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Continuous exposure to α-glycosyl isoquercitrin from developmental stage facilitates fear extinction learning in rats

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ABSTRACT

This study investigated the effect of exogenously administered polyphenolic antioxidant, α-glycosyl isoquercitrin (AGIQ), on neural function in rats following dietary exposure at 0.5% from gestational day 6 to postnatal day 77, in comparison with nonpolyphenolic α-lipoic acid (ALA) at 0.2%. AGIQ-exposed offspring revealed enhanced fear extinction learning at the 3rd trial and transcript upregulation of *Fos* and *Kif21b* in the hippocampal dentate gyrus and *Grin2d* in the amygdala. AGIQ also increased the number of FOS-immunoreactive $^{(+)}$ hippocampal granule cells. While ALA showed fear extinction learning-related transcript expression changes, it changed neither fear extinction learning nor FOS^+ cell numbers. The increases of FOS^+ cells and *Grin2d* transcripts by AGIQ suggested an association with increase of synaptic plasticity, leading to enhancement of fear extinction learning. AGIQ may also upregulate KIF21B, a recently identified memory-rewriting molecule. The present study findings suggest a possible therapeutic use of AGIQ in mitigating some stress disorders.

1. Introduction

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) production and antioxidant defence system in an organism and involved in a variety of disorders. It has been utilized to explain the increased incidence of cancer and heart diseases, but the brain may be even more vulnerable to oxidative stress, since it exhibits reduced free radical scavenging ability and utilizes high amounts of oxygen (Cantuti-Castelvetri, Shukitt-Hale, & Joseph, 2003). Previous experiments have examined the effects of diets rich in fruits and vegetables in reducing certain types of cancer and cardiovascular diseases (Ferro-Luzi & Branca, 1995), and evidence emerging from such experiments suggests that these kinds of dietary modifications may be beneficial in altering neuronal/behavioural deficits, as well. These kinds of diets are particularly rich in antioxidants such as vitamins A, C and E, and bioflavonoids (Ferro-Luzi & Branca, 1995), and thus, there may be synergistic effects among them. Therefore, it might be important to examine the impact of the antioxidants on various neuronal

Abbreviations: AGIQ, α-glycosyl isoquercitrin; ALA, α-lipoic acid; ARC, activity-regulated cytoskeleton-associated protein; BW, body weight; CALB2, calbindin-D–29K; COX2, cyclooxygenase 2; C_T, threshold cycle; DAB, 3,3′-diaminobenzidine; DCX, doublecortin; FOS, Fos proto-oncogene, AP-1 transcription factor subunit; GABA, γ-aminobutyric acid; GCL, granule cell layer; GD, gestational day; GFAP, glial fibrillary acidic protein; IEG, immediate-early gene; LTP, long-term potentiation; NeuN, neuronal nuclei; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; PCNA, proliferating cell nuclear antigen; p-ERK1/2, phosphorylated extracellular signal-regulated kinase 1/2 (MAP kinase1/2); PFA, paraformaldehyde; PND, postnatal day; PTSD, post-traumatic stress disorder; PVALB, parvalbumin; RELN, reelin; ROS, reactive oxygen species; RT, reverse transcription; SGZ, subgranular zone; SOX2, SRY (sex determining region Y)-box 2; SST, somatostatin; TBR2, T-box brain protein 2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling

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and behavioural parameters (e.g., signal transduction, cognitive behaviour and motor behaviour) and determine whether these parameters can be altered.

The hippocampus is an important temporal lobe brain structure involved in cognition, learning and memory. The hippocampus postnatally generates new neurons within the subgranular zone (SGZ) of the dentate gyrus, a subregion of the hippocampus, which is termed "adult neurogenesis" (Kempermann, Jessberger, Steiner, & Kronenberg, 2004). This neuronal production consists of multistep processes, including a number of developmental phases, such as self-renewal of stem cells, the facilitation of continued division of precursor cells to produce new granule cells, and subsequent differentiation and migration of these new cells into the granule cell layer (GCL; McDonald & Wojtowicz, 2005). In the hilus of the dentate gyrus, subpopulations of γ-aminobutyric acid (GABA)-ergic interneurons innervate granule cell lineage populations to control neurogenesis in the SGZ (Masiulis, Yun, & Eisch, 2011). In addition to GABAergic neuronal inputs, various types of neurons outside the SGZ also create a synaptic connection with neurons in the dentate gyrus, such as glutamatergic neurons in the entorhinal cortex providing axonal projections to the dentate gyrus (Fonnum, Karlsen, Malthe-Sørenssen, Skrede, & Walaas, 1979) and cholinergic neurons originating from the septal nucleus and nucleus of the diagonal band of Broca innervating neurons in the dentate hilus (Amaral & Kurz, 1985). Glutamatergic inputs to the SGZ are important for maintaining proper proliferation and differentiation of the granule cell lineage (Freund & Buzsáki, 1996). Importantly, SGZ cells in the dentate gyrus generate ROS, because these cells have a high cellular activity for proliferation and differentiation requiring high oxygen demand (Walton et al., 2012). Therefore, oxidative stress arises more in the dentate gyrus than in other brain regions.

Synaptic plasticity is the ability of synapses to strengthen or weaken over time, in response to increases or decreases in their activity. Since memories are postulated to be represented by vastly interconnected neural circuits in the brain, synaptic plasticity is one of the important neurochemical foundations of learning and memory (Herszage & Censor, 2018). It is known that excessive oxidative stress responses in the hippocampus impair synaptic plasticity (An & Zhang, 2014), and administration of antioxidant is effective for amelioration of cognitive deficits by protecting against oxidative damage (Wu, Ying, & Gomez-Pinilla, 2010).

Alpha-glycosyl isoquercitrin (AGIQ), also known as enzymatically modified isoquercitrin, is a polyphenolic flavonol glycoside derived by enzymatic glycosylation of rutin, which is contained in natural products such as citrus fruits, red beans, and buck-wheat. AGIQ is a mixture of quercetin glycoside, consisting of isoquercitrin and its α-glucosylated derivatives, with 1–10 or more of additional linear glucose moieties (Akiyama, Washino, Yamada, Koda, & Maitani, 2000). AGIQ has high water solubility in addition to antioxidant potential (Formica & Regelson, 1995). AGIQ has been reported to exert antioxidant effects (Kangawa, Yoshida, Maruyama, et al., 2017; Kangawa, Yoshida, Abe, et al., 2017; Morita et al., 2011; Nishimura et al., 2010; Shimada et al., 2010), as well as having anti-inflammatory (Kangawa, Yoshida, Maruyama, et al., 2017; Kangawa, Yoshida, Abe, et al., 2017), antihypertensive (Gasparotto et al., 2011), anti-allergic (Makino et al., 2013) and tumour suppressive (Fujii, Kimura, et al., 2013; Hara et al., 2014; Kimura et al., 2013) properties. It has been found to be safe in many toxicity studies (Nyska et al., 2016), as well as in vivo genotoxicity assays (Hobbs et al., 2018), and has been approved for use as a food additive under the Japan Food Sanitation Law (Ministry of Health and Welfare of Japan, 1996). AGIQ has been self-affirmed as Generally Recognized as Safe (GRAS) for use in specific foods in the United States. The US FDA concluded that AGIQ is GRAS as an antioxidant based on information provided in the GRAS Notice for AGIQ (GRN 000220) (U.S. FDA, 2007). AGIQ was also affirmed as GRAS by the Expert Panel of the Flavor and Extract Manufacturers Association (Adams et al., 2007; Smith et al., 2005). On the other hand, α-lipoic acid (ALA), a natural compound that is chemically named 5-(1,2-dithiolan-3-yl)pentanoic acid and is also known as thioctic acid (Gruzman, Hidmi, Katzhendler, Haj-Yehie, & Sasson, 2004), is nonpolyphenolic metabolic antioxidant. ALA also functions as an antioxidant that increases intracellular glutathione levels and regenerates other antioxidants such as vitamins C and E (Packer, Witt, & Tritschler, 1995). ALA has been reported to prevent or ameliorate several ailments such as diabetes, polyneuropathy, cataract, neurodegeneration and nephropathies (Takaoka, Ohkita, Kobayashi, Yuba, & Matsumura, 2002) because of its antioxidant properties (Winiarska, Malinska, Szymanski, Dudziak, & Bryla, 2008).

