Effects of low concentration of fluoride exposure during fetal on behavior and neurotransmitters in adult mice

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Abstract. Fluoride (F) naturally occurs in water in China and India, and in excess, can cause skeletal fluorosis and mottled teeth. Chronic exposure to F during gestation can affect the development of the brain, reducing intelligence quotient and inducing autism spectrum disorder-like behavior. In the present study, it was aimed to clarify the effects of chronic exposure to low concentrations of F *in utero* on brain function. The behavior was assessed, the levels of brain neurotransmitters were measured in mice and their relationships were analyzed. ICR mice consumed water containing sodium fluoride (F concentrations: 0, 15, or 30 ppm) from 3 weeks of age until the weaning of their pups (F1). The pups then consumed water containing the same concentration of F as their parents from weaning. At 8-weeks old, the F1 mice underwent behavioral testing using the Y-maze, elevated plus maze, Barnes maze (BM) and open-field test (OFT). At 10 weeks of age, they were euthanized, their brains were collected, and the levels of neurotransmitters were measured. Grooming events in the OFT were more frequent in F-exposed groups than in the control group, indicating that F exposure causes anxiety-like behavior. In the BM, the time taken to reach the escape box and the number of errors were higher during the training and test, suggesting spatial memory impairment. Cerebellar glutamate (Glu) concentrations were significantly lower in the F-exposed groups than in the control group. Low Glu concentration was associated with greater grooming frequency in the OFT, lower mean speed and more errors in the BM, and a delay in reaching the escape box. In the F-exposed groups, the midbrain noradrenaline concentrations were significantly lower and the number of errors in the BM was larger than in controls. Thus, F-exposed mice showed poorer spatial memory and differences in the levels of neurotransmitter, suggesting that F is an environmental contributor to disease.

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Introduction

Fluoride (F) is an element that is naturally present in the environment, and World Health Organization (2017) guidelines state that the allowable concentration of F in drinking water is 1.5 ppm. However, in some areas, such as India, where groundwater containing high concentrations of F is supplied as drinking water, concentrations can exceed 48 ppm (1), and this is associated with widespread skeletal fluorosis and mottling of the teeth, which are serious public health problems. In addition, 25 countries fluoridate their tap water to prevent tooth decay (GOV.UK, 2022). In recent years, there has been concern that F exposure during pregnancy in countries where people consume fluoridated tap water or groundwater with high F concentrations may reduce the intelligence quotients (IQs) of children, and increase their risks of memory and learning disorders, attention deficit hyperactivity disorder and autism spectrum disorder (ASD) (2-4).

In general, it is considered that both environmental and genetic factors are involved in the etiology of developmental abnormalities (5), and if F exposure causes brain dysfunction, it is possible that it is a significant environmental factor. In some European Union member states, the fluoridation of tap water was banned between the 1970s and 1990s, and it has been reported that the incidence of ASDs is low in these countries (2,6). Worldwide, ~240 million children have developmental disorders (7). In particular, the prevalence of ASD is ~1/100, and this has rapidly increased over the past 20 years, such that these disorders have become a serious problem (WHO, 2023).

In an epidemiologic study of pregnant women living in areas of Canada with fluoridated or non-fluoridated drinking water, the IQ scores of boys were found to decrease as their urinary F concentrations increased (3). In a study of Indian adolescents who were consuming well water with F concentrations of 5-10 ppm, their IQ scores, attention, concentration, verbal memory and spatial memory were found to decrease

with increasing F concentration (8). Most of the F absorbed into the body accumulates in the bones and teeth (1,9), but there is concern that trace amounts may cross the blood-brain barrier and accumulate in brain tissue, where it is neurotoxic (10). Furthermore, it has been suggested that when women are exposed to F during pregnancy, the concentrations of F in the placenta, plasma/serum and umbilical cord blood increase in direct proportion to its consumption (11,12). The placenta not only transports nutrients and gases, but also contains neurotransmitters such as serotonin, dopamine and norepinephrine/epinephrine, and there is concern that exposure to various risk factors may affect fetal brain development and be involved in the development of ASD (13). If F crosses the blood-brain barrier, it accumulates in brain tissue, and although it is not involved in the synthesis of the neurotransmitter, it can impair their synthesis. As a result, brain development may be impaired, and subsequent behavior may be affected (14). Water fluoridation has been discussed worldwide, and although no definitive conclusions have been reached, F has been suggested to be a neurotoxin (15,16). Previous studies of experimental animals have shown nerve damage, neurodegeneration, a lack of muscle coordination, chronic fatigue, attention deficits and memory impairments caused by the accumulation of F in brain regions associated with long-term exposure to high concentrations (50-100 ppm) of F (17-21).

In the present study, the effects of F on brain function were evaluated, including the characteristics of ASD, by chronically exposing mice to relatively low concentrations of F during pregnancy and subsequently, and the relationships between the results of behavioral testing and the levels of brain neurotransmitters were evaluated.

Materials and methods

Animals and treatment. Male and female 21-day-old ICR mice (F0) were purchased from Sankyo Laboratory Services, Inc. They were housed in an air-conditioned room under a 12/12-h light/dark cycle (lights off at 20:00; rearing temperature at 24˚C), with males and females under standard rearing conditions, three per cage. Information on the F0 mice is shown in Table I. The purchased mice were randomly allocated to a control group consuming tap water and sodium fluoride (Nacalai Tesque, Inc.) exposure groups consuming 15 mg F ions/l (15 ppm) or 30 mg F ions/l (30 ppm). The sodium fluoride was dissolved in ultrapure water (Organo Corp.). Female and male F0 mice were exposed to the sodium fluoride-containing drinking water from 3 weeks of age. F0 mice were mated at 8 weeks-old and continued to consume tap water or water containing sodium fluoride during pregnancy and until weaning on postnatal day 21. After weaning, the pups (F1 mice) were exposed to the same concentrations of F as F0 through their drinking water. In the present study, behavioral testing and brain analysis were performed exclusively on male F1 offspring. Only male F1 mice were included in the analysis because the prevalence of ASD in males is higher than in females (22). Behavioral tests began at 8 weeks of age to assess neurodevelopmental effects and concluded by 10 weeks. After testing, the F1 mice were euthanized, and their brains were collected. Neurotransmitter concentrations in the cerebellum, midbrain and hippocampus were measured using liquid chromatography-tandem mass spectrometry (LC/MS/MS). Exposure to sodium fluoride continued for the F1 mice until euthanasia. This choice is in line with the research content, which focuses on the neurodevelopmental consequences associated with ASD. The experimental protocol was approved by the Juntendo University Center for Biomedical Resources (approval no. 1231; Tokyo, Japan). The experimental procedure, including sodium fluoride exposure, behavioral testing, and euthanasia/brain collection, for the F0 and F1 mice is shown in Fig. 1.

