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Original Article

Lack of combined effect of continuous exposure to α-glycosyl isoquercitrin from fetal stages to adulthood and voluntary exercise or environmental enrichment on learning and behaviors in rats

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ABSTRACT — We previously reported that continuous exposure to α-glycosyl isoquercitrin (AGIQ) from the fetal stage to adulthood facilitates fear-extinction learning in rats. The present study investigated the combined effect of continuous exposure to AGIQ with voluntary exercise or environmental enrichment on learning and behaviors in rats. For this purpose, maternal Long-Evans rats were either untreated or treated with 0.5% AGIQ in basal diet from gestational day 6 to day 21 post-delivery. Offspring in both groups were weaned on postnatal day 21 and reared thereafter either in a standard cage, a wheel cage or an environmental enrichment cage until the end of the experiment with or without exposure to AGIQ. Fear memory, locomotor activity and anxiety-like behavior in open field test, spatial memory and nonspatial memory were assessed in adulthood. Environmental enrichment without AGIQ exposure, as well as AGIQ exposure in standard cage, showed a tendency for facilitation of fear-extinction learning. However, exposure to AGIQ and environmental enrichment did not act synergistically. Voluntary exercise only decreased the total distance traveled in the open field test in the condition with or without AGIQ exposure, suggesting induction of anxiety-like behavior. Body weight from lactation period to adulthood, body and brain weights at the end of the experiment did not change by exposure to AGIQ under any cage condition. Therefore, there was no beneficial or detrimental effect of voluntary exercise and environment enrichment on the outcome of behavior or general conditions by continuous AGIQ exposure from the fetal stage.

Key words: Alpha-glycosyl isoquercitrin, Behavior, Environmental enrichment, Learning, Rat, Voluntary exercise

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INTRODUCTION

Alpha-glycosyl isoquercitrin (AGIQ), also known as enzymatically modified isoquercitrin, is a polyphenolic flavonol glycoside derived by enzymatic glycosylation of rutin, with 1-10 or more of additional linear glucose moieties (Akiyama *et al*., 2000). AGIQ is a mixture of quercetin glycoside, consisting of isoquercitrin and its α-glucosylated derivatives. AGIQ is highly water-soluble and has an antioxidant potential (Formica and Regelson, 1995). AGIQ has been reported to exert antioxidant effects (Kangawa *et al.*, 2017) as well as having anti-inflammatory (Kangawa *et al*., 2017), anti-hypertensive (Gasparotto *et al.*, 2011), anti-allergic (Makino *et al*., 2013) and tumor suppressive (Fujii *et al*., 2013) properties. It has been found to be safe in a 90-day toxicity study (Nyska *et al*., 2016) and in genotoxicity assays (Hobbs *et al*., 2018).

We recently reported that continuous exposure to 0.5% AGIQ in diet from the fetal stage through adulthood in rats facilitated fear-extinction learning on contextual fear conditioning test (Okada *et al.*, 2019). We further found up-regulation of synaptic plasticity-related genes or proteins in brain regions related to fear-extinction learning after the last trial of fear-extinction test (Masubuchi *et al*., in press). This suggested that enhancement of synaptic plasticity in the learning-related neural circuit might be involved in the facilitation of fear-extinction learning. On the other hand, AGIQ exposure showed no effects on anxiety-like behavior, object recognition memory, and acquisition of fear memory (Masubuchi *et al*., in press).

It has been reported that voluntary exercise and environmental enrichment improve learning and memory of animals, such as spatial memory (van Praag *et al*., 1999; Hullinger *et al*., 2015) and nonspatial memory (Hopkins *et al*., 2011; Bruel-Jungerman *et al*., 2005). Brain-derived neurotrophic factor (BDNF)-mediated signaling has been reported to be one of the crucial mechanisms mediating the positive effect of voluntary exercise and environmental enrichment (Cotman *et al*., 2007; Novkovic *et al*., 2015). BDNF-mediated signaling is essential for the hippocampal function, synaptic plasticity, learning and modulation of depression (Kuipers and Bramham, 2006). Therefore, it might be possible that BDNF-mediated signaling by voluntary exercise or environmental enrichment further enhances synaptic plasticity induced by AGIQ exposure. It might also be possible to enhance the effect on learning and memory or behavior of animals by combining AGIQ exposure with voluntary exercise or environmental enrichment.