Oral intake of AGIQ instantly increased the plasma level of quercetin metabolites and enzymatic α-oligoglucosylation to the sugar moiety is effective for enhancing the bioavailability of quercetin glucosides in humans (Murota et al., 2010). On the other hand, plasma pharmacokinetics of ALA in humans revealed that orally administered ALA was absorbed rapidly and eliminated rapidly (Teichert, Hermann, Ruus, & Preiss, 2003). Both AGIQ and ALA can pass the blood-brain barrier and are distributed in the brain (Gilgun-Shrki, Melamed, & Offen, 2001; Valentová, Vrba, Bancířová, Ulrichová, & Křen, 2014). While there are no available studies reporting transplacental or translactational transfer of these antioxidants to offspring, ameliorating effect on disease conditions has been revealed in offspring by developmental exposure to quercetin or quercetin glucoside (Tan, Meng, Reece, & Zhao, 2018; Wu et al., 2014), or ALA (Al-Matubsi, Oriquat, Abu-Samak, Al Hanbali, & Salim, 2016; Sugimura et al., 2009; Takeda et al., 2017).

Recently, some polyphenolic antioxidants, such as flavonoids and stilbenoids, have shown to exert ameliorating effect on post-traumatic stress disorder (PTSD) in animal models (Lee, Shim, Lee, & Hahm, 2018; Li et al., 2018; Sanz-García et al., 2016; Wu, Ni, Shao, & Cui, 2017; Zhang et al., 2017). Therefore, it is possible that AGIQ may have a potential in ameliorating PTSD, and it is reasonable to examine whether AGIQ alters neurobehavioural functions in normal animals before investigating in PTSD models. In the present study, we tested maternal exposure effect of AGIQ with the dietary dose to exert apparent antioxidant effect on spatial learning and long-term associative memory of rat offspring by exposure from gestational stage until adult stage. For this purpose, we used ALA as a strong nonpolyphenolic antioxidant for comparison. By means of real-time reverse transcription (RT)-PCR, we evaluated modifying effect of antioxidant agents on synaptic plasticity changes in terms of glutamatergic and cholinergic signals, neurotrophin action and immediate-early gene (IEG) responses in the hippocampal dentate gyrus, amygdala and prefrontal cortex. We also immunohistochemically examined changes in neurogenesis and IEG products in the hippocampal dentate gyrus.

2. Materials and methods

2.1. Chemicals and animals

Alpha-glycosil isoquercitrin (AGIQ; purity: > 97%) was provided by San-Ei Gen F.F.I. Inc. (Osaka, Japan). DL-alpha-lipoic acid (ALA; CAS No. 1077-28-7; purity: ≥99.0%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan; Catalog No.: L0058). Mated female Slc:SD rats purchased from Japan SLC, Inc. (Hamamatsu, Japan), at gestational day (GD) 1 (appearance of vaginal plugs was designated as GD 0) were individually housed with their offspring in plastic cages with paper bedding until postnatal day (PND) 21. Animals were kept in an air-conditioned animal room (temperature: 23 ± 2 °C, relative humidity: 55 ± 15%) with a 12-h light/dark cycle. Mated female rats were provided *ad libitum* a pelleted basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) during the experimental period, and with tap water until the start of exposure to test compound. From PND 21 (where PND 0 was the day of delivery) onwards, offspring were reared and provided the CRF-1 diet with or without test compound and tap

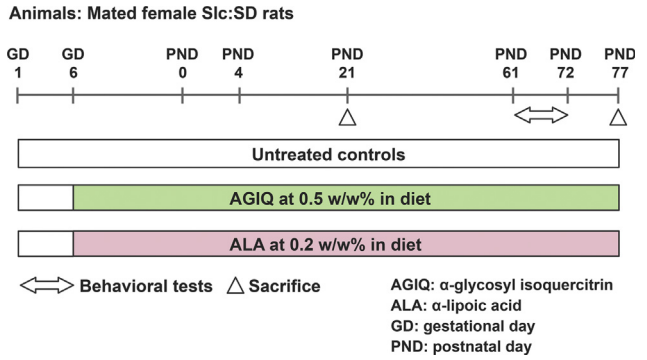

Fig. 1. Experimental design of continuous exposure to α-glycosyl isoquercitrin (AGIQ) or α-lipoic acid (ALA) from developmental stage in rats. Mated female rats were fed a diet containing AGIQ at 0.5 w/w% or ALA at 0.2 w/w% or a basal diet (untreated controls) from gestational day (GD) 6 to day 21 post-delivery. At postnatal (PND) 4, the litters were randomly culled, leaving 6 male and 2 female offspring per dam. After the interim kill of both male and female offspring for other experimental purposes, the remaining offspring were fed a diet containing AGIQ or ALA at the same dose to dams (AGIQ or ALA group) or a basal diet (untreated controls) until PND 77. Neurobehavioural tests were performed using 7 male offspring per group (1 offspring per dam) during the period from PND 61 to PND 72. On PND 77, 10 male offspring per group (1 offspring per dam) were subjected to perfusion fixation for brain immunohistochemistry. For transcript expression analysis, brain samples from 6 male offspring per group (1 male offspring per dam) were prepared. Animals used for neurobehavioural analyses were excluded from those for immunohistochemical and transcript expression analyses. The remaining brain samples from offspring were prepared for other purposes of analysis.

water *ad libitum* throughout the experimental period.

2.2. Experimental design

Mated female rats were randomly divided into three groups of untreated controls (13 animals), AGIQ group (19 animals) and ALA group (19 animals) (Fig. 1). These animals were treated from GD 6 to day 21 post-delivery with AGIQ at 0.5% (w/w) or ALA at 0.2% (w/w) in diet. The chosen dosage of AGIQ or ALA has been shown to suppress the promotion of hepatic preneoplastic lesions in rats (Kimura et al., 2013; Fujii, Kimura, et al., 2013; Fujii, Segawa, et al., 2013).