Behavioral testing for developmental disorders. Behavioral characteristics of developmental disorders were evaluated in 8-week-old male mice (n=10 for each of the control, 15 ppm F, and 30 ppm F groups) using the four behavioral tests described below. These behaviors were automatically recorded using a CCD camera and DVTrack Video Tracking System (Compact VAS/DV; Muromachi Kikai Co., Ltd.). In addition, behaviors in the Barnes maze (BM) were assessed visually after recording. To avoid interference between tests, each set of apparatus was washed with 70% ethyl alcohol after each test.

Y-maze. Voluntary behavior was evaluated using a Y-maze (Muromachi Kikai Co). The Y-maze apparatus was made of gray polyvinyl chloride and consisted of three arms, with 120˚ angles between adjacent arms. Each arm was 4x41.5x10 cm [width (W) x length (L) x height (H)] in size. The spontaneous alternation rate and spontaneous locomotor activity (locomotor activity) were measured over 8 min.

Elevated plus maze (EPM). Anxiety-like behavior was evaluated using an EPM (Panlab, S.L., Harvard Apparatus). The apparatus was made of methacrylic resin and aluminum, with black floors and gray walls. The EPM was placed 45 cm above the floor and consisted of open and closed arms, each of size 6x29.5 cm [W x depth], with an H of 15 cm for the open arm. The number of entries into the open arm and the time spent in the open arm were measured over 8 min.

BM. Hippocampus-dependent spatial learning and memory were evaluated using a BM (Panlab, S.L.). The BM consisted of boxes, a white circular platform (92 cm in diameter) placed 90 cm above the ground, with 20 equally-spaced holes of 5 cm in diameter through which fake target boxes and a single black escape box could be accessed. The latter served as the goal throughout the training period and memory test. The training consisted of five trials of 1 min per day for 5 consecutive days, during which the time taken to reach the escape box (latency) and the number of errors made were measured. On the sixth day, a 1-min test was performed to evaluate memory retention.

Open field test (OFT). Exploratory and anxiety-like behaviors were evaluated using an OFT (Muromachi Kikai Co.). It was made of gray polyvinyl chloride, was 50x50 cm in size, and had walls 40 cm high. The distance traveled (cm), the traveling time (sec), the time spent in the central zone (s), the mean speed (cm/sec), and the grooming frequency were measured over 15 min. Several visible objects were placed in plain sight of the mouse to cue spatial learning.

Table I. Information of F0 mice (weight, food, water, and number of births). Data represents the mean ± SEM (n=3) for body weight, food, and water intake, and the number of births represents the sum of each group.

	Body weight of 21day-old	Daily Food intake per animal	Daily water intake per animal
Male			
Control	20.1±0.2	3.1±0.3	4.4±1.2
15 ppm	20.2±0.1	3.3±0.3	3.9±1.5
30 ppm	20.1±0.1	3.3±0.2	4.1±1.8
Female			
Control	20.3±0.1	3.2±0.2	4.5±1.1
15 ppm	20.1±0.2	3.1±0.4	4.4±0.7
30 ppm	20.2±0.2	3.0±0.3	4.0±1.3
Number of births			
	Male	Female	
Control	11	12	
15 ppm	11	17	
30 ppm	18	15	

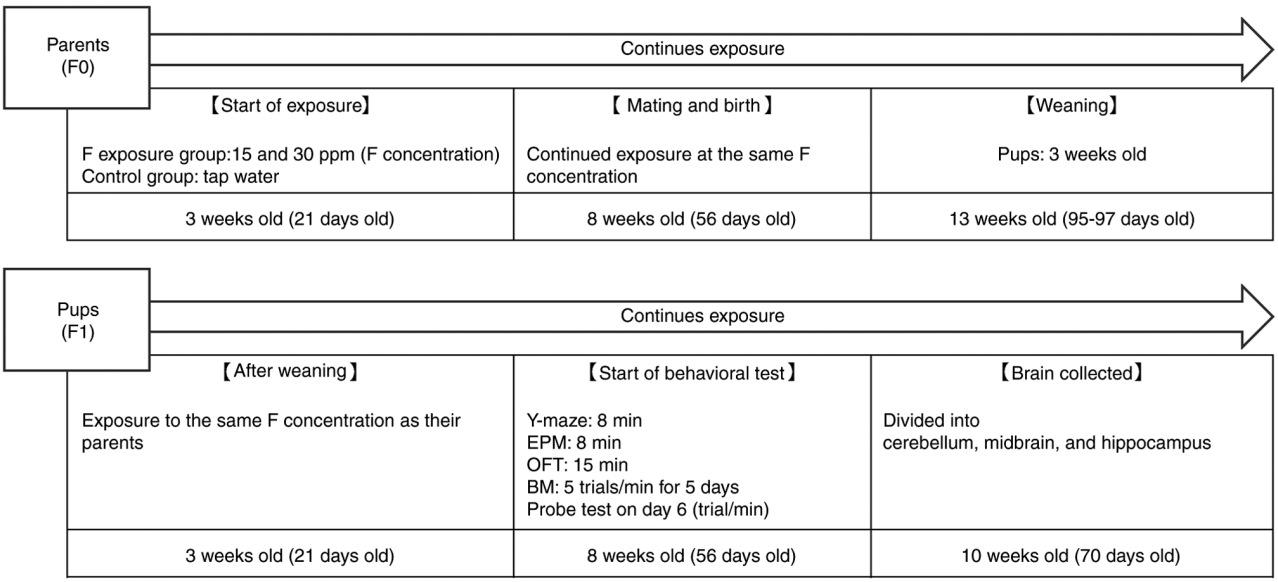

Figure 1. Prenatal sodium fluoride exposure and effects on brain function. The figure shows chronic sodium fluoride exposure.

