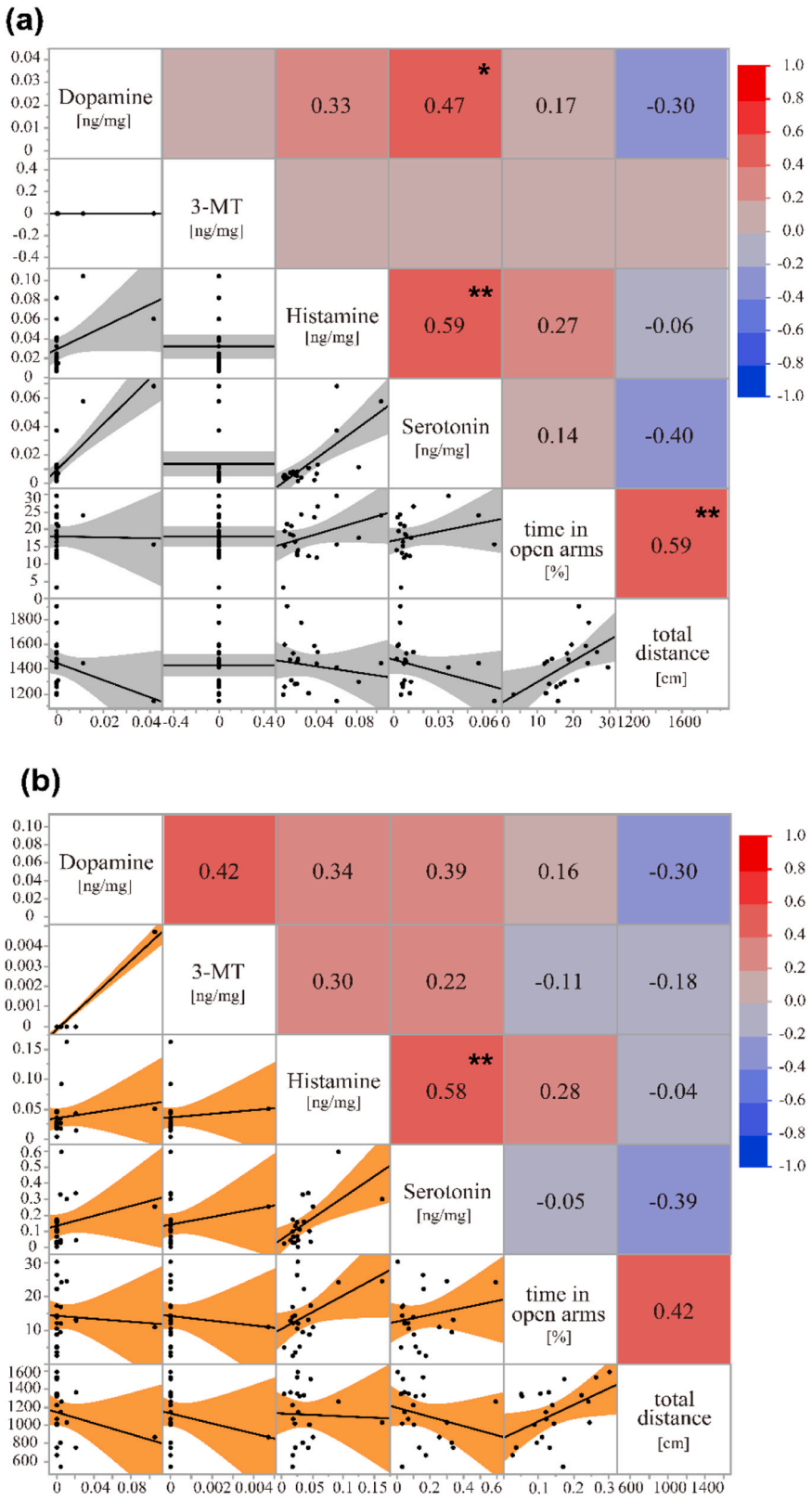


Fig. 7. Scatterplot matrix of correlation between the results of EZM test and MA concentrations in the brainstem. The lower left half of the figures show the scatterplot matrix and the upper right half show a heat map of the correlation coefficients. (a) In the control group ($n = 20$ each), three correlations were significant. (b) In the ACE group ($n = 21$ each), only one correlation was significant. * $P < 0.05$, ** $P < 0.01$, Spearman's rank correlation coefficient test.