Dams were subjected to measurements of body weight (BW), as well as food and water consumption, every 3–4 days between GD 6 and day 21 post-delivery. At PND 4, the litters were randomly culled, leaving 6 male and 2 female offspring per dam. The offspring were weighed every 3 or 4 days until PND 21. Dams were euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anaesthesia and subjected to necropsy at day 21 after delivery. In the present study, male offspring were selected for neurobehavioural analyses, as well as immunohistochemical and gene expression analyses, because neurogenesis is influenced by circulating levels of steroid hormones during the estrous cycle (Pawluski, Brummelte, Barha, Crozier, & Galea, 2009).

On PND 21, 32 or 34 male and 11 or 12 female offspring per group (2–3 male offspring and 1 female offspring per dam) were either subjected to perfusion fixation or euthanized by exsanguination from the abdominal aorta under CO_2/O_2 anaesthesia and subjected to necropsy for brain tissue sampling for other purposes of analysis. The remaining offspring were fed a diet containing AGIQ or ALA at the same dose to dams (AGIQ or ALA group) or a basal diet (untreated controls) until PND 77 (2 or 3 animals/cage); BW, as well as food and water consumption, was measured once weekly.

On PND 77, 10 male offspring per group (1 male offspring per dam) were subjected to perfusion fixation for brain immunohistochemistry through the left cardiac ventricle with cold 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) at a flow rate of 35 mL/min under CO_2/O_2 anaesthesia. For transcript expression analysis, brain samples from 6 male offspring per group (1 male offspring per dam) were prepared. The remaining brain samples from 16 or 18 male and 11 or 12 female offspring per group (1–2 male offspring per dam and 1 female offspring per dam) were prepared for other purposes of analysis. Animals used for neurobehavioural analyses were excluded from those for immunohistochemical and transcript expression analyses.

All dams and offspring were checked for general conditions in terms of the appearance of abnormal gait and behaviours everyday until PND 21, and then, offspring were checked at the time of BW measurement.

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. All efforts were made to minimize animal suffering.

2.3. Neurobehavioural tests

2.3.1. Y-maze test

Y-maze test was performed on PND 61 and PND 62 to assess the short-term spatial memory of rats. The Y-maze consisted of matt grey polyvinyl plastic and had three arms (600 mm long, 250 mm high, 60 mm wide at the bottom, 250 mm wide at the top) at angles of 120° (YM-3001; O'Hara & Co., Ltd., Tokyo, Japan). The illumination was set at 5 Lux at the centre of Y-maze. Rats were transferred from the animal room to the behaviour test room 1 h before the start of the test and acclimatized to the behaviour test room. Rats were placed at the end of one arm and allowed to move freely for 5 min. Apparatus was cleaned with 70% ethanol solution before each test. The number of arm entries and movement distance were recorded by a CCD camera (WAT-902B; Watec Co., Ltd., Tsuruoka, Japan) mounted above the maze and evaluated by automatic video tracking (TimeYM1 software; O'Hara & Co., Ltd.). In video tracking analysis, object-centred coordinates were tracked to judge whether animals were moved to any of the arm regions or centre region. Spontaneous alternation behaviour was defined as successive entries into the three arms, in overlapping triplet sets. The percent alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries -2×100). All experiments were conducted during the period from 12:00p.m. to 18:00 p.m., and the order of animal selection for test among groups was counter-balanced across test time to avoid any bias in the trial time of each group.

2.3.2. Contextual fear conditioning test

Contextual fear conditioning testing was performed during PND 64 and PND 72. Conditioning and testing took place in a rodent observation cage ($30 \times 37 \times 25$ cm) that was placed in a sound-attenuating chamber (CL-4211; O'Hara & Co., Ltd.). The side walls of the observation cage (CL-3001; O'Hara & Co., Ltd.) were constructed of Plexiglas, and the door was also constructed of Plexiglas. The floor consisted of 21 steel rods through which a scrambled shock from a shock generator (SGA-2020; O'Hara & Co., Ltd.) could be delivered. Each observation cage was cleaned with a 70% ethanol solution before each session. During the animal test, the chamber was ventilated, kept at a background white noise level of 50 dB, and illuminated at 200 Lux by white light emitting diode bulbs.

On the conditioning and each testing day, each rat was transported from the animal room to the behavioural test room one hour before the start of treatment and placed in the conditioning chamber.

Contextual fear conditioning (Day 1): Rats were moved to the testing chamber and after 138 s of exposure to the cage, they received two 2 s footshocks (0.5 mA intensity) with a 100 s interval between the two successive footshocks. Rats were removed from the conditioning chamber 60 s after the second shock and returned to their home cages. Thus, it took 5 min for a trial.

Fear acquisition (Day 2): The fear acquisition testing was performed

24 h after the conditioning. Animals were placed back into the original training context for 5 min, during which no footshock was delivered.

Fear extinction (Day 5, 7, 9): The following fear extinction testing was performed at 4, 6 and 8 days after the conditioning. Animals were placed back into the original training context for 5 min, during which no footshock was delivered.

The animals' behaviour was video recorded by a CCD camera (902B; O'Hara & Co., Ltd.) and analysed using a automatic video tracking system (TimeFZ2 software; O'Hara & Co., Ltd.), and freezing time was measured. All experiments were conducted between 8:00 a.m. and 16:00. p.m. to avoid the influence of circadian hormonal fluctuations, and the order of animal selection for test among groups was counterbalanced across test time to avoid any bias in the trial time of each group.

2.4. Immunohistochemistry and apoptotic cell detection

For immunohistochemistry analysis, perfusion-fixed brains were additionally fixed with 4% (w/v) PFA in 0.1 M phosphate buffer (pH 7.4) overnight. Coronal slices were prepared at −3.5 mm from bregma in PND 77 offspring brains ($N = 10$/group). Brain slices were further fixed with the same fixative overnight at 4 °C. Brain slices were routinely processed for paraffin embedding and were sectioned into 3-μm-thick sections.

Brain sections from offspring at PND 77 were subjected to immunohistochemistry using primary antibodies against the following listed in Table S1: glial fibrillary acidic protein (GFAP), which is expressed in type-1 stem cells (radial glial cells) in the SGZ and astrocytes (Kempermann et al., 2004); SRY (sex determining region Y)-box 2 (SOX2), which is expressed in type-1 stem cells and type-2a progenitor cells in the SGZ (Steiner et al., 2006); T-box brain protein 2 (TBR2), expressed in type-2b progenitor cells in the SGZ (Lugert et al., 2012); doublecortin (DCX), which is expressed in type-2b and type-3 progenitor cells and immature granule cells in the SGZ and GCL (Kempermann et al., 2004); neuronal nuclei (NeuN), which is expressed in postmitotic neurons of both immature and mature granule cells in the SGZ and GCL (Lugert et al., 2012); and reelin (RELN), parvalbumin (PVALB), calbindin-D-29K (CALB2) and somatostatin (SST), which are expressed in GABAergic interneurons in the hilus of the dentate gyrus (Freund & Buzsáki, 1996); proliferating cell nuclear antigen (PCNA), a cell proliferation marker in the SGZ; activity-regulated cytoskeleton-associated protein (ARC), Fos proto-oncogene, AP-1 transcription factor subunit (FOS), which are members of the IEGs involved in synaptic plasticity (Guzowski, 2002) in the GCL; phosphorylated p44/42 MAPK (ERK1/2) (p-ERK1/2), a member of MAPK family that is activated by phosphorylation to promote transcriptional programs leading to the induction of *Arc* and *Fos* (Brami-Cherrier, Roze, Girault, Betuing, & Caboche, 2009); and cyclooxygenase 2 (COX2), which is expressed in hippocampal granule cells and is known to regulate synaptic plasticity (Chen, Magee, & Bazan, 2002). The respective primary antibodies were applied to brain sections for incubation overnight at 4 °C. One section per animal was subjected to immunohistochemistry of each molecule.