Measurement of levels of neurotransmitters. At the end of the behavioral study (at 10 weeks of age), male F1 mice were euthanized by cervical dislocation, and their brains were collected. The brains were then divided into the cerebellum, midbrain, and hippocampus and stored at -80˚C. The cerebellum, midbrain, and hippocampus masses were measured, then these components were homogenized in a 10-mM hydrochloric acid solution and diluted to a final concentration of 0.25 g/ml and centrifuged at 10,000 x g for 5 min, then the supernatants were collected. After pretreatment, the cerebellum, midbrain and hippocampus were analyzed for their glycine (Gly; pmol/g), gamma-aminobutyric acid (GABA; pmol/g), glutamic acid (Glu; pmol/g), tryptophan (Trp; nmol/g), tyrosine (Tyr; nmol/g), homovanillic acid (HVA; pmol/g), 5-hydroxytryptamine (5-HT; pmol/g), 3,4-dihydroxyphenylacetic acid (DOPAC; pmol/g), dopamine (DA; pmol/g) and noradrenaline (NA; pmol/g) contents using liquid chromatography-mass spectrometry (LC-MS/MS). LC-MS/MS analyses were conducted using an LCMS-8045 mass spectrometer equipped with a Nexera UHPLC and a SIL-30AC autosampler (Shimadzu Corporation). Chromatographic separation was achieved using a SunShell C18 column (2.1x150 mm, 2.6-μm particles; ChromaNik Technologies, Inc.) fitted with a SecurityGuard Ultra C18 (2.1x2 mm, 2-μm particles; Phenomenex; https://www.phenomenex.com/) guard column. The mobile phase consisted of 10 mM ammonium formate buffer (pH 3.6) and acetonitrile. The total assay time was 25 min and the following multistep gradient was used: 0-5 min, 15-80% B (linear gradient); 5-10 min, 80-95% B (linear gradient); 10-20 min, 95% B (isocratic); 20-20.1 min, 95-15% B (linear gradient); 20.1-25 min, 15% B (isocratic). The mobile phase flow rate was 0.2 ml/min, the column temperature was 40˚C,

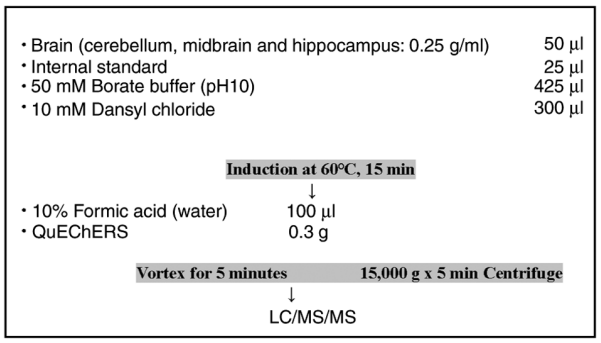

Figure 2. Sample preparation. This shows the flow of analysis of neurotransmitters in the cerebellum, midbrain, and hippocampus. LC-MS/MS, liquid chromatography-mass spectrometry.

and the injection volume was 5 µl. Electrospray ionization was employed in both positive and negative ion modes, and selective reaction monitoring was used for analyte quantification. The interface voltage was set at 4.0 kV. Drying and nebulizing gases were supplied at flow rates of 10.0 and 3.0 l/min, respectively. The temperature was set to 250˚C, and the heat block temperature was set at 400˚C (23) (Fig. 2).

Statistical analysis. The Shapiro-Wilk test was used to assess the normality of each dataset. For normally distributed continuous data, one-way analysis of variance (ANOVA) was used to compare the mean values of groups, and if a significant difference was detected, the Tukey-Kramer *post hoc* test was used to identify specific differences. For variables that were not normally distributed, the Kruskal-Wallis test was used to compare the three groups, and following the identification of a significant result, pairwise comparisons were conducted using the Dunn-Bonferroni *post hoc* test. The significance level (α) was set at 0.05, and P-values for the *post hoc* tests were adjusted using the Bonferroni correction to account for multiple comparisons. Spearman's rank correlation coefficients were calculated to assess the relationships between behavioral test results and the levels of neurotransmitters. No prior sample size calculation was performed, but a *post hoc* power analysis was conducted to evaluate the ability of the analysis to detect differences in the mean values obtained for each behavioral test of groups of 10 mice. This analysis indicated a statistical power of $1-\beta=0.22$, assuming an effect size of $f=0.30$ and a significance level of $\alpha=0.05$. All statistical analyses were conducted using SPSS version 29 (IBM Corp.).

Results

Effects of prenatal sodium fluoride exposure on body mass. Data confirmed normal distribution ($P<0.05$). At weaning (3 weeks old), the body masses were 21.7±0.8 g for the control group (n=10), 20.9±0.6 for the 15-ppm group (n=10), and 15.5±0.8 for the 30-ppm group (n=10). That of the 30-ppm group was significantly lower than those of the control and 15-ppm groups ($P<0.001$). Before euthanasia (10 weeks old), the body masses of the mice were as follows: control group, 40.8±0.8 g; 15-ppm group, 42.8±0.4 g; and 30-ppm group, 41.2±2.8 g; with no significant differences.

Effects of prenatal sodium fluoride exposure on developmental disease-like behavior. Data confirmed normal distribution ($P<0.05$). The Y-maze, EPM and OFT results are shown in Fig. 3A-I. There were no significant differences between the F-exposure and control groups in the Y-maze (Fig. 3A and B) or in the EPM (Fig. 3C and D). In the OFT, there were significantly higher grooming frequencies in the 15 and 30-ppm groups than in the control group (Fig. 3I). However, there were no significant differences in the other parameters associated with the OFT (Fig. 3E-H). The results of the BM analysis are shown in Fig. 4A-D. During the 5-day training period, the 15 and 30-ppm groups showed significantly longer latencies and more errors than the control group (Fig. 4A and B). In the test, the 15 and 30-ppm groups also exhibited significantly longer latencies and more errors (Fig. 4C and D).

