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Artificially reared mice exhibit anxiety-like behavior in adulthood

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Abstract: It is important to establish experimental animal techniques that are applicable to the newborn and infant phases for nutrition and pharmacological studies. Breeding technology using the artificial suckling method without breast milk is very effective for the study of newborn nutrition. Using this method, we separated newborn mice from dams within 48 h of birth and provided them with artificial milk. We evaluated mouse anxiety levels after early postnatal maternal separation. Artificially reared mice were subjected to elevated plus-maze tests to assess emotional behavior at 9 weeks of age. Artificially reared mice showed a significantly lower frequency of entries and dipping into the open arms of the maze compared with dam-reared mice. This result indicates that the anxiety level of artificially reared mice was higher than that of dam-reared mice. Moreover, the concentration of monoamines in the brain was determined after the behavioral experiment. The hippocampal norepinephrine, serotonin, and 5-hydroxyindoleacetic acid levels in the artificially reared mice were significantly higher than those of the dam-reared mice. These results suggest that maternal-offspring interactions are extremely important for the emotional development of newborn infants during the lactation period. In future studies, it is necessary to consider the environmental factors and conditions that minimize the influence of artificial rearing on emotional behavior.
Key words: artificial rearing, anxiety-like behavior, elevated plus maze, mouse

Introduction

The toxic and functional effects of various materials or dietary components are generally evaluated in experimental adult animals. Mice are a useful evaluation system for methods to prevent and treat many diseases. However, it is difficult to directly apply the results of adult animal experiments to animals in the lactation period, as it is a formative stage of development, including brain development. Therefore, it is necessary to evaluate materials using newborns. A mouse artificial rearing system is a suitable research model to determine the nutrients required by premature human infants since rodents are born at a more immature embryological stage than humans. Conventional artificial rearing of rodents involves compulsory feeding of nutrients by a tube inserted into the stomach from about 4 days of age [2, 8, 27]. However, the insertion of tubes directly into the stomach has several problems in breeding conditions. The surgery presents a physical burden to newborns and they cannot receive physical contact from dams or other pups. In addition, newborns cannot perform normal

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suckling behavior. To account for these limitations, a new artificial rearing system for rodents within 48 h of birth has been developed [21]. This nursing system is structured such that newborns can suckle artificial milk directly from a nipple, reducing mental and physical stress. However, this method is limited because individual growth is lower due to the lower amount of care lactation times are determined by the voluntary feeding behavior of newborns. Therefore, artificial rearing with hand-feeding has been established to reduce stress during lactation, and the spontaneous feeding volume of artificial milk can be measured for each individual [10]. Additionally, the nutrients in artificial rodent milk have been standardized to purified artificial milk via an analysis of the secreted milk [26], and there are differences in body weight between artificially reared and dam-reared groups [15, 26]. This combination of artificial milk and artificial rearing is useful and has been examined with respect to the immune system, memory, and learning [11, 15, 25]. There were only two reports for the behavioral evaluation of rodents that were weaned by our artificial rearing method [7, 15]. The several researchers have reported that the development of social behavior is inhibited when bond formation between dams and pups fails due to early weaning [1, 14, 17–20]. Therefore, the breeding environment also has a significant effect on brain function after maturation, although neonates need to consume sufficient nutrients for growth and development during the lactation period.

In this study, we evaluated the behavioral differences between dam-reared mice and artificially reared mice based on motor activity and the elevated plus-maze (EPM) test. After the behavioral experiments, the levels of norepinephrine, 5-hydroxytryptamine (5-HT), dopamine, and their metabolites in various brain regions were also measured.

Materials and Methods

Study design

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Azabu University.

Individuals in the dam-reared group (Dam group) were reared in normal conditions by their dam in an artificially regulated environment at 23 ± 3°C, 55 ± 10% humidity, and a 12-h light/dark cycle (lights on between 07:00 and 19:00). Dam-reared pups were thinned out to 8 pups/litter to ensure sufficient growth at 2 days of age. The conditions for the artificially reared (AR) group are described below (see *Artificial rearing system*).