To block endogenous peroxidase, deparaffinized sections were incubated in 0.3% (v/v) H_2O_2 solution in absolute methanol for 30 min. The antigen retrieval conditions that were applied for some antibodies are listed in Table S1. Immunodetection was conducted using a Vectastain® Elite ABC kit (PK-6101 and PK-6102; Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine (DAB)/H_2O_2 as the chromogen. Haematoxylin counterstaining was then performed, and coverslips were mounted on immunostained sections for microscopic examination.

To evaluate apoptosis in the SGZ of the dentate gyrus in the offspring, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) assay was performed using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (S7100; Merck Millipore, Burlington, MA, USA) according to the manufacturer's instructions, with DAB/H_2O_2 as the chromogen. One section per animal was subjected to TUNEL assay.

Table 1
Changes in short-term spatial memory in Y-maze test.

	Control	AGIQ	ALA
No. of animals examined	7	7	7
Alternation rate (%)	76.39 ± 13.78[a]	67.57 ± 10.52	74.26 ± 15.75
Total arm entries	13.14 ± 4.78	15.43 ± 4.35	15.71 ± 2.29

Abbreviations: AGIQ, α-glycosyl isoquercitrin; ALA, α-lipoic acid.
[a] Mean ± SD.

2.5. Evaluation of immunoreactive cells and apoptotic cells

Immunoreactive cells, i.e., $GFAP^+$, $SOX2^+$, $TBR2^+$, DCX^+, $NeuN^+$, ARC^+, FOS^+, p-$ERK1/2^+$, $COX2^+$, and $PCNA^+$, or $TUNEL^+$ apoptotic cells, in the SGZ and/or GCL were bilaterally counted and normalized for the length of the SGZ (Fig. S1). Immunoreactive cells distributed within the hilus of the hippocampal dentate gyrus, i.e., $RELN^+$, $PVALB^+$, $CALB2^+$ or SST^+ cells were bilaterally counted and normalized per area unit of the hilus area. These immunolocalized and apoptotic cells were analysed by blind trial for the treatment conditions. Immunoreactive neurons located inside of the Cornu Ammonis region 3, consisting of large pyramidal neurons that can be morphologically distinguished from relatively small interneurons, were excluded from counting immunoreactive cells in the hilus of the dentate gyrus. Number of each immunoreactive cellular population (except for $NeuN^+$ cells in the GCL) or $TUNEL^+$ apoptotic cells was manually counted under microscopic observation using a BX53 microscope (Olympus Corporation, Tokyo, Japan). In case of $NeuN^+$ cells in the GCL, digital photomicrographs at ×100-fold magnification were taken using a BX53 microscope attached to a DP72 Digital Camera System (Olympus Corporation), and positive cell counting was performed applying the WinROOF image analysis software package (version 5.7; Mitani Corporation, Fukui, Japan). The length of the SGZ and the hilar area were measured in microscopic images at ×40-fold magnification by applying the cellSens image analysis software package (standard package 1.9; Olympus Corporation).

2.6. Transcript expression analysis

Transcript expression levels in the hippocampal dentate gyrus, amygdala, infralimbic cortex and prelimbic cortex were examined using real-time RT-PCR in offspring on PND 77. Brain tissues were dissected and processed using the methacarn solution as previously described (Akane et al., 2013). In brief, 2-mm-thick coronal cerebral slices were prepared at the position of −3.6 mm from bregma to collect tissue samples of the hippocampal dentate gyrus and amygdala using a punch-biopsy device with a pore size of 1 mm in diameter (BP-10F; Kai Industries Co., Ltd., Gifu, Japan) (Fig. S2). In case of sampling of infralimbic cortex and prelimbic cortex tissues, 1-mm-thick coronal cerebral slices were prepared at the position of +2.7 mm from bregma (Fig. S2). Total RNA was extracted from brain tissue samples of all groups (n = 6 per group) using the RNeasy Mini kit (Catalog No.: 74104; Qiagen). First-strand cDNA was synthesized using SuperScript® III Reverse Transcriptase (Catalog No.: 18080085; Thermo Fisher Scientific, Waltham, MA, USA) in a 20-μL total reaction mixture with 1 μg of total RNA. Analysis of the transcript levels for gene targets shown in Table S2 was performed using PCR primers designed with Primer Express software (Version 3.0; Thermo Fisher Scientific). Real-time PCR with Power SYBR™ Green PCR Master Mix (Catalog No.: 4368702; Thermo Fisher Scientific) was conducted using a StepOnePlus™ Real-time PCR System (Thermo Fisher Scientific). The relative differences in gene expression between the untreated controls and AGIQ or ALA group were calculated