Effects of fetal sodium fluoride exposure on subsequent neurotransmitters. Levels of neurotransmitters have been measured as indicators of developmental disorders; therefore, neurotransmitters were also measured (Table II). The levels of Gly, Glu, Trp and Tyr in the cerebellum; Gly, HVA, NA and DA in the midbrain; and Gly and HVA in the hippocampus showed a normal distribution ($P<0.05$). Therefore, ANOVA was performed, followed by the Tukey-Kramer test as a post hoc analysis. On the other hand, the Kruskal-Wallis test was used for GABA, HVA, 5-HT and NA in the cerebellum; GABA, Glu, Trp, Tyr, Dopac, 5-HT and NA in the midbrain' GABA, Glu, Trp, Tyr, Dopac, 5-HT and NA in the hippocampus; and DA, as normality was not observed ($P>0.05$). For post hoc analysis, the Dunn-Bonferroni test was performed to determine significant differences between groups whereas the Kruskal-Wallis test yielded statistically significant results. The cerebellar Glu was significantly lower in the 15 and 30 ppm groups compared with the control group ($P<0.01$).

Correlations between behavioral test outcomes and levels of brain neurotransmitters. Although the mechanisms of ASD are not fully understood, human studies have reported decrease in the neurotransmitters 5-HT, DA (24), Glu and GABA (25). In addition, it has been reported that the effects of sodium fluoride exposure on the behavior of experimental animals and humans include a decrease in cognitive function (26,27), anxiety-like and depressive behavior (28). If ASD-like behavior is being observed, it was considered that it might be related to neurotransmitters; thus the relationship between the two was calculated. The characteristics of ASD include delayed social interaction, communication and repetitive behaviors. In addition, it has been reported that the levels of the following neurotransmitters in the brain are decreased with ASD: 5-HT (abnormal control of emotions and social behavior), Glu (excitatory neurotransmitter) (24), GABA (inhibitory neurotransmitter) and DA (abnormal reward processing) (25). If F-exposure is a factor in the development of ASD, it was considered that there may be abnormalities in neurotransmitters related to behavior, therefore both behavior and neurotransmitters were analyzed. Therefore, the effects of sodium fluoride exposure on behavior and neurotransmitters were investigated using experimental animals during the fetal period. As a result, it was found that exposure to sodium fluoride affects behavior tests and neurotransmitters in the brain. Neurotransmitters

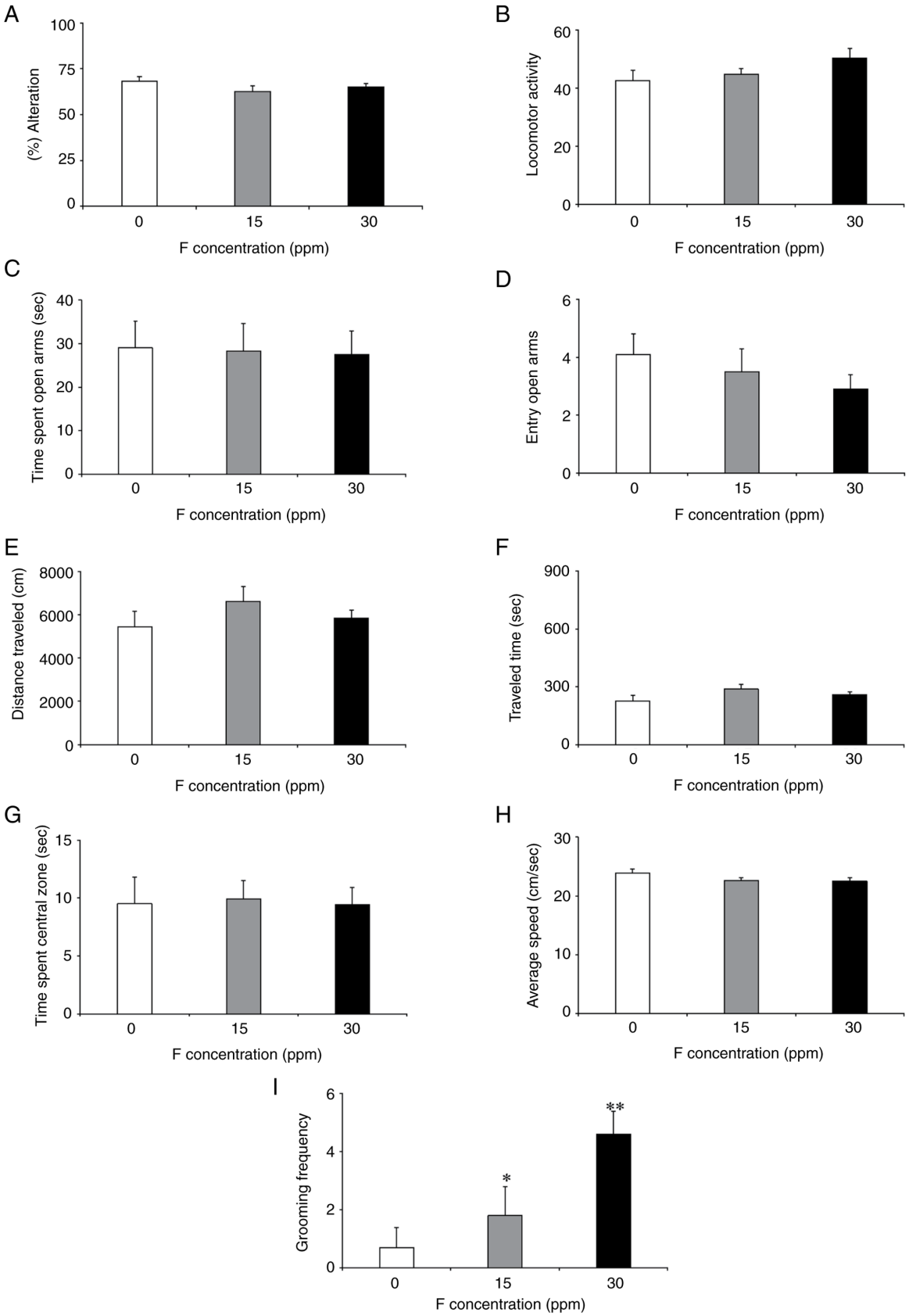

Figure 3. Effects of fetal period sodium fluoride exposure on behavior test. (A-H) Data represents the mean ± SEM (n=10). An ANOVA was conducted, and post hoc comparisons were performed using the Tukey-Kramer test. (I) In the open-field test, there was a significant increase in the grooming frequency in the 15 and 30 ppm groups compared with the control group ($P<0.05$ and $P<0.01$, respectively. $^{*}P<0.05$ and $^{**}P<0.01$.

are chemicals that transmit information in the brain, and are therefore involved in a variety of behaviors. The identification of the effects of sodium fluoride exposure on behavior (for example, memory, learning, anxiety-like behavior) and levels of

Table II. Effects of fetal period exposure to sodium fluoride on neurotransmitters.