Table 1. Composition of experimental diets[a)]

Component	Amount (g/100 g diet)
Crude protein	23.1
Carbohydrates	55.3
Minerals	5.8
Crude fat	5.1
Dietary fiber	2.8

[a)]The experimental diet was MF, obtained from Oriental Yeast Co., Tokyo, Japan.

When AR mice and dam-reared mice were 9 weeks of age, their spontaneous motor activities and anxiety levels were measured using running wheels and the EPM test, respectively. Their brains were dissected into the frontal cortex, hippocampus, striatum, and hypothalamus. The components were analyzed for serotonin, dopamine, and norepinephrine as monoamines, and metabolite levels were estimated using high-performance liquid chromatography (HPLC) (Fig. 1).

Animals and experimental diets

Pregnant CD-1 mice (Charles River Japan, Inc., Yokohama, Japan) were bred on custom pelleted diets (MF, Oriental Yeast Co., Ltd., Tokyo, Japan, Table 1), and male offspring were used in this study. The mice were maintained on the pelleted diet after weaning.

Artificial mice milk

The artificial milk formula was developed based on the methods of Yajima *et al.* and Hussein *et al.* [11, 26], with slight modifications. Table 2 shows the ingredients as well as their commercial sources. Casein and whey protein were used as protein sources and lactose was used as the carbohydrate. The milk was homogenized two times under high pressure (800–1000 bar) using a high-pressure homogenizer (Panda PLUS 2000; Niro Soavi S.p.A., Parma, Italy), resulting in emulsified, sterilized, and smoothed milk. The homogenized milk was stored at −80°C.

Artificial rearing system

For the artificial rearing procedure, the hand-feeding technique was used with nursing bottles [10]. The artificial rearing system consisted of a custom-made nursing bottle, a small plastic cage, an electronic hot pad, and

Table 2. Composition of artificial milk[b)]

	Ingredient	Amount (weight / 100 ml milk)
Protein (g)	Whey protein isolate	4.0
	Whey protein hydrolyzed	5.0
	Casein	4.0
	Serine	0.02875
	Cystine	0.3
	Tryptophan	0.027
	Methionine	0.0045
Carbohydrate (g)	Lactose	1.89
Fat (g)	MCT	1.25
	Palm oil	8.25
	Coconut oil	2.5
	Corn oil	0.5
	Soybean oil	2.75
	Linseed oil	0.75
	Cholesterol	0.04
Minerals (mg)	NaOH	25
	KOH	150
	$GlyCaPO_4$	800
	$MgCl_2\ 6H_2O$	190
	$CaCl_2\ 2H_2O$	170
	$CaCo_3$	184
	Ca-Citrate	120
	Na_2HPO_4	80
	KH_2PO_4	8
	$FeSO_4$	24
	Citrate H_2O	0.5
	$ZnSO_4$	6
	$CuSO_4$	1.5
	$MnSO_4$	0.25
	NaF	0.155
	KI	0.25
	K_2SO_4	163.5
	$Na_2SiO_3\ 9H_2O$	5.075
	Na_2O_4Se	0.035
	$H_8MoN_2O_4\ 4H_2O$	0.0275
	$CrH_{24}KO_{20}S_2$	0.9625
	Li_2CO_3	0.06
	H_3BO_3	0.285
	$NiCO_3$	0.1125
	NH_4VO_3	0.0225
Vitamins (mg)	Vitamin mix	400
	Vitamin C	200
	Vitamin K3	1.9825
	Vitamin A	0.1284
	Vitamin D	23.46
	Vitamin E	0.0025
Others (mg)	Carnitine	4.0
	Picolinate	2.0
	Ethanolamine	3.5
	Taurine	15.0
	Tricholine Citrate	147.0