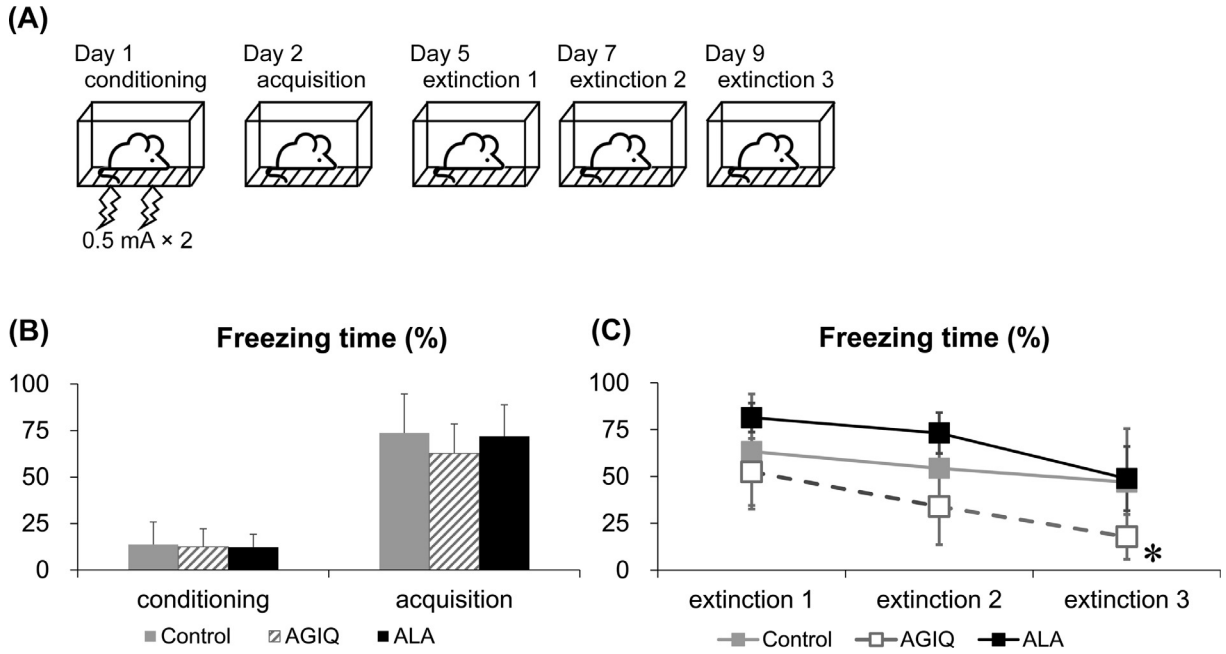

Fig. 2. Contextual fear conditioning test. Graphs show the freezing time in the fear conditioning, fear acquisition and fear extinction in untreated controls and each exposure group. (A) Summary of experimental design. (B) Freezing time in the fear acquisition and fear conditioning. (C) Freezing time in the 1st, 2nd and 3rd trials of fear extinction. Values are expressed as mean + SD or mean ± SD. N = 7 in each group. $^{*}P < 0.05$, compared to untreated controls by Dunnett's or Steel's test.

Table 2
Number of immunoreactive cells or TUNEL^{+} cells in the SGZ/GCL or hilar region of the hippocampal dentate gyrus.

	Control	AGIQ	ALA
No. of animals examined	10	10	10
Granule cell lineage subpopulations (No./mm SGZ length)			
GFAP	2.78 ± 0.43 [a]	3.06 ± 1.02	3.42 ± 1.19
SOX2	17.58 ± 2.41	18.98 ± 3.06	16.80 ± 2.26
TBR2	2.13 ± 0.88	3.09 ± 1.82	2.50 ± 1.15
DCX	7.64 ± 2.86	7.87 ± 3.83	6.18 ± 2.88
NeuN	505.03 ± 59.80	511.68 ± 70.75	525.21 ± 52.72
Cell proliferation and apoptosis (No./mm SGZ length)			
PCNA	2.02 ± 0.54	2.17 ± 0.59	2.07 ± 1.04
TUNEL	0.79 ± 2.50	0.80 ± 0.96	0.68 ± 1.43
Interneuron subpopulations (No./mm^2 hilar region)			
RELN	48.35 ± 10.18	57.60 ± 16.54	52.04 ± 28.57
PVALB	12.92 ± 4.53	14.60 ± 8.17	15.65 ± 10.01
CALB2	3.85 ± 2.42	2.58 ± 2.71	2.01 ± 1.68
SST	34.94 ± 6.34	34.72 ± 12.98	35.02 ± 10.27
Synaptic plasticity-related molecules (No./mm SGZ length)			
ARC	1.10 ± 0.65	1.52 ± 0.87	1.34 ± 0.78
FOS	0.74 ± 0.32	1.33 ± 0.40*	0.85 ± 0.43
COX2	10.56 ± 4.30	13.63 ± 4.11	8.90 + 2.67
p-ERK1/2	1.28 ± 1.12	1.30 ± 0.99	1.25 ± 0.66

Abbreviations: AGIQ, α-glycosyl isoquercitrin; ALA, α-lipoic acid; ARC, activity-regulated cytoskeleton-associated protein; CALB2, calbindin-D–29K; COX2, cyclooxygenase 2; DCX, doublecortin; FOS, Fos proto-oncogene, AP-1 transcription factor subunit; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; PCNA, proliferating cell nuclear antigen; p-ERK1/2, phosphorylated extracellular signal-regulated kinase 1/2 (MAP kinase1/2); PVALB, parvalbumin; RELN, reelin; SGZ, subgranular zone; SOX2, SRY (sex determining region Y)-box 2; SST, somatostatin; TBR2, T-box brain protein 2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.

[a] Mean ± SD.

* $P < 0.05$, significantly different from untreated controls by Dunnett's test or Steel's test.

using threshold cycle (C_T) values that were first normalized to *Hprt1* or *Gapdh*, which served as endogenous controls in the same sample, and then relative to a control C_T value using the $2^{-\Delta\Delta C}{}_T$ method (Livak & Schmittgen, 2001).

2.7. Statistical analysis

Maternal data, such as BW, brain weights, and food and water consumption were analysed using the individual animal as the experimental unit. Offspring data, such as BW and brain weight at necropsy, the number of immunoreactive cells for each antigen, number of apoptotic cells, and the transcript expression level, were analysed using the litter as the experimental unit. Differences between untreated controls, AGIQ group and ALA group were evaluated as follows. Data were analysed using Bartlett's test for homogeneity of variance. If the variance was homogenous, numerical data were evaluated using Dunnett's test to compare between untreated controls and AGIQ or ALA group. For heterogeneous data, the Steel's test was used. All analyses were conducted using an Excel Statistics 2013 software package version 2.02 (Social Survey Research Information Co. Ltd., Tokyo, Japan), and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Maternal parameters

One non-pregnant animal in each of the untreated controls and ALA group and three animals which had abortions as confirmed by examination of implantation sites and the number of live offspring at birth, one in the AGIQ group and two in the ALA group, were excluded from the experiment. Therefore, the effective numbers of dams were 12, 18 and 16 for untreated controls, AGIQ and ALA group, respectively. The number of implantation sites and live offspring and male ratio were not different between the untreated controls and each of the AGIQ and ALA groups (Table S3). Dams in AGIQ group showed no significant changes in BW during gestation and lactation periods compared with untreated controls (Fig. S3). In contrast, dams in ALA group showed significantly lower BW from PND 1 onwards. Food consumption in