	Gly	GABA	Glu	Trp	Tyr	HVA	DOPAC	5-HT	DA	NA
Cerebellum (mean ± SEM)										
0 ppm	1.1±0.1	2.0±0.5	3.9±0.8	27.3±4.5	132.7±18.5	89.0±28.7	14.2±3.1	111.6±58.4	Not detected	130.5±19.6
15 ppm	1.4±0.2	2.7±0.6	1.4±0.2[a]	34.1±7.5	155.0±28.9	94.5±28.5	15.8±2.0	78.7±60.8		94.1±6.8
30 ppm	1.3±0.3	2.5±0.7	1.3±0.3[a]	40.1±9.3	155.2±30.5	93.1±26.9	11.2±2.8	17.7±12		104.9±6.2
Midbrain (mean ± SEM)										
0 ppm	1.6±0.2	2.4±0.5	2.1±0.6	37.3±6.9	164.9±26.8	278.5±69.2	384.3±72.9	214.8±39.6	21.4±5.2	326.1±52.6
15 ppm	2±0.3	4.1±0.9	2.6±1	53.1±12.8	222.9±46	352.0±81	304.3±62.2	87.5±69.6	22.7±6.7	154.6±15.5
30 ppm	1.9±0.3	3.4±0.8	2.9±0.7	49.1±13.1	202.8±48.2	305.8±54.2	318.2±62.2	200.2±42.3	14.1±2.4	235.8±42.7
Hippocampal (mean ± SEM)										
0 ppm	1.1±0.1	2.4±0.4	4.4±1	28.7±3.9	157.7±17.1	211.2±36.8	314.3±124.5	285.4±37.9	39.4±14.7	269.7±49.2
15 ppm	1.3±0.1	3.2±0.6	5.9±1.8	34.1±5.2	175.0±23.4	264.7±58.8	198.2±35.9	225.9±44.5	13.9±5.4	171.4±19.5
30 ppm	1.2±0.2	2.9±0.8	6.8±1.7	30.8±6.4	153.2±24.9	206.4±41.7	206.4±55.7	232.5±29.1	24.8±6.5	257.8±17.8

Data represents the mean ± SEM (n=10). The levels of Gly, Glu, Trp and Tyr in the cerebellum; Gly, HVA, NA and DA in the midbrain; and Gly and HVA in the hippocampus showed a normal distribution ($P<0.05$). Therefore, an ANOVA was performed, followed by the Tukey-Kramer test as a post hoc analysis. On the other hand, the Kruskal-Wallis test was used for GABA, HVA, 5-HT and NA in the cerebellum; GABA, Glu, Trp, Tyr, Dopac, 5-HT and NA in the midbrain, GABA, Glu, Trp, Tyr, DOPAC, 5-HT and NA in the hippocampus, and DA, as normality was not observed ($P>0.05$). For post hoc analysis, the Dunn-Bonferroni test was performed to determine significant differences between groups whereas the Kruskal-Wallis test yielded statistically significant results. Glu concentrations in the cerebellum were significantly lower in the sodium fluoride exposed group than in the control group (vs. 15 and 30 ppm; $P<0.01$). [a]$P<0.01$. SEM, standard error of the mean; HVA, homovanillic acid; NA, noradrenaline; DA, dopamine; GABA, gamma-aminobutyric acid; 5-HT, 5-hydroxytryptamine.