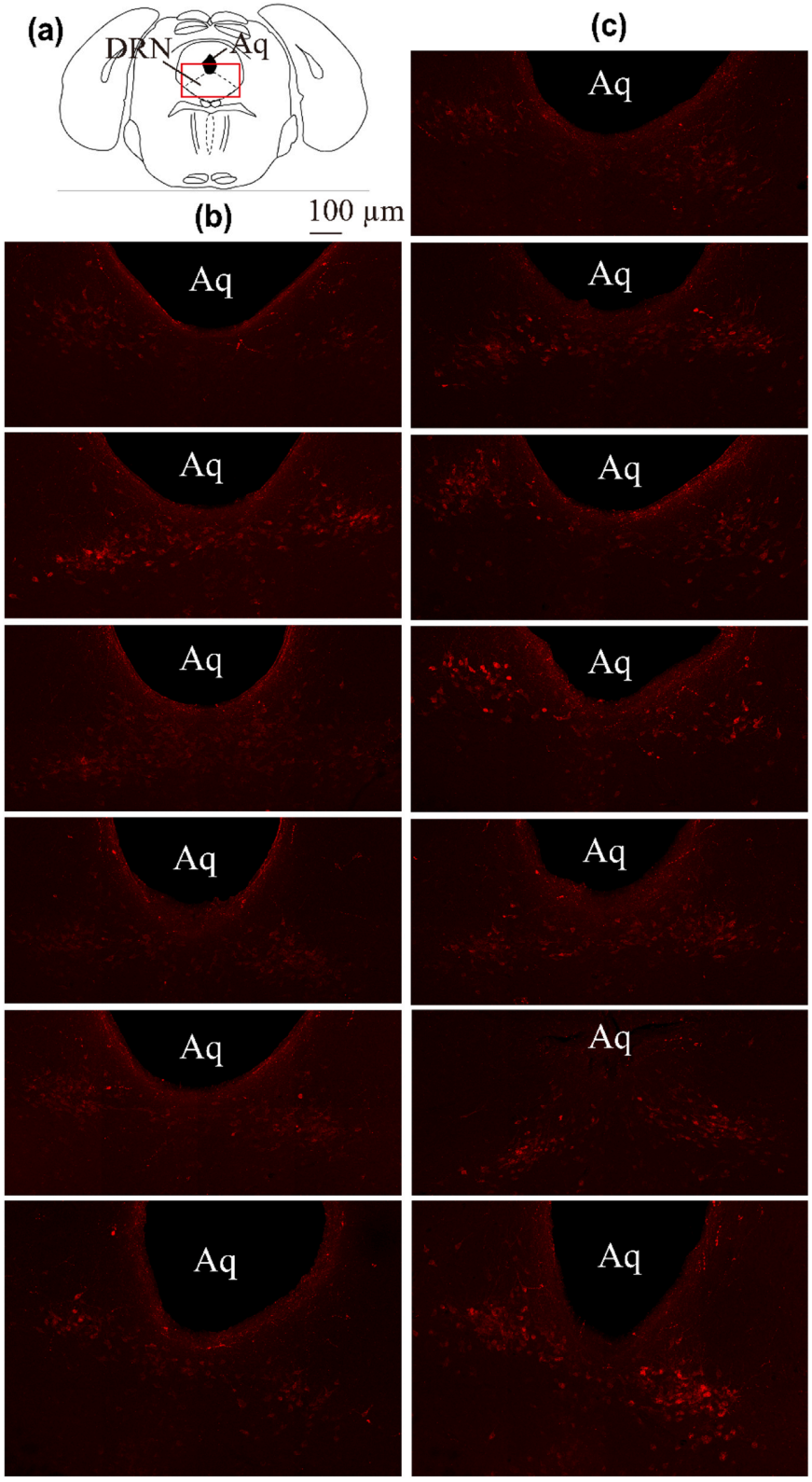


Fig. 8. Anti-serotonin staining in the DRN. (a) Observed area was circled in red. The maximum intensity projections of 8 z-stack red fluorescence images of the (b) control ($n = 6$) and (c) ACE ($n = 7$) groups are shown. Aq: cerebral aqueduct.

increased in the ACE group, suggesting that ACE acts on the RN to increase serotonin levels.

However, a limitation of this study is that not all brain samples analyzed necessarily contained the RN because the RN is a very small region of the brainstem and was technically difficult to separate visually. Therefore, anti-serotonin staining, which was performed in this study for a more reliable examination of DRN serotonin activity, revealed that the number of stained neurons was increased in the ACE group. This suggests that ACE treatment increased serotonin activity in the DRN. Further, in the ACE group, there was a significant decrease in total

distance traveled in the EZM; as activation of the serotonergic pathway from the DRN to the inferior colliculus has been reported to cause freezing (da Silva Soares et al., 2019), the results of this behavioral test may reflect freezing rather than a decrease in activity. Further studies on effects on the serotonergic system, as well as dose-response and sex differences, will provide more detailed information on the mechanisms by which ACE affects behavior.

5. Conclusions

In conclusion, ACE induced anxiety-like behavior 30 min after oral administration, and the results suggest that hippocampal serotonin and striatal dopamine play an important role in this behavioral change. Specifically, it is hypothesized that activation of serotonergic neurons in the DRN increases serotonin levels in projection regions, such as the hippocampus, which in turn modulates dopamine concentrations.

This study is the first to examine the effects of NNs on behavior using EZM and, to my knowledge, this is the first paper to evaluate these correlations, although the effects of NNs on MA or behavior are beginning to be investigated. Little attention has been paid to the effects of NNs on the serotonergic system in mammals, but further studies on the serotonergic system will be helpful in elucidating the mechanism of NNs-induced anxiety-like behavior.

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CRediT authorship contribution statement

Kei Nomiyama: Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Akifumi Eguchi:** Writing – review & editing, Software, Methodology, Formal analysis, Conceptualization. **Mai Tamba:** Writing – review & editing, Methodology, Conceptualization. **Yared Beyene Yohannes:** Writing – review & editing, Methodology, Conceptualization. **Nimako Collins:** Writing – review & editing, Methodology, Conceptualization. **Yoshinori Ikenaka:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Anri Hirai:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chitoku Toda:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Shouta M.M. Nakayama:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Mayumi Ishizuka:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Nobuhiko Hoshi:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Tetsushi Hirano:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

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.

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ARRIVE Statement

In this study, all animal experiments were conducted in accordance with experimental designs approved by the Experimental Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval date: 17th March 2022, approval number: 22–0024). This committee has been fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International since 2007. It is ensured that all approved animal experiments comply with the Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2024.153839.

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