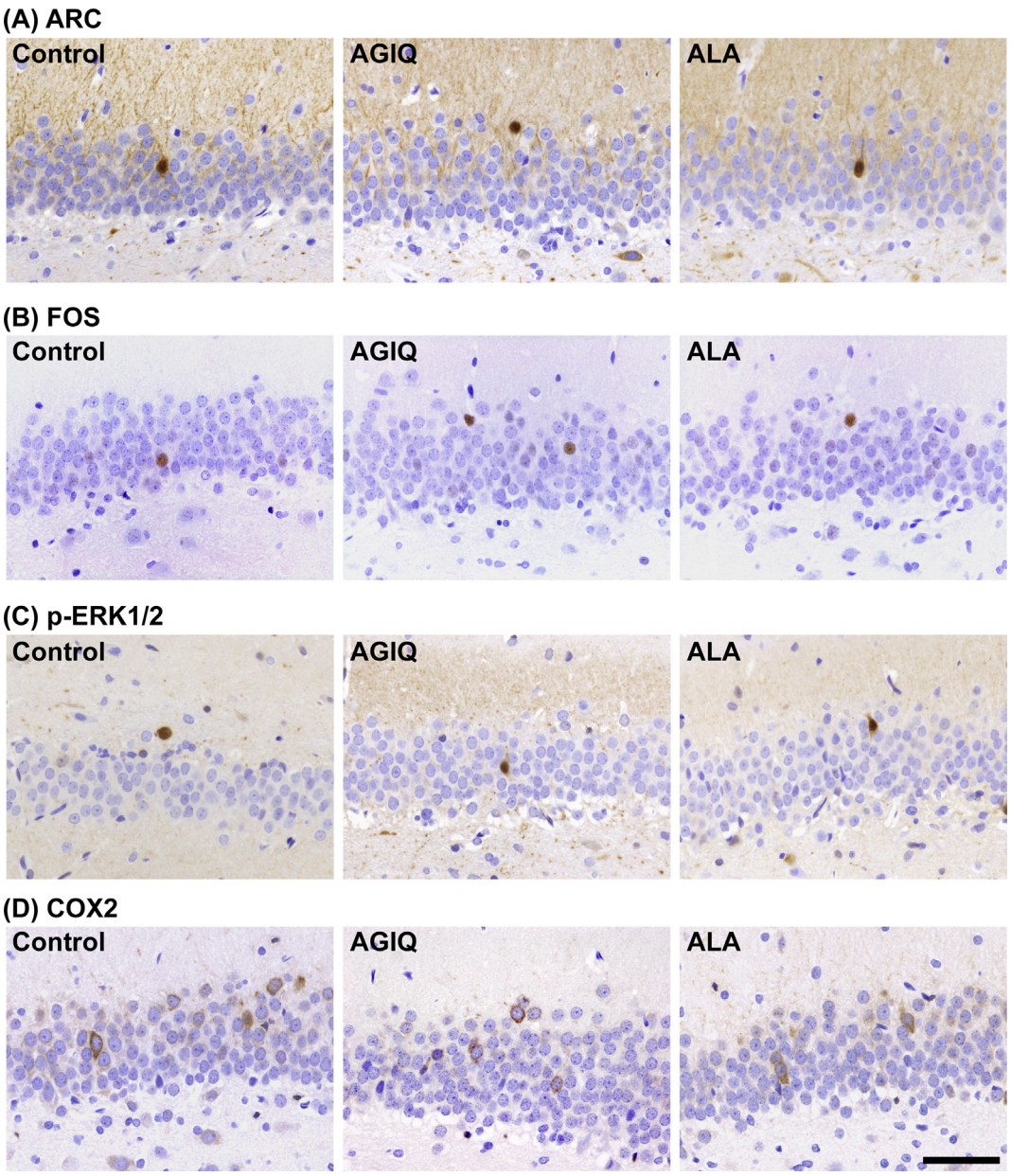

Fig. 3. Distribution of immunoreactive cells for synaptic plasticity-related molecules in the granule cell layer of the hippocampal dentate gyrus. (A) ARC. (B) FOS. (C) p-ERK1/2. (D) COX2. Representative images from untreated controls (left), AGIQ group (middle) and ALA group (right) on PND 77. Magnification ×400; bar = 50 μm.

AGIQ group was significantly decreased on GD 9 and then increased on PND 9 and PND 16 compared with untreated controls (Fig. S3). In the ALA group, food consumption was significantly decreased from GD 6 to GD 21, PND 6, PND 13 and PND 21. Water consumption in AGIQ group was significantly increased at GD 9, PND 9 and PND 16 compared with untreated controls (Fig. S3). In the ALA group, water consumption was significantly decreased at GD 5, GD 6, GD 14, PND 1, PND 6, PND 13, PND 20 and PND 21 compared with untreated controls. There were no abnormalities in the gait and behaviours of dams in any group. At necropsy on PND 21, there was no change in BW and absolute and relative brain weights in AGIQ group compared with untreated controls (Table S3). In contrast, BW was significantly decreased in ALA group compared with untreated controls. Both absolute and relative brain weights in ALA group were unchaged compared with untreated controls. Based on mean values of food consumption, dams in the AGIQ and ALA groups received 306.0 and 105.3 mg/kg BW/day AGIQ and ALA, respectively, during the gestation period. Dams took 650.6 and 228.0 mg/kg BW/day AGIQ and ALA during the lactation period, respectively.

3.2. In life parameter data and necropsy data of offspring

No abnormalities in the gait and behaviours were observed in offspring of any group before necropsy on PND 21 and after weaning. BW of male offspring of the ALA group significantly decreased from PND 4 to PND 77 compared with untreated controls (Table S4). BW of female offspring of the ALA group from PND 4 to PND 21 significantly decreased compared with untreated controls (Table S4). Food consumption was significantly decreased in AGIQ group on PND 28, PND 35, PND 49 and PND 56 and in ALA group from PND 28 to PND 69 compared with untreated controls (Fig. S4). Water consumption was significantly decreased on PND 28 and then significantly increased on PND 35 in AGIQ group compared with untreated controls (Fig. S4). Water consumption was significantly decreased from PND 28 onwards in ALA group compared with untreated controls. At necropsy on PND 21, both

Table 3
Transcript expression changes in the hippocampal dentate gyrus.

	Control		AGIQ		ALA	
Normalization control	*Gapdh*	*Hprt1*	*Gapdh*	*Hprt1*	*Gapdh*	*Hprt1*
No. of animals examined	6	6	6	6	6	6
Cholinergic receptors						
Chrm1	1.09 ± 0.50 [a]	1.27 ± 0.92	1.03 ± 0.29	0.73 ± 0.29	0.75 ± 0.20	0.56 ± 0.17
Chrm2	1.04 ± 0.33	1.12 ± 0.58	2.08 ± 0.35**	2.02 ± 0.83*	2.29 ± 0.48**	1.92 ± 0.36
Chrna7	1.04 ± 0.29	1.07 ± 0.43	0.87 ± 0.17	0.83 ± 0.30	0.81 ± 0.31	0.68 ± 0.26
Glutamate receptors						
Gria1	1.03 ± 0.28	1.04 ± 0.31	0.96 ± 0.13	0.91 ± 0.29	0.77 ± 0.34	0.64 ± 0.28
Gria2	1.02 ± 0.19	1.05 ± 0.36	0.94 ± 0.16	0.88 ± 0.27	0.82 ± 0.35	0.68 ± 0.29
Grin2a	1.03 ± 0.29	1.04 ± 0.33	0.92 ± 0.23	0.89 ± 0.36	0.80 ± 0.28	0.68 ± 0.25
Grin2d	1.11 ± 0.54	1.13 ± 0.55	1.91 ± 0.85	1.89 ± 1.30	1.88 ± 0.21	1.60 ± 0.28
Glutamate transporters						
Slc17a7	1.02 ± 0.25	1.05 ± 0.35	1.15 ± 0.20	1.11 ± 0.44	1.20 ± 0.51	1.01 ± 0.42
Slc17a6	1.53 ± 1.64	1.56 ± 1.37	5.88 ± 1.83**	5.75 ± 3.13**	5.98 ± 1.03**	5.11 ± 1.28*
Synaptic plasticity-related						
Fos	1.04 ± 0.34	1.04 ± 0.30	1.82 ± 0.50**	1.82 ± 1.01	1.72 ± 0.28*	1.46 ± 0.31*
Arc	1.05 ± 0.38	1.05 ± 0.34	0.73 ± 0.13	0.72 ± 0.35	0.71 ± 0.24	0.59 ± 0.19*
Neurotrophin-related						
Bdnf	1.04 ± 0.31	1.07 ± 0.40	0.94 ± 0.10	0.90 ± 0.31	1.07 ± 0.45	0.90 ± 0.38
Ntrk2	1.01 ± 0.15	1.07 ± 0.39	1.31 ± 0.14*	1.26 ± 0.45	1.45 ± 0.26**	1.22 ± 0.21
Motor protein						
Kif21b	1.02 ± 0.20	1.04 ± 0.29	1.48 ± 0.21*	1.42 ± 0.52	1.34 ± 0.45	1.13 ± 0.39