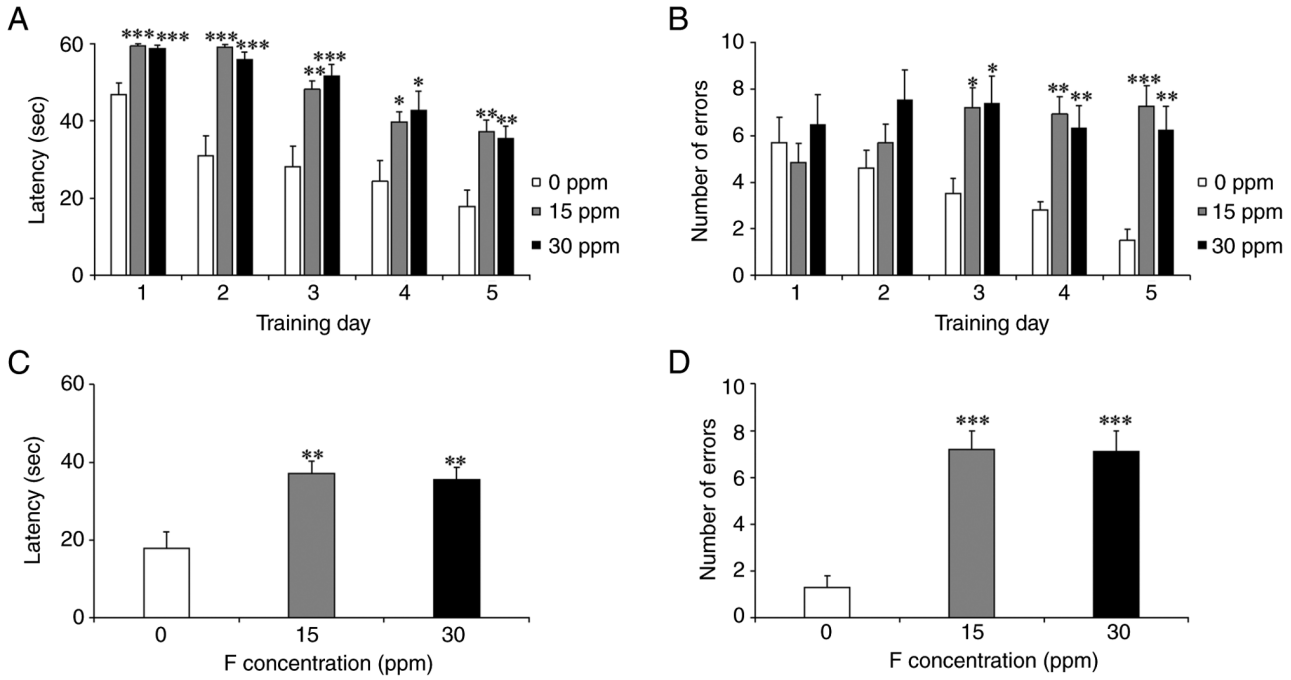

Figure 4. Effect of sodium fluoride exposure on developmental disease-like behavior. The number of errors and latency in the 5-day training and probe test in the Barnes maze were compared between the control and fetal period sodium fluoride exposure groups. Data represents the mean ± SEM (n=10). An ANOVA was conducted, and post hoc comparisons were performed using the Tukey-Kramer test. (A and B) During the 5 days of training, the 15 and 30 ppm groups required significantly longer time to latency compared with the control group (0 ppm vs. 15 ppm: D1; P<0.001, D2; P<0.001, D3; P<0.01, D4; P<0.05, D5; P<0.01, 0 ppm vs. 30 ppm: D1; P<0.001, D2; P<0.001, D3; P<0.001, D4; P<0.05, D5; P<0.01, and significantly more errors (0 ppm vs. 15 ppm: D3; P<0.05, D4; P<0.001, D5; P<0.001, 0 ppm vs. 30 ppm: D3; P<0.05, D4; P<0.01, D5; P<0.001. (C and D) In the probe test, both the 15 and 30 ppm groups had significantly longer latencies (0 ppm vs. 15 ppm; P<0.01, 0 ppm vs. 30 ppm; P<0.01) and more errors than the control group (0 ppm vs. 15 ppm; P<0.001, 0 ppm vs. 30 ppm; P<0. 001. *P<0.05, **P<0.01 and ***P<0.001.

neurotransmitters provoked us to perform correlation analysis to determine the extent to which these parameters were related. The correlations between the levels of cerebellar neurotransmitters and behavioral test outcomes are demonstrated in Fig. 5. There was a negative correlation between grooming frequency in the OFT and the Glu concentration (Fig. 5A). There were also positive correlations between the mean speed and the Glu concentration (Fig. 5B); the distance traveled (Fig. 5C) and the traveling time (Fig. 5D) during the OFT with the DOPAC concentration. For the BM, there were significant negative correlations of the number of errors (Fig. 5E) and the latency (Fig. 5F) with the Glu concentration. The correlations between the levels of neurotransmitters in the midbrain and the behavioral test outcomes are shown in Fig. 6. In the OFT, there were significant positive correlations of mean speed with the NA (Fig. 6A) and DOPAC (Fig. 6B) concentrations. In the BM, there was a negative correlation between the number of errors and the NA concentration (Fig. 6C). The correlations between the levels of hippocampal neurotransmitters and the behavioral test outcomes are shown in Fig. 7. In the Y-maze, there was a positive correlation between the 5-HT concentration and locomotor activity (Fig. 7A). In addition, in the OFT, there was a positive correlation between the NA concentration and average speed (Fig. 7B).

Discussion

At present, there is particular concern about the possibility of damage to the central nervous system being caused by excitotoxicity resulting from exposure to F (29). However, the mechanisms underlying the relationship between exposure to F and brain dysfunction remain unclear. In the present study, it was aimed to determine whether exposure to sodium fluoride *in utero* causes subsequent brain dysfunction, such as ASD. To this end, an experiment was conducted in mice to characterize the relationships between exposure to sodium fluoride *in utero* and subsequently, the behavior of the mice in adulthood, and the neurotransmitters that determine such behavior. Behavioral testing is an important means of evaluating neurobiological function, but these tests are known to be susceptible to individual differences, environmental factors and stress, and therefore can yield variable results (30). For this reason, it can be difficult to accurately assess neurophysiologic changes and the underlying molecular mechanisms using behavioral testing alone. The use of a combination of behavioral testing and neurotransmitter measurements makes it possible to identify the neurobiological mechanisms underlying the observed behavior and correct for the variability in behavioral test outcomes (31,32). This combination provides multilayered information that cannot be obtained from a single behavioral test and improves the reproducibility of findings (33). The main causes of brain dysfunction, including ASD, are considered to involve complex interactions of genetic and environmental factors (34,35). The characteristics of ASD include delayed social interaction, communication and repetitive behaviors (36). In addition, it has been reported that the levels of the following neurotransmitters in the brain are decreased with ASD: 5-HT (abnormal control of emotions and social