[b)]The artificial milk formula was made following the method of Yajima *et al.*

an infant incubator developed for humans (Neo-Servo Incubator V-2100G; Atom Medical Co., Ltd., Tokyo, Japan). The nursing bottles were composed of nipples, a milk inflow tube, a milk overflow tube, and a refill syringe (Fig. 2). The cage was placed in an infant incubator initially set at 33–34°C. The temperature was decreased by 0.5°C/day starting on day 10 until it reached 30°C, and it was maintained at this level until day 14. The infant incubator was then maintained at 26–28°C until weaning (day 21). The humidity was set at 70–80% until day 14, and was slowly reduced until it reached 50%. A 12-h light/dark cycle was used (lights on between 08:00 and 20:00). The newborns in the AR group were placed in a small plastic cage with wood chips for bedding within 48 h of birth. To minimize maternal effects, all pups of the experimental groups were obtained from different litters. High pressure-treated artificial milk was loaded into the nursing bottle using a sterilized syringe. Pups were capable of suckling from silicon nipples connected to the nursing bottles. They were separated from their dams on postnatal day 2 and fed artificial milk using a nursing bottle by hand every 3 h, 5 times/day. The nursing bottles with silicon nipples and fresh milk were stored at 4°C after feeding to reduce bacterial growth. From day 14, pups were fed artificial milk from a nursing bottle combined with infant formula. The infant formula was made by mixing a powder diet with milk. The powder diet was made by crushing the standard diet. Pups in both the AR and Dam groups were weaned to the pelleted diet at day 21.

Motor activity test

Spontaneous motor activity was measured using cages (19 × 30 × 13 cm) equipped with wireless dish type running wheels (Wireless Low Profit Running Wheel, ENV-044 Wheel and SOF-860 software; Neuroscience Co., Ltd., Tokyo, Japan). Mice were assessed individually by recording the number of wheel rotations over a 30-min period without the practice [9].

Elevated plus maze test (EPM)

To measure anxiety-related behavior, an EPM was used. The EPM was elevated 50 cm above the floor and consisted of two open and two closed arms of equal sizes (35 × 5 cm); closed arms were surrounded by walls that were 15 cm high. The arms consisted of gray acrylic boards extending from a central platform (5 × 5 cm) to form a plus sign. A mouse was placed on the central