Abbreviations: AGIQ, α-glycosyl isoquercitrin; ALA, α-lipoic acid; *Arc*, activity-regulated cytoskeleton-associated protein; *Bdnf*, brain-derived neurotrophic factor; *Chrm1*, cholinergic receptor, muscarinic 1; *Chrm2*, cholinergic receptor, muscarinic 2; *Chrna7*, cholinergic receptor nicotinic alpha 7 subunit; *Fos*, Fos proto-oncogene, AP-1 transcription factor subunit; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Gria1*, glutamate ionotropic receptor AMPA type subunit 1; *Gria2*, glutamate ionotropic receptor AMPA type subunit 2; *Grin2a*, glutamate ionotropic receptor NMDA type subunit 2A; *Grin2d,* glutamate ionotropic receptor NMDA type subunit 2D; *Hprt1*, hypoxanthine phosphoribosyltransferase 1; *Kif21b*, kinesin family member 21B; *Ntrk2*, neurotrophic receptor tyrosine kinase 2; *Slc17a7*, solute carrier family 17 member 7; *Slc17a6*, solute carrier family 17 member 6.

[a] Mean ± S.D.

* $P < 0.05$.

** $P < 0.01$, significantly different from untreated controls by Dunnett's test or Steel's test.

male and female offspring of the ALA group showed significantly decreased BW and absolute brain weight and significantly increased relative brain weight compared with untreated controls (Table S5). At necropsy on PND 77, male offspring of the ALA group showed significantly decreased BW and significantly increased relative brain weight compared with untreated controls. Offspring in the AGIQ and ALA groups received 417.9 and 196.6 mg/kg BW/day AGIQ and ALA, respectively.

3.3. Neurobehavioural testing scores of male offspring

3.3.1. Y-maze test

With regard to the Y-maze test, there were no significant differences in the alternation rate and total arm entries between untreated controls and each exposure group (Table 1).

3.3.2. Contextual fear conditioning test

With regard to the contextual fear conditioning test, there were no significant differences in the freezing time (%) in the fear acquisition at 24 h after fear conditioning between untreated controls and each exposure group (Fig. 2, Table S6). There were no significant differences in the freezing time in the 1st and 2nd trials of fear extinction between untreated controls and each exposure group. With regard to the freezing time in the 3rd trial of fear extinction, AGIQ group showed a significantly lower rate compared to untreated controls, while ALA group did not alter the rate compared to untreated controls.

3.4. Number of granule cell lineage subpopulations in the SGZ and/or GCL

There were no significant differences in the number of $GFAP^+$, $SOX2^+$ and $TBR2^+$ cells in the SGZ, and DCX^+ and $NeuN^+$ cells in the SGZ and GCL between untreated controls and each exposure group (Table 2, Fig. S5).

3.5. Number of interneuron subpopulations in the hilus of the dentate gyrus

There were no significant differences in the densities of $RELN^+$, $PVALB^+$, $CALB2^+$ and SST^+ cells in the hilus of the dentate gyrus between untreated controls and each exposure group (Table 2, Fig. S6).

3.6. Proliferating and apoptotic cells in the SGZ

There were no significant differences in the number of $PCNA^+$ proliferating cells or $TUNEL^+$ apoptotic cells in the SGZ between untreated controls and each exposure group (Table 2, Fig. S7).

3.7. Distribution of synaptic plasticity-related molecule-immunoreactive cells in the GCL

There were no significant differences in the number of ARC^+, $COX2^+$ or p-$ERK1/2^+$ cells in the GCL between untreated controls and each exposure group (Table 2, Fig. 3). With regard to the FOS^+ cells, AGIQ group showed a significantly higher number compared with untreated controls, while ALA group did not alter the number compared with untreated controls.

3.8. Transcript expression in the hippocampal dentate gyrus, amygdala and prefrontal cortex

In the hippocampal dentate gyrus, transcript level of *Chrm2* was

Table 4
Transcript expression changes in the amygdala.

	Control		AGIQ		ALA	
Normalization control	*Gapdh*	*Hprt1*	*Gapdh*	*Hprt1*	*Gapdh*	*Hprt1*
No. of animals examined	6	6	6	6	6	6
Cholinergic receptors						
Chrm1	1.06 ± 0.39 [a]	1.04 ± 0.29	0.96 ± 0.36	1.67 ± 0.99	0.95 ± 0.23	1.42 ± 0.68
Chrm2	0.98 ± 0.39	1.02 ± 0.22	1.43 ± 0.50	1.30 ± 0.37	1.01 ± 0.11	1.06 ± 0.19
Chrna7	1.02 ± 0.22	1.00 ± 0.11	1.29 ± 0.37	1.20 ± 0.30	1.44 ± 0.41	1.49 ± 0.48
Glutamate receptors						
Gria1	1.06 ± 0.41	1.02 ± 0.24	1.08 ± 0.42	0.99 ± 0.32	0.86 ± 0.27	0.88 ± 0.24
Gria2	1.08 ± 0.48	1.04 ± 0.30	1.18 ± 0.56	1.04 ± 0.21	1.08 ± 0.26	1.12 ± 0.33
Grin2a	1.02 ± 0.25	1.03 ± 0.25	1.02 ± 0.34	0.93 ± 0.21	0.94 ± 0.27	0.98 ± 0.35
Grin2d	1.09 ± 0.46	1.07 ± 0.36	1.64 ± 0.37 *	1.61 ± 0.66	1.34 ± 0.30	1.39 ± 0.38
Glutamate transporters						
Slc17a7	1.22 ± 0.58	1.26 ± 0.64	1.84 ± 0.89	1.61 ± 0.54	2.04 ± 0.72	2.13 ± 0.79
Slc17a6	0.98 ± 0.33	1.04 ± 0.30	1.43 ± 0.68	1.58 ± 1.39	1.73 ± 0.21 *	1.81 ± 0.39 **
Synaptic plasticity-related						
Fos	1.04 ± 0.32	1.04 ± 0.30	0.81 ± 0.33	0.72 ± 0.19	0.70 ± 0.15	0.72 ± 0.19
Arc	1.11 ± 0.38	1.03 ± 0.26	1.11 ± 0.71	0.92 ± 0.21	0.78 ± 0.22	0.81 ± 0.24
Neurotrophin-related						
Bdnf	1.10 ± 0.41	1.12 ± 0.47	1.81 ± 1.01	1.55 ± 0.36	1.88 ± 0.35 *	1.93 ± 0.31 **
Ntrk2	1.08 ± 0.46	1.04 ± 0.33	1.59 ± 0.81	1.38 ± 0.37	1.03 ± 0.18	1.06 ± 0.14
Motor proteins						
Kif21b	1.07 ± 0.38	1.03 ± 0.27	0.86 ± 0.31	0.79 ± 0.24	0.93 ± 0.31	0.99 ± 0.46

Abbreviations: AGIQ, α-glycosyl isoquercitrin; ALA, α-lipoic acid; *Arc*, activity-regulated cytoskeleton-associated protein; *Bdnf*, brain-derived neurotrophic factor; *Chrm1*, cholinergic receptor, muscarinic 1; *Chrm2*, cholinergic receptor, muscarinic 2; *Chrna7*, cholinergic receptor nicotinic alpha 7 subunit; *Fos*, Fos proto-oncogene, AP-1 transcription factor subunit; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Gria1*, glutamate ionotropic receptor AMPA type subunit 1; *Gria2*, glutamate ionotropic receptor AMPA type subunit 2; *Grin2a*, glutamate ionotropic receptor NMDA type subunit 2A; *Grin2d,* glutamate ionotropic receptor NMDA type subunit 2D; *Hprt1*, hypoxanthine phosphoribosyltransferase 1; *Kif21b*, kinesin family member 21B; *Ntrk2*, neurotrophic receptor tyrosine kinase 2; *Slc17a7*, solute carrier family 17 member 7; *Slc17a6*, solute carrier family 17 member 6.