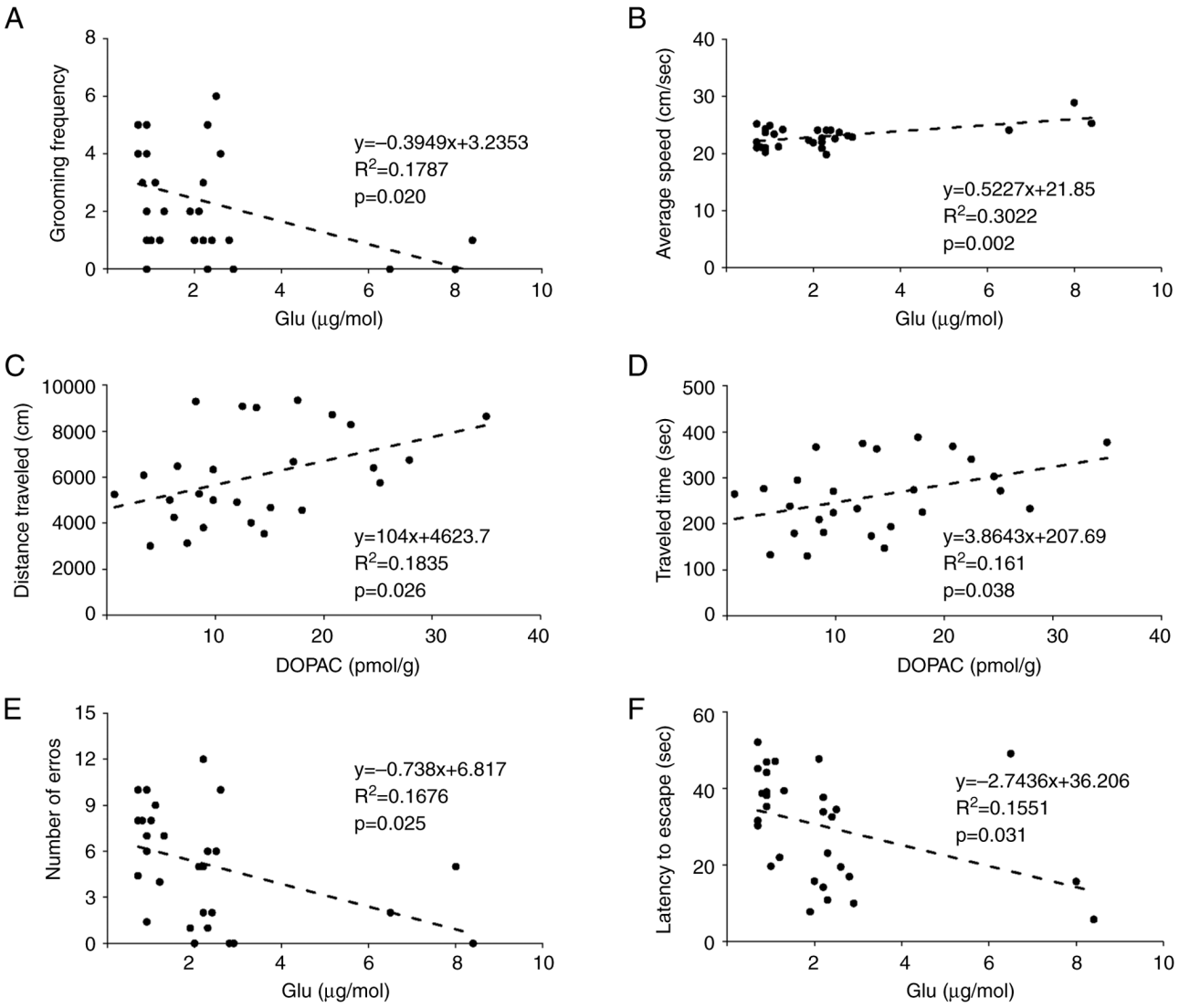

Figure 5. Correlation between neurotransmitters in the cerebellum of mice exposed to sodium fluoride during the fetal period and behavioral tests. (A) The OFT showed a negative correlation between grooming frequency and Glu (r=-0.423; P=0.02). (B) A positive correlation was found between average speed and Glu (r=0.55; P=0.002). (C and D) There was also a positive correlation between (C) distance traveled and (D) traveled time in correlation to DOPAC in the OFT (r=0.428; P=0.026 and r=0.378; P=0.038, respectively). (E and F) In the Barnes maze, there was a significantly negative correlation between the (E) number of errors and (F) latency in correlation to Glu (r=-0.409; P=0.025 and r=-0.394; P=0.031, respectively). OFT, open-field test; DOPAC, 3,4-dihydroxyphenylacetic acid.

behavior), Glu (excitatory neurotransmitter), GABA (inhibitory neurotransmitter) and DA (abnormal reward processing) (24). If F exposure is a factor in the development of ASD, then there may be abnormalities in the neurotransmitters that are related to behavior. It was found that low Glu concentrations in the cerebella of the mice were associated with more anxiety-like behavior, less locomotor activity and poorer cognitive function (Fig. 5A, B, E and F). The cerebellum has neural circuits that connect to the amygdala and prefrontal cortex, and it is considered that it affects emotion and anxiety through interactions with these regions (37,38). Glu is an excitatory neurotransmitter and mediates signal transmission to the deep cerebellar nuclei. The low Glu concentration may have reduced the transmission of information from the cerebellum to the prefrontal cortex and amygdala, thereby affecting emotion, cognitive function and locomotor activity (39,40). In addition, Glu contributes to synaptic plasticity, such as long-term potentiation and long-term depression, and it has been reported that a low Glu concentration inhibits these processes, leading to poorer learning and adaptive behavior (41,42). In ASD and schizophrenia, abnormalities in the cerebellum have been reported to cause anxiety and emotional instability (43). The results of the present and previous studies suggest that the cerebellum plays important roles, not only in locomotor activity, but also in the control of anxiety and emotion. In addition, it was found that the lower the DOPAC concentration in the cerebellum is, the more impaired the locomotor activity is (Fig. 5C and D). However, further investigation is needed regarding the lower locomotor activity associated with a low DOPAC concentration, because there have been no studies to date regarding low DOPAC concentrations in the cerebellum.

It was found that low concentrations of NE and DOPAC in the midbrain were associated with poorer motor function (Fig. 6A and B). It has previously been reported that low NE and