The present study investigated whether the combination of continuous AGIQ exposure from the fetal stage with voluntary exercise or environmental enrichment has a modifying effect on learning and memory or behavior of rats. Behavioral tests such as open field test, object recognition test, object location test and contextual fear conditioning test were performed in adulthood.

MATERIALS AND METHODS

Chemicals and animals

AGIQ (purity: > 97%) was provided by San-Ei Gen F.F.I. Inc. (Osaka, Japan). Mated female Iar: Long-Evans rats were purchased from Japan SLC, Inc. (Shizuoka, Japan), at gestational day (GD) 3 (appearance of vaginal plugs was designated as GD 0) and individually housed with their offspring in polycarbonate cages with paper bedding until day 21 post-delivery. Rats were kept in an air-conditioned animal room (temperature: 23 ± 2°C, relative humidity: 55 ± 15%) with a 12 hr light/dark cycle, and provided *ad libitum* powdered basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) until exposure to AGIQ began and tap water during the experiment. Offspring were weaned at postnatal day (PND) 21 (where PND 0 was the day of delivery) and reared two animals per cage thereafter.

Experimental design

Mated female rats were randomly divided into two groups of untreated controls (12 animals) and AGIQ group (12 animals) (Fig. 1). Maternal animals in the AGIQ group were treated with AGIQ at 0.5% (w/w) in their powdered basal diet from GD 6 to day 21 post-delivery. The dosage chosen has been shown to facilitate fear-extinction learning with continuous exposure from fetal stages to adulthood in rats (Okada *et al*., 2019).

We measured body weight (BW), food and water consumption of dams every 3-4 days from GD 6 to day 21 post-delivery. On PND 4, litters were randomly culled to preserve 5 or 6 male and 2 or 3 female offspring per dam (a total of 8 offspring per dam). Offspring were weighed every 3 or 4 days until PND 21. Dams and female offspring were euthanized by exsanguination through the abdominal aorta under CO_2/O_2 anesthesia and subjected to necropsy on day 21 post-delivery. Male offspring were selected for behavioral tests because animal behavior is influenced by circulating levels of steroid hormones during the estrous cycle (Milad *et al*., 2009; Mora *et al*., 1996; Paris and Frye 2008).

From PND 21 onwards, male offspring were either untreated or treated with 0.5% AGIQ identically with their dams. The following six groups were set: basal diet

+ standard cage group (BD + SC; 28 animals), basal diet + wheel cage group (BD + WC; 16 animals), basal diet + environmental enrichment cage group (BD + EC; 18 animals), AGIQ + standard cage group (AGIQ + SC; 30 animals), AGIQ + wheel cage group (AGIQ + WC; 16 animals), and AGIQ + environmental enrichment cage group (AGIQ + EC; 18 animals) (Fig. 1). The standard cage condition consisted of a standard cage (inner dimension, 204 mm width × 363 mm depth × 196 mm height) with paper bedding but no enrichment items. The wheel cage condition consisted of a wheel cage (inner dimension, 200 mm width × 355 mm depth × 195 mm height, Melquest Ltd., Toyama, Japan) contained a running wheel (85 mm width × 310 mm diameter) and paper bedding, and rats reared in this cage were allowed to exercise freely. The environmental enrichment cage condition consisted of a large-sized cage (inner dimension, 440 mm width × 275 mm depth × 180 mm height) equipped with two kinds of paper bedding, shelters, nesting materials, enrichment items (plastic toys, wooden toys, chains, ropes, rattles or ladders), and the items were replaced once weekly to new ones to maintain novelty of the environment. Rats were weighed once weekly thereafter, and the amounts of food and water consumption were also measured.

On PND 76 and PND 81, animals subjected to behavioral tests (16-18 animals/group) were subjected to brain sampling after the last trial of fear extinction test for other experimental purposes. On PND 77, the remaining 10 male offspring in BD + SC group and 12 male offspring in AGIQ + SC group were subjected to brain sampling for other experimental purposes.

All dams and offspring were checked for general conditions (abnormal gait and behaviors) every day. All procedures in this study were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and according to the protocol approved by the Animal Care and Use Committee of The Tokyo University of Agriculture and Technology (Approved No.: 30-107). All efforts were made to minimize animal suffering.