[a] Mean ± S.D.

* $P < 0.05$.

** $P < 0.01$, significantly different from the untreated controls by Dunnett's test or Steel's test.

increased in AGIQ group after normalization with *Gapdh* and *Hprt1* and in ALA group after normalization with *Gapdh* compared with untreated controls (Table 3). Transcript level of *Slc17a6* was increased in AGIQ and ALA groups after normalization with *Gapdh* and *Hprt1* compared with untreated controls. Transcript level of *Fos* was increased in AGIQ group after normalization with *Gapdh* and in ALA group after normalization with *Gapdh* and *Hprt1* compared with untreated controls. Transcript level of *Arc* was decreased in ALA group after normalization with *Hprt1* compared with untreated controls. Transcript level of *Ntrk2* was increased in AGIQ and ALA groups after normalization with *Gapdh* compared with untreated controls. Transcript level of *Kif21b* was increased in AGIQ group after normalization with *Gapdh*.

In the amygdala, transcript level of *Grin2d* was increased in AGIQ group after normalization with *Gapdh* compared with untreated controls (Table 4). Transcript level of *Slc17a6* and *Bdnf* was increased in ALA group after normalization with *Gapdh* and *Hprt1* compared with untreated controls.

In the infralimbic cortex, transcript level of *Chrm1* was increased in ALA group after normalization with *Hprt1* compared with untreated controls (Table 5). Transcript level of *Chrna7* was increased in AGIQ group after normalization with *Hprt1* compared with untreated controls. Transcript level of *Gria1* was increased in ALA group after normalization with *Gapdh* and *Hprt1* compared with untreated controls. Transcript level of *Grin2a* was increased in ALA group after normalization with *Gapdh* compared with untreated controls. Transcript level of *Grin2d* was increased in ALA group after normalization with *Hprt1* compared with untreated controls. Transcript level of *Fos* was decreased in AGIQ group after normalization with *Gapdh* compared with untreated controls. Transcript level of *Bdnf* was increased in ALA group after normalization with *Gapdh* and *Hprt1* compared with untreated controls. Transcript level of *Ntrk2* was increased in ALA group after normalization with *Gapdh* compared with untreated controls. Transcript level of *Kif21b* was increased in ALA group after normalization with *Gapdh* compared with untreated controls.

In the prelimbic cortex, transcript level of *Fos* was decreased in ALA group after normalization with *Gapdh* compared with untreated controls (Table 6). Transcript level of *Ntrk2* was increased in ALA group after normalization with *Hprt1* compared with untreated controls.

4. Discussion

In the present study, we examined neurobehavioural effects of AGIQ in comparison with ALA on offspring by oral exposure to dams during the gestation and lactation and to offspring from weaning until adult stage. As a result, both of dams and offspring of ALA group decreased body weight and food and water consumption. In contrast, dams of AGIQ group only showed temporarily high food or water consumption. Offspring in this group showed decreased food consumption; however, the magnitude of the decreases was weaker than that of ALA. With regard to neurobehavioural testing scores of male offspring, there were no changes in Y-maze test in both AGIQ and ALA. However, AGIQ group showed facilitation of fear extinction at the 3rd trial in the contextual fear conditioning test. Immunohistochemically, there were no number changes of cells consisting of hippocampal neurogenesis in both AGIQ and ALA. However, AGIQ group showed increase of FOS^+ granule cells among synaptic plasticity-related molecule-immunoreactive cells, while ALA group did not change the number of any type of cells. Both AGIQ and ALA upregulated transcript level of several genes including *Fos* in the hippocampal dentate gyrus, while AGIQ-alone upregulated *Kif21b*. In the amygdala and infralimbic cortex, AGIQ

Table 5
Transcript expression changes in the infralimbic cortex.

	Control		AGIQ		ALA	
Normalization control	*Gapdh*	*Hprt1*	*Gapdh*	*Hprt1*	*Gapdh*	*Hprt1*
No. of animals examined	6	6	6	6	6	6
Cholinergic receptors						
Chrm1	1.03 ± 0.26 [a]	1.14 ± 0.66	0.96 ± 0.40	0.82 ± 0.44	1.96 ± 0.83	1.26 ± 0.27 *
Chrm2	1.06 ± 0.20	1.01 ± 0.19	0.88 ± 0.16	1.13 ± 0.20	1.45 ± 0.53	1.08 ± 0.26
Chrna7	1.03 ± 0.28	1.01 ± 0.16	1.00 ± 0.18	1.30 ± 0.20 *	1.45 ± 0.64	1.03 ± 0.20
Glutamate receptors						
Gria1	1.05 ± 0.32	1.03 ± 0.23	0.87 ± 0.16	1.12 ± 0.19	1.91 ± 0.75 *	1.43 ± 0.38 *
Gria2	1.05 ± 0.33	1.01 ± 0.18	0.95 ± 0.25	1.21 ± 0.19	1.78 ± 0.77	1.26 ± 0.19
Grin2a	1.02 ± 0.19	1.01 ± 0.18	0.95 ± 0.17	1.23 ± 0.22	1.72 ± 0.60 *	1.29 ± 0.36
Grin2d	1.04 ± 0.32	1.04 ± 0.28	0.82 ± 0.17	1.06 ± 0.15	2.06 ± 1.03	1.44 ± 0.32 *
Glutamate transporters						
Slc17a7	1.03 ± 0.25	1.00 ± 0.09	0.79 ± 0.27	0.98 ± 0.24	1.72 ± 0.73	1.22 ± 0.13
Slc17a6	1.04 ± 0.18	1.02 ± 0.20	1.04 ± 0.17	1.38 ± 0.47	1.42 ± 0.67	1.02 ± 0.24
Synaptic plasticity-related						
Fos	1.00 ± 0.08	1.01 ± 0.14	0.78 ± 0.16 *	1.01 ± 0.14	1.29 ± 0.53	1.19 ± 0.60
Arc	1.03 ± 0.28	1.02 ± 0.24	0.99 ± 0.55	1.24 ± 0.52	1.48 ± 0.39	1.15 ± 0.36
Neurotrophin-related						
Bdnf	1.03 ± 0.26	1.02 ± 0.23	0.84 ± 0.