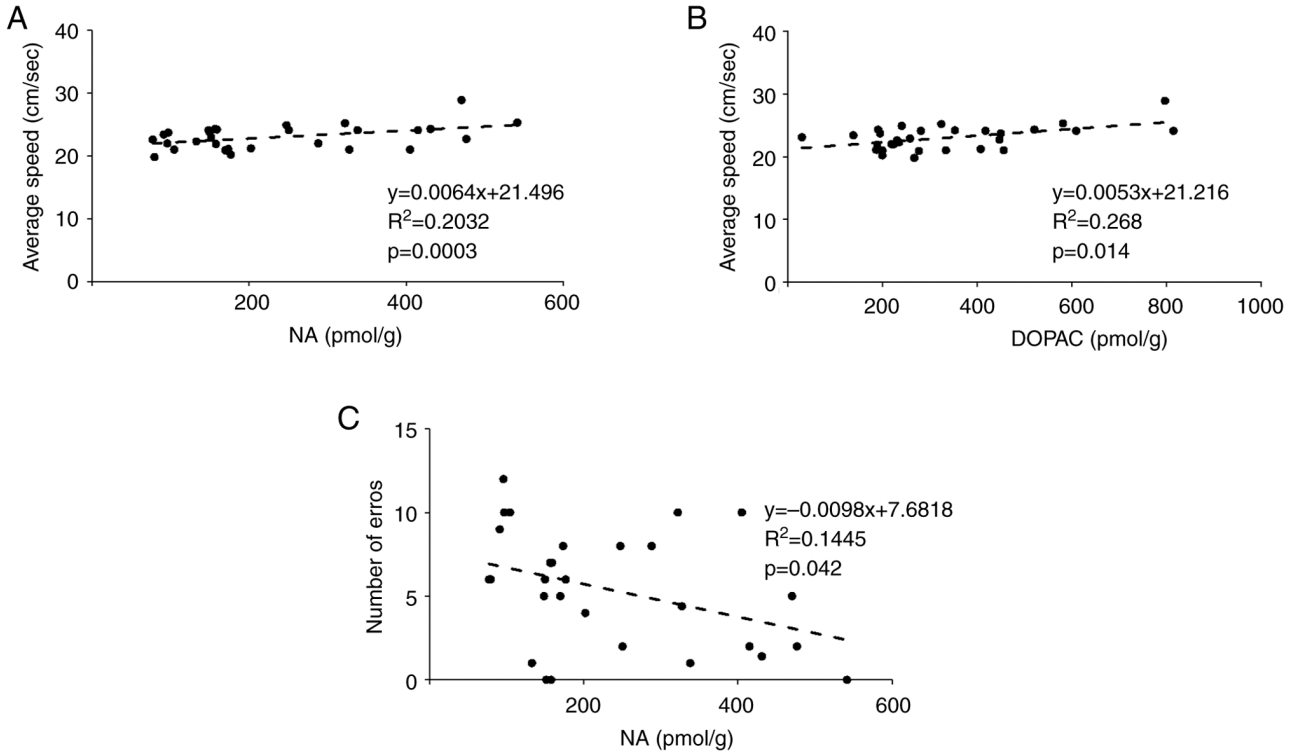

Figure 6. Correlation between neurotransmitters in the midbrain of mice exposed to sodium fluoride during the fetal period and behavioral tests. (A and B) The open-field test showed a significantly positive correlation between average speed and (A) DOPAC and (B) NA (r=0.518; P=0.014 and r=0.451; P=0.003, respectively). (C) The Barnes maze showed a negative correlation between the number of errors and NA (r=-0.3820; P=0.042). DOPAC, 3,4-dihydroxyphenylacetic acid; NA, noradrenaline.

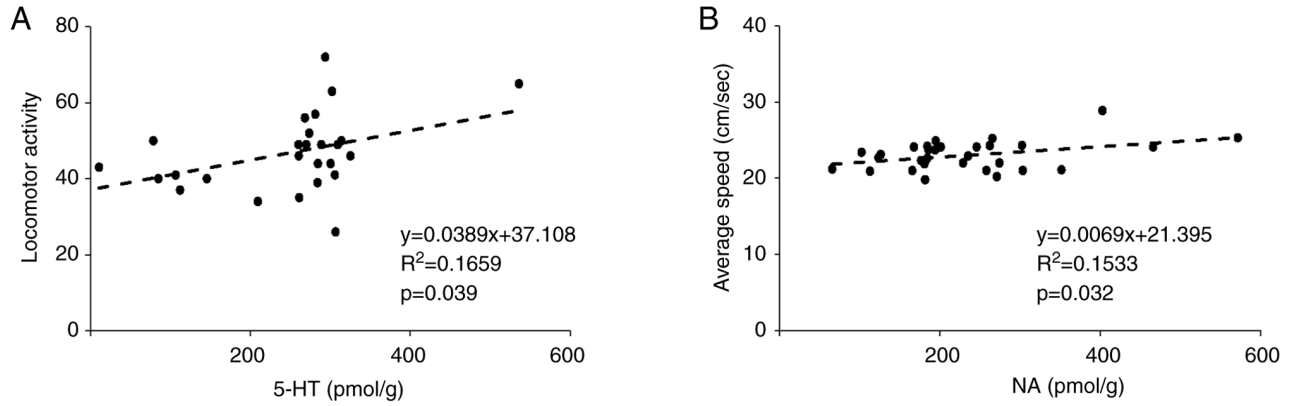

Figure 7. Correlation between neurotransmitters in the hippocampus of mice exposed to sodium fluoride during the fetal period and behavioral tests. (A) In the Y maze, there was a positive correlation between 5-HT and locomotor activity (r=0.407; P=0.039). (B) The open-field test had a positive correlation between NA and average speed (r=0.392; P=0.032). 5-HT, 5-hydroxytryptamine; NA, noradrenaline.

DA concentrations in the midbrain, as well as low concentrations of their metabolites, such as DOPAC, are associated with poorer locomotor activity (44). Furthermore, low NA concentrations in the midbrain have been shown to be associated with a larger number of errors in the BM (Fig. 6C). Furthermore, low NA concentrations in the midbrain, and particularly in the locus coeruleus (LC), are associated with impaired memory and cognitive function (45). The LC is the main source of norepinephrine in the brain and plays an important role in the regulation of various cognitive processes (46,47). In the present study, the correlations obtained between locomotor activity and levels of neurotransmitter concentrations suggested that poor locomotor activity may be the result of low concentrations of 5-HT and NA (Fig. 7A and B). This suggests that the concentrations of 5-HT and NA, which are involved in emotion and cognitive function, may also contribute to motor control. The effects of 5-HT on emotion and cognitive function have been widely reported, and it is considered that these may also affect locomotor activity (48,49). NA has been reported to promote neurogenesis and synaptic plasticity in the hippocampus (50,51), and there have also been reports that hippocampal neurogenesis is related to motor learning (52). Thus, the present results suggest that low hippocampal concentrations of 5-HT and NA may indirectly affect locomotor

activity *via* cognition and emotion. Correlation analysis of the relationships between the levels of neurotransmitters and behavioral data generated relatively weak correlations (the R^2 values were mostly <0.3). For correlation coefficients <0.3, the statement 'a trend was indicated' is used because it is a weak correlation. Indeed, a single neurotransmitter does not determine behavior, but instead interacts with other neurotransmitters, hormones, environmental factors, learning experiences and genetics to function in this way (53). Individual differences, environmental conditions, technical errors in the measurement of behavioral parameters, and the levels of neurotransmitters may have weakened these correlations (54). In the future, it will be necessary to construct more precise models and evaluate the interactions between neurotransmitters and behavior, considering multiple variables. In addition, the current research results showed that chronic exposure of the fetus to sodium fluoride caused weight loss after weaning. In a study in which pregnant rats were exposed to F, it was reported that F passed through the placental barrier and accumulated in the amniotic fluid and fetal plasma, that the osmotic pressure of the amniotic fluid decreased on day 20 of gestation, and that the rate of delayed fetal development was high in F0 fetuses (55). It has been suggested that F1s after weaning may decrease in weight because of F exposure to fetal development (55). However, the mechanism remains unknown.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MH conceptualized the study, developed methodology, conducted investigation, wrote the original draft and acquired funding. YI conducted investigation, and wrote, reviewed and edited the manuscript. AS and TT wrote, reviewed and edited the manuscript, supervised the study, and confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal experiments were approved (approval no. 1241) by the Juntendo University Center for Biomedical Resources (Tokyo, Japan) and were performed by according to appropriate ethical standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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