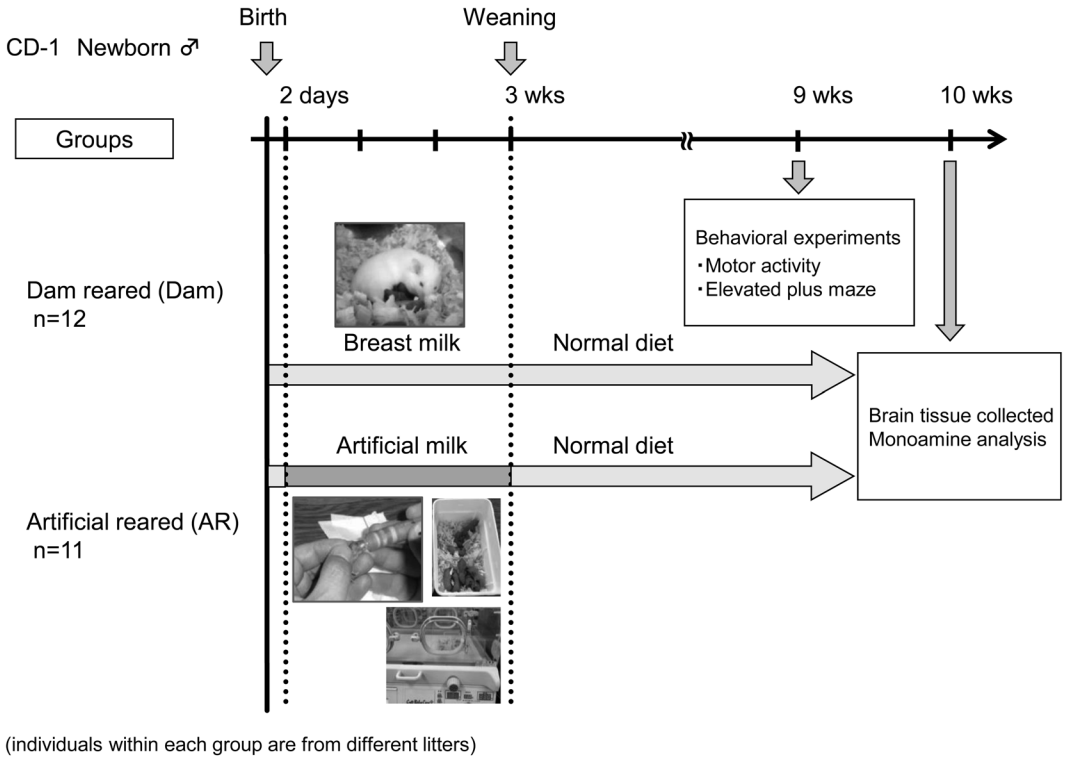

Fig. 1. Schematic diagram illustrating the study design.

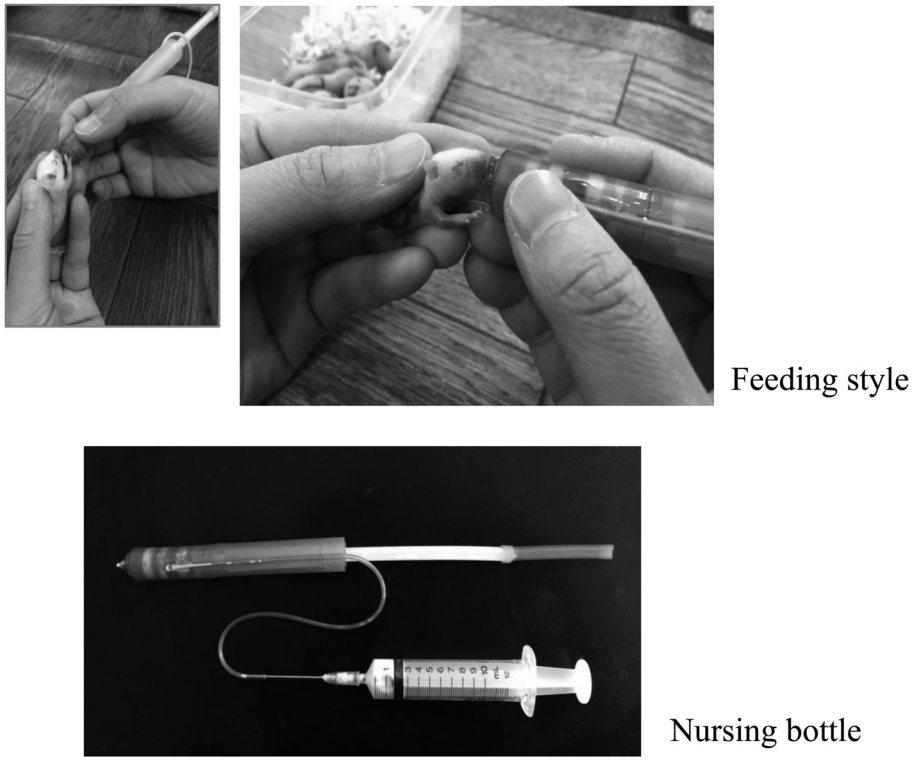

Fig. 2. Feeding style and nursing bottle.

platform in a shade bottle for 2 min. The session was initiated by removing the bottle from the mouse. The session was recorded by a video camera (HDR-CX550V; SONY, Tokyo, Japan) for 5 min. The parameters measured were the time spent on open arms, the number of entries into open arms, and the count of head dipping over the sides of the open arm toward the floor [22, 23].