Behavioral tests

Animals in the BD + SC (18 animals), BD + WC (16 animals), BD + EC (18 animals), AGIQ + SC (18 animals), AGIQ + WC (16 animals) and AGIQ + EC groups (18 animals) were subjected to the open field test, object recognition test, object location test and contextual fear conditioning test. In each behavior experiment, animals were transported from the animal room to the

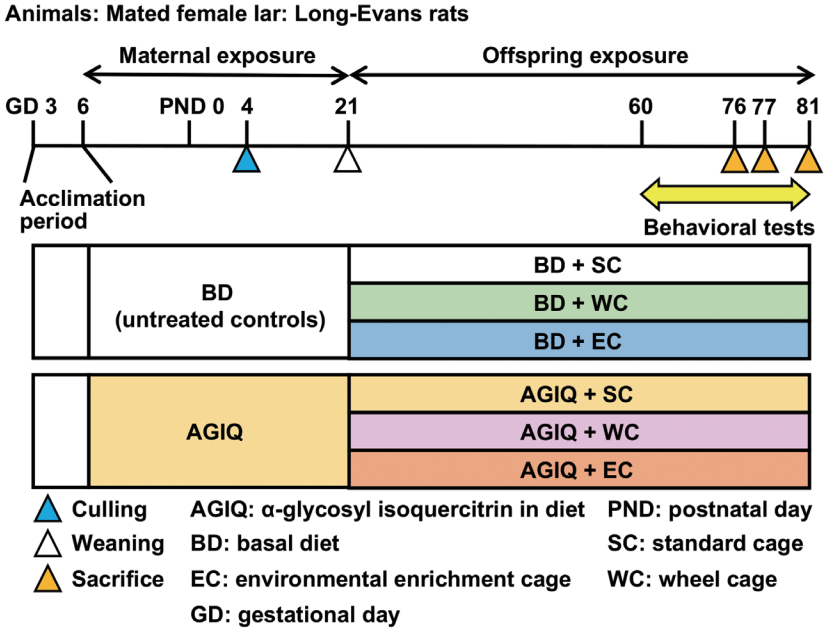

Fig. 1. Experimental design for combination of continuous exposure to α-glycosyl isoquercitrin (AGIQ) from fetal stages to adulthood and voluntary exercise or environmental enrichment in rats.

behavioral test room 1-2 hr before starting the tests. After the end of each behavioral test, animals were promptly returned to the home cage and transferred to the animal room. Apparatuses were cleaned with 70% ethanol solution before and after each test. All experiments were conducted between 08:00 and 19:00, and the order of animal selection for tests among groups was counter-balanced across the test time to avoid any bias in the trial times of each group.

The open field test was performed on PND 60, PND 64 and PND 68 to assess locomotor activity and anxiety-like behavior and to habituate rats to the arena that would be used in the object recognition test and object location test on the following day (acclimation phase), according to the methods and apparatuses reported previously (Masubuchi *et al*., in press).

The object recognition test was performed on PND 61, PND 62, PND 65, PND 66, PND 69 and PND 70 to assess nonspatial object memory. The object location test was performed on PND 63, PND 67 and PND 71 to assess spatial object memory. Experiments were conducted in the same arena that was used in the open field test, according to the methods and apparatuses reported previously (Masubuchi *et al*., in press). The test comprised four steps: acclimation, sample phase, test phase (object recognition test; 24 hr after sample phase) and test phase (object location test; 24 hr after test phase of object recognition test).

The contextual fear conditioning test was performed during PND 72 and PND 76 and during PND 77 and PND 81 to assess fear memory and fear extinction learning, according to the methods and apparatuses reported previously (Masubuchi *et al*., in press). The test comprised three steps: conditioning, acquisition test (24 hr after conditioning) and three times of extinction test (24 hr after acquisition test, thereafter, 2nd and 3rd trials were performed at 24 hr intervals).

Statistical analysis

Offspring data until PND 21 (BWs) were analyzed using the litter as the experimental unit. Offspring data from PND 21 onwards (BWs, brain weights at necropsy and parameters on the behavioral tests) were analyzed using the individual animals as the experimental unit. Data from six groups were analyzed using Levene's test for homogeneity of variance. If the variance was homogenous, numerical data were evaluated using Tukey-Kramer's multiple comparison test. For heterogeneous data, Steel-Dwass's test was used. Statistical comparison was made between BD + SC and BD + WC, BD + EC, AGIQ + SC, AGIQ + WC or AGIQ + EC, between AGIQ + SC and AGIQ + WC or AGIQ + EC, between BD + WC and AGIQ + WC or between BD + EC and AGIQ + EC. Data from two groups were analyzed using *F*-test for homogeneity of variance. When the variance was homogenous, Student's *t*-test was applied. When data were heterogeneous, Aspin-Welch's *t*-test was used.

