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...
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].

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 norepinephrine as monoamines, and metabolite levels were estimated using high-performance liquid chromatography (HPLC) (Fig. 1).

### 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).

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).

#### 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.

### Table 1. Composition of experimental diets

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

The experimental diet was MF, obtained from Oriental Yeast Co., Tokyo, Japan.

### 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...
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

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**Table 2. Composition of artificial milk**

| 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                          |
| GlyCaPO4        | 800                          |
| MgCl2 6H2O      | 190                          |
| CaCl2 2H2O      | 170                          |
| CaCO3          | 184                          |
| Cu-Citrate      | 120                          |
| Na2HPO4        | 80                           |
| KH2PO4         | 8                            |
| FeSO4          | 24                           |
| Citrate H2O     | 0.5                          |
| ZnSO4          | 6                            |
| CuSO4          | 1.5                          |
| MnSO4          | 0.25                         |
| NaF            | 0.155                        |
| KI             | 0.25                         |
| K2SO4          | 163.5                        |
| Na2SO4 9H2O    | 5.075                        |
| Na2O2Se        | 0.035                        |
| H2MoO4O4 4H2O  | 0.0275                       |
| CrH3KO2S2      | 0.9625                       |
| Li2CO3         | 0.06                         |
| H3BO3          | 0.285                        |
| NiCO3          | 0.1125                       |
| NH4VO3         | 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                |
| Picolinat      | 2.0                          |
| Ethanolamine   | 3.5                          |
| Taurine        | 15.0                         |
| Tricholine Citrate | 147.0                      |

*The artificial milk formula was made following the method of Yajima et al.*
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 mea-
sured 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-4hy-
droxyphenylethylamine) in the brain were measured by
the HPLC method described by Eicom (Kyoto, Japan),
with slight modifications. The brain samples were ho-
genized 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 separa-
tion 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). Wa-
ters 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 Stu-
dent’s t-tests. A P-value of less than 0.05 was considered
significant.

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 ex-
eriments (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 hippo-
campus, hypothalamus, frontal cortex, and striatum are
sumarized in Table 4. The norepinephrine concentra-
tions in the hippocampus of the AR group were signifi-
cantly 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 (hip-
locampus, P<0.05; frontal cortex, P<0.01). Addition-
ally, 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,
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-
of newborns, with feeding via artificial milk to minimize
laxative suppression of feeding paradigm. Also it will be impor-
tant to use ovariectomized parous mice, which take care
in future studies, it needs to add other behavioral ex-
mines bond formation between pups and dams [13, 14].
cause the breeding environment during lactation deter-
moves bond formation between pups and dams [13, 14].

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 | 124.8 ± 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 Bonferroni 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.

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

The stress on newborn pups and to evaluate the formation of brain function using artificial rearing methods.

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