Measurements of monoamine

After the behavioral tests, mice in each group (Dam, n=12; AR, n=11) were sacrificed and the brains were quickly removed and placed on an ice-cooled plate. The hippocampus, hypothalamus, striatum, and frontal cortex were dissected, immediately frozen, and stored at −80°C until analysis. The concentrations of monoamines and their metabolite levels (norepinephrine and metabolite 3-methoxy-4hydroxyphenylglycol [MHPG], 5-HT and metabolite 5-hydroxyindole acetic acid [5-HIAA], and DA and metabolites 3,4-dihydroxyphenylacetic acid [DOPAC], homovanillic acid, and 3-methoxy-4hydroxyphehylethylamine) in the brain were measured by the HPLC method described by EICOM (Kyoto, Japan), with slight modifications. The brain samples were homogenized in 200 μl of 0.2 M perchloric acid containing 100 μM EDTA-2Na and 10 ng of isoproterenol as an internal standard. The homogenate was kept on ice for 30 min and centrifuged at 20,000 × *g* for 15 min at 0°C. The supernatant was filtered through a centrifugal filter (Ultrafree-MC, 0.45-μm filter unit; Millipore, Bedford, MA, USA) at 15,000 × *g* for 3 min at 0°C. The HPLC equipment consisted of a Waters Alliance e2695 separation module (Waters Corporation, Milford, MA, USA) equipped with a Waters 2465 electrochemical detector. The chromatographic separation was performed using an EICOMPAK SC-5ODS column (3.0 × 150 mm) linked to a precolumn (EICOM PREPAK, 4.0 × 4.0 mm). Waters Empower 2 software was used for data collection and analysis. The mobile phase consisted of 0.1 M citric acid buffer (pH 3.5) containing 17% methanol, 190 mg/L sodium 1-octanesulfonate, and 5 mg/L EDTA-2Na; the flow rate was 0.2 ml/min. The potential applied was +750 mV over an ISAAC reference electrode. The column temperature was maintained at 25°C.

Statistical analysis

Data are expressed as means ± SEM. Body weight and all parameters in the behavioral test were analyzed using Student's *t*-tests (two-tailed). For each monoamine in each of the four brain regions, Bonferroni corrections were applied to avoid multiple testing errors after Student's *t*-tests. A *P*-value of less than 0.05 was considered significant.

Table 3. Motor activity counts and the body weight at 9 wks of age

Group	No. of mice	Count (n)	Body weight (g)
Dam	12	527.8 ± 54.4	36.3 ± 0.59
AR	11	622.6 ± 75.5	32.6 ± 0.43**

The parameter is presented as the mean ± SEM. ** P <0.01 for the comparison between the Dam and AR group using the Student's *t*-test.

Results

Behavioral experiments

In this study, all mice in AR group were weaned by artificial rearing. Also it was difficult to compare the body weight between AR and Dam groups, because AR was no nursing in the night-time. Therefore the body weight in each group was compared at the time of the evaluation of behavioral experiments after weaning. The body weight of the Dam group was larger than that of the AR group at 9 weeks of age, before behavioral experiments (P<0.01; Table 3). However, there was no difference in spontaneous motor activity over a 30-min period between the AR and Dam groups (Table 3). In the EPM test, the time spent on the open arm tended to be shorter for the AR group than the Dam group (P=0.09, Fig. 3A). Furthermore, the numbers of entries into open arms and head dips from the open arms of the maze were significantly lower in the AR group than the Dam group (P<0.01, Fig. 3B; P<0.05, Fig. 3C).

Brain monoamine levels

The monoamines and their metabolites in the hippocampus, hypothalamus, frontal cortex, and striatum are summarized in Table 4. The norepinephrine concentrations in the hippocampus of the AR group were significantly higher than those of the Dam group (P<0.01). The MHPG levels in the hippocampus and the frontal cortex were lower in the AR group than the Dam group (hippocampus, P<0.05; frontal cortex, P<0.01). Additionally, the 5-HT concentrations in the hippocampus and the hypothalamus of the AR group were significantly higher than those of the corresponding regions in the Dam group (hippocampus, P<0.05; hypothalamus,

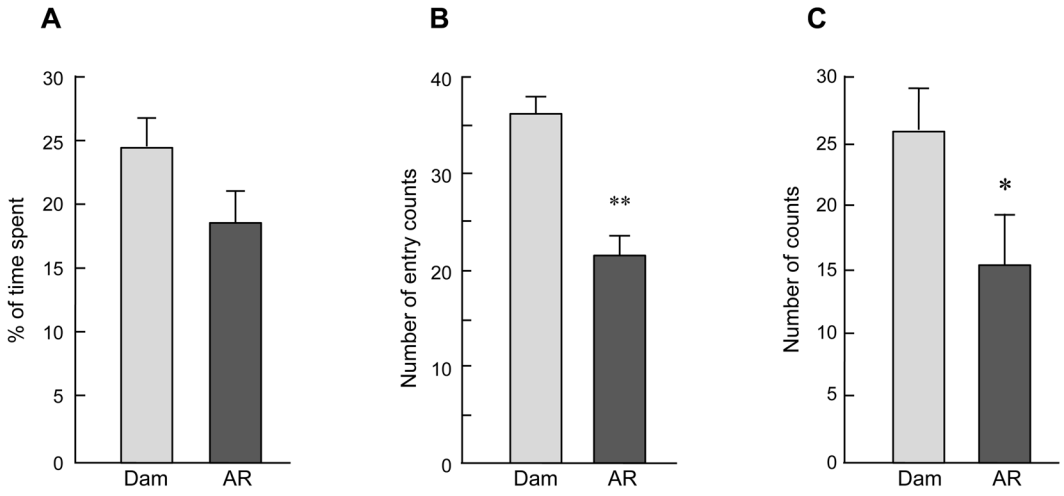

Fig. 3. The influence of dam or artificial rearing on the anxiety level of mice after 5 min in the elevated plus maze. Panel A shows the time spent on the open arm and Panel B shows the number of entries to the open arm. C shows the head dipping counts. Each value represents a mean ± SEM. **P*<0.05, ** *P* <0.01 vs. Dam (Student's *t*-test).

P<0.05). The 5-HIAA levels in the hypothalamus were higher in the AR group than the Dam group (*P*<0.05), and the DOPAC levels in the hypothalamus were significantly lower in the AR group than the Dam group (*P*<0.05). However, the monoamines in the striatum did not differ significantly between groups.

Discussion

Before the behavioral experiments, there was a significant difference in body weight between mice in the AR and Dam groups. However, in some studies that have used artificial milk with a similar composition and materials, there was no detectable difference in body weight between groups [15, 26]. In this study, we used the elevated plus-maze test in order to observe emotional behavior under the natural behavior without other stress factors in dam-reared and artificially reared mice. Also the spontaneous motor activity was measured in order to avoid the evaluation of the change in the apparent based on the excitement or the sedation. The spontaneous motor activity was slightly higher in the AR group than the Dam group, although there was not a significant difference between the two groups. Accordingly, we inferred that there were no functional differences during the developmental stage between mice in the two groups in this study. However, in the EPM test, both the number of entries and the time spent on the open arms in the AR group were lower than those of the Dam group, despite a lack of a difference in spontaneous motor activity between the two groups. This result suggested that the AR group exhibited anxiety-like behavior under the novel environmental conditions, and had a poorer ability to adapt to the new environment.

The levels of monoamines and their metabolites in brains showed that artificial rearing mainly affected the noradrenergic and serotonergic systems in the hippocampus and hypothalamus. Several studies have reported that increased amounts of 5-HT are released from the hippocampus and hypothalamus after stress in rats [16, 24]. Our study also confirmed the increase of 5-HT in the hippocampus and hypothalamus after artificial rearing. Additionally, serotonergic systems are activated in response to physical and psychological stress [3, 12]. It is well known that a variety of stressful events, including emotional stress, cause marked increases in norepinephrine release in several brain regions (e.g., the amygdala, hippocampus, and hypothalamus) [4]. Consistent with previous data, our results showed that the NE content was significantly increased in the hippocampus. Moreover, the norepinephrine metabolite MHPG decreases in the frontal cortex of maternally separated rat pups after restraint stress [5]. We observed increased 5-HT and norepinephrine and decreased MHPG in the hippocampus and hypothalamus of the AR group, consistent with these previous studies. These data indicate that artificial rearing exerts a profound effect on neurotransmitter contents in various regions of the brain. In addition, physical contact with dams affects not only mice, but also humans. It has been reported that children are re-

Table 4. Monoamine levels in mouse brain

Group	Dam (n = 12)	AR (n = 11)
Hippocampus		
NE	111.4 ± 5.4	140.6 ± 17.6**
MHPG	88.2 ± 3.7	74.3 ± 2.1*
5-HT	118.4 ± 7.8	147.1 ± 6.1*
5-HIAA	190.1 ± 7.9	214.2 ± 4.3
DA	13.2 ± 4.7	13.5 ± 0.8
DOPAC	101.8 ± 10.9	86.3 ± 3.6
3MT	31.4 ± 2.7	29.2 ± 1.2
HVA	14.6 ± 2.5	13.1 ± 0.7
Hypothalamus		
NE	611.7 ± 18.4	674.4 ± 19.6
MHPG	155.1 ± 6.4	142.8 ± 2.6
5-HT	148.1 ± 20.1	222.1 ± 11.7*
5-HIAA	516.0 ± 23.5	431.5 ± 12.4*
DA	211.6 ± 20.3	243.0 ± 15.4
DOPAC	562.8 ± 33.1	448.9 ± 17.2*
3MT	53.9 ± 1.9	58.7 ± 1.86
HVA	85.3 ± 5.4	73.7 ± 2.6
Frontal cortex		
NE	179.9 ± 16.8	131.9 ± 21.4
MHPG	379.2 ± 30.3	200.4 ± 33.0**
5-HT	168.0 ± 25.5	151.5 ± 27.0
5-HIAA	410.0 ± 47.4	350.0 ± 44.4
DA	584.0 ± 175.1	620.0 ± 206.3
DOPAC	1,458.2 ± 273.2	1,360.3 ± 331.5
3MT	73.7 ± 6.9	62.4 ± 6.9
HVA	345.6 ± 68.3	304.3 ± 77.4
Striatum		
NE	62.2 ± 7.7	76.6 ± 9.2
MHPG	73.2 ± 2.4	67.9 ± 1.5
5-HT	141.3 ± 6.1	139.7 ± 7.7
5-HIAA	223.2 ± 8.9	204.5 ± 10.5
DA	2,902.8 ± 155.1	3,183.7 ± 130.0
DOPAC	3,064.9 ± 226.7	3,261.8 ± 181.1
3MT	468.5 ± 19.4	498.9 ± 13.8
HVA	594.4 ± 17.7	584.8 ± 16.3

Unit=pg/mg brain tissue (mean ± SEM). Each monoamine was adjusted by the Bonferonni corrections avoiding the error of multiple testing after Student's *t*-test. *P<0.05, **P<0.01 for the comparison between the Dam and AR groups.

laxed and have low heart rates after nestling with their mothers [6].

Our results demonstrated that anxiety-like behavior in the AR group continues to be elevated after weaning, compared with the Dam group. The artificial rearing method is the most severe model of early weaning because the breeding environment during lactation determines bond formation between pups and dams [13, 14]. In future studies, it needs to add other behavioral experiment for the evaluation of emotion, such as the novelty suppressed feeding paradigm. Also it will be important to use ovariectomized parous mice, which take care of newborns, with feeding via artificial milk to minimize the stress on newborn pups and to evaluate the formation of brain function using artificial rearing methods.

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