Because some animals were resistant to fear-extinction learning, Smirnov-Grubbs's test was performed to detect the outliers in each group. Identified outliers were excluded as abnormal animals from the fear-extinction test analysis.

All analyses were performed using Excel Statistics 2013 software package version 2.02 (Social Survey Research Information Co. Ltd., Tokyo, Japan), and $P < 0.05$ was considered statistically significant.

RESULTS

Maternal intake of AGIQ

Based on the mean values of food consumption, dams in the AGIQ group received 426.6 and 843.7 mg/kg BW/day of AGIQ during the gestation and lactation period, respectively.

Clinical signs, BWs and necropsy data of male offspring

No abnormality in gait and behavior was observed in the AGIQ group during the lactation period. There were no significant changes in BW between untreated controls and AGIQ group from PND 5 to PND 21 (Table 1).

No abnormality in gait and behavior was observed in each treatment group from postweaning to the end of the experiment. In comparison with BD + SC, BD + WC showed a significant decrease in BW on PND 75 (Table 2). In comparison with AGIQ + SC, AGIQ + WC showed a significant decrease in BW on PND 26

Table 1. Body weight changes of male offspring from PND 5 to PND 21.

	Ctrl	AGIQ
No. of offspring examined	63	69
Body weight (g)		
PND 5	13.5 ± 0.6	14.1 ± 1.0
PND 8	19.9 ± 1.0	21.0 ± 1.6
PND 12	30.6 ± 1.9	31.4 ± 2.4
PND 15	37.8 ± 2.2	38.5 ± 2.8
PND 19	47.6 ± 2.9	48.7 ± 3.1
PND 21	56.2 ± 3.5	57.6 ± 3.3

Abbreviations: AGIQ, α-glycosyl isoquercitrin; Ctrl, untreated controls; PND, postnatal day.
Data are expressed as mean ± S.D.

Table 2. Body weight changes of male offspring from PND 26 to PND 75 and body and brain weights of male offspring at the terminal necropsy on PND 81.

	BD + SC	BD + WC	BD + EC	AGIQ + SC	AGIQ + WC	AGIQ + EC
No. of offspring examined	28	16	18	30	16	18
Body weight (g)						
PND 26	83.9 ± 4.5	81.1 ± 5.8	82.7 ± 7.0	86.4 ± 6.3	81.2 ± 4.7†	87.7 ± 4.5
PND 33	137.7 ± 10.7	134.6 ± 9.6	135.0 ± 12.2	138.1 ± 8.3	136.4 ± 7.1	141.9 ± 8.0
PND 40	197.6 ± 14.6	194.8 ± 15.5	191.5 ± 18.0	194.4 ± 13.3	197.5 ± 9.7	199.6 ± 12.1
PND 47	255.0 ± 18.7	250.3 ± 19.5	248.2 ± 22.4	252.6 ± 17.8	258.1 ± 14.2	259.4 ± 17.7
PND 54	302.0 ± 21.6	293.1 ± 20.7	295.1 ± 23.1	301.6 ± 21.9	301.3 ± 17.3	309.1 ± 22.5
PND 61	343.1 ± 24.6	328.3 ± 22.0	336.5 ± 26.1	346.1 ± 24.2	337.1 ± 21.2	353.6 ± 26.2
PND 68	378.3 ± 27.8	358.6 ± 23.5	371.8 ± 27.3	383.5 ± 27.3	370.0 ± 21.3	388.3 ± 29.7
PND 75	409.0 ± 31.2	380.1 ± 27.5*	402.2 ± 28.3	417.2 ± 29.0	394.6 ± 23.9	417.9 ± 33.0
No. of offspring examined	8	6	8	8	6	8
Body weight (g)						
PND 81	426.3 ± 28.4	383.5 ± 28.5	414.9 ± 27.5	439.7 ± 28.8	414.1 ± 18.5	434.6 ± 31.0
Brain weight (g)						
PND 81	2.10 ± 0.03	2.18 ± 0.06	2.18 ± 0.06	2.17 ± 0.07	2.16 ± 0.09	2.16 ± 0.05

Abbreviations: AGIQ, α-glycosyl isoquercitrin; BD, basal diet; EC, environmental enrichment cage; PND, postnatal day; SC, standard cage; WC, wheel cage. Data are expressed as mean ± S.D. *$P < 0.05$, significantly different from BD + SC by Tukey-Kramer's test or Steel-Dwass's test. †$P < 0.05$, significantly different from AGIQ + SC by Tukey-Kramer's test or Steel-Dwass's test.

(Table 2). There were no significant differences between the groups of any other combination at any time point.

There were no significant differences in BW and brain weight on PND 81 between the groups of any combination (Table 2).

Based on the mean values of food consumption, male offspring in AGIQ + SC, AGIQ + WC and AGIQ + EC received 400.6, 488.8 and 417.4 mg/kg BW/day of AGIQ, respectively.

Behavioral testing scores of male offspring

Open field test

Total distance significantly decreased in BD + WC compared with BD + SC, and significantly decreased in AGIQ + WC compared with AGIQ + SC (Table 3). Total distance showed no significant difference between the groups of any other combination. Center area rate showed no significant difference between the groups of any combination (Table 3).

Object recognition test and object location test

In the object recognition test and the object location test, total distance and discrimination index in the test phase after 24 hr interval showed no significant difference between the groups of any combination (Table 3).

Contextual fear conditioning test

There were no significant differences in the freezing rate in the fear acquisition and fear-extinction tests between the groups of any combination (Table 3).

DISCUSSION

In the present study, while the change is not statistically significant, we observed facilitation of fear-extinction learning at the 3rd trial after continuous exposure to AGIQ with standard cage condition. However, continuous exposure to AGIQ with wheel cage condition or environmental enrichment condition showed little or no facilitation on fear-extinction learning as compared to AGIQ-untreated counterpart. In contrast, environmental enrichment condition with or without AGIQ-exposure revealed facilitation of fear-extinction at the all trials as compared to AGIQ-untreated animals with standard cage condition, while the changes were not statistically significant. These results suggest that both continuous exposure to AGIQ and environmental enrichment condition facilitate fear-extinction learning but they would not act synergistically.

In the present study, wheel cage condition revealed lower values of the total distance traveled in the open field test as compared to standard cage condition, irrespective of the presence or absence of AGIQ-exposure. It has been reported that voluntary exercise causes anxiety-like behavior of animals including fewer exploring of open field (Burghardt *et al*., 2004). Therefore, these results in

Table 3. Changes in parameters of the behavioral tests of male offspring.

	BD + SC	BD + WC	BD + EC	AGIQ + SC	AGIQ + WC	AGIQ + EC
Open field test						
No. of offspring examined	18	16	18	18	16	18
Total distance (cm)	5614 ± 551	4531 ± 978**	5707 ± 1113	5831 ± 615	4787 ± 1054††	5560 ± 793
Center area rate (%)	10.7 ± 4.1	14.0 ± 8.8	13.6 ± 6.2	13.1 ± 4.1	10.4 ± 5.5	13.3 ± 5.3
Object recognition test						
No. of offspring examined	18	16	18	18	16	18
Total distance (cm)						
sample phase	3326 ± 514	2987 ± 627	3526 ± 391	3730 ± 489	3269 ± 564	3529 ± 451
test phase	1563 ± 226	1363 ± 338	1634 ± 294	1602 ± 174	1486 ± 333	1626 ± 221
Discrimination index						
sample phase	0.47 ± 0.10	0.48 ± 0.13	0.49 ± 0.09	0.47 ± 0.06	0.47 ± 0.11	0.46 ± 0.09
test phase	0.54 ± 0.14	0.60 ± 0.13	0.57 ± 0.16	0.58 ± 0.10	0.48 ± 0.17	0.57 ± 0.12
Object location test						
No. of offspring examined	18	16	18	18	16	18
Total distance (cm)						
test phase	1489 ± 204	1364 ± 344	1614 ± 195	1647 ± 155	1443 ± 279	1311 ± 399
Discrimination index						
test phase	0.49 ± 0.16	0.55 ± 0.14	0.50 ± 0.12	0.48 ± 0.12	0.47 ± 0.16	0.52 ± 0.23
Contextual fear conditioning test						
No. of offspring examined	18	16	18	18	16	18
Freezing rate (%)						
Conditioning	40.8 ± 8.7	33.8 ± 12.1	39.5 ± 9.7	40.7 ± 11.2	34.0 ± 11.8	39.9 ± 12.2
Acquisition	74.2 ± 13.5	63.6 ± 20.2	72.7 ± 13.9	58.8 ± 22.6	61.5 ± 14.3	67.6 ± 17.2
No. of offspring examined	18 (17)‡	16	18 (16)§	18	16 (15)‖	18 (17)¶
Freezing rate (%)						
Fear extinction #1	33.4 ± 18.1	33.9 ± 20.9	24.9 ± 14.0	30.0 ± 23.0	28.9 ± 19.9	27.6 ± 15.9
Fear extinction #2	18.0 ± 14.1	16.6 ± 9.2	10.7 ± 6.7	14.7 ± 13.5	12.7 ± 9.3	12.1 ± 6.7
Fear extinction #3	13.4 ± 12.3	14.3 ± 7.7	5.9 ± 7.0	8.8 ± 7.3	12.1 ± 7.8	6.9 ± 5.8

Abbreviations: AGIQ, α-glycosyl isoquercitrin; BD, basal diet; EC, environmental enrichment cage; SC, standard cage; WC, wheel cage. Data are expressed as mean ± SD. **$P < 0.01$, significantly different from BD + SC by Tukey-Krammer's test or Steel-Dwass's test. ††$P < 0.01$, significantly different from AGIQ + SC by Tukey-Krammer's test or Steel-Dwass's test. ‡An outlier was detected by Smirnov-Grubbs's test. It was excluded from data analysis. Number in parenthesis is effective number of animals. §Two outliers were detected by Smirnov-Grubbs's test. They were excluded from data analysis. Number in parenthesis is the effective number of animals. ‖An outlier was detected by Smirnov-Grubbs's test. It was excluded from data analysis. Number in parenthesis is the effective number of animals. ¶An outlier was detected by Smirnov-Grubbs's test. It was excluded from data analysis. Number in parenthesis is the effective number of animals.

the present study are consistent with the previous report and considered to be an effect of voluntary exercise. Voluntary exercise also has been reported to show beneficial effects on learning and memory, such as spatial memory (van Praag *et al*., 1999), nonspatial memory (Hopkins *et al*., 2011), and fear memory (Lin *et al*., 2012). However, wheel cage condition did not exert any effects on other behavioral parameters examined, while the reason was not clear.

In the present study, environmental enrichment condition did not show any change on behavioral parameters examined as compared with standard condition except for a facilitating tendency in fear-extinction learning. Environmental enrichment has been reported to show beneficial effects on learning and memory of animals, such as spatial memory (Hullinger *et al*., 2015) and nonspatial memory (Bruel-Jungerman *et al*., 2005). In the environmental enrichment paradigm, the definition and method of the environmental enrichment are varying considerably across the laboratories (Simpson and Kelly, 2011). Furthermore, it has been reported that locomotor activity of animals changes depending on the degree of the environmental enrichment (Xie *et al*., 2013). Therefore, the effect of environmental enrichment on learning and memory function may be related to the degree of the enrichment.

In the present study, BW of animals with wheel cage

condition showed lower values with or without statistical significance relative to animals with standard cage condition, irrespective of the presence or absence of AGIQ-exposure. The lowered BW may be simply due to energy consumption by voluntary exercise.

In the present study, BWs from PND 5 to PND 21 did not show any change by continuous exposure to AGIQ. During PND 26 and PND 75, AGIQ also did not alter BW in each cage condition. At the terminal necropsy, AGIQ also did not alter BW and brain weight in each cage condition. Additionally, AGIQ did not show changes in any parameters of the behavioral tests in any of the cage condition, except for a tendency for facilitation of fear-extinction learning. These results suggest that continuous exposure to 0.5% AGIQ in diet from the fetal stage does not affect general health condition of animals under each cage condition.

In conclusion, a combination of continuous exposure to AGIQ from the fetal stage with voluntary exercise or environmental enrichment did not show any modifying effect on learning and memory or behavior of animals. BW, brain weight and behavioral test data revealed that AGIQ exposure showed no beneficial or detrimental effect in combination with voluntary exercise or environmental enrichment.

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Conflict of interest---- Mihoko Koyanagi and Shim-mo Hayashi are employed by a food additive manufacturer whose product lines include α-glycosyl isoquercitrin. Robert R. Maronpot is a scientific consultant at the aforementioned food additive manufacturer. The views and opinions expressed in this article are those of the authors and not necessarily those of their respective employers. Yasunori Masubuchi, Satomi Kikuchi, Hiromu Okano, Yasunori Takahashi, Kazumi Takashima, Ryota Ojiro, Qian Tang, Toshinori Yoshida, and Makoto Shibutani declare that no competing interests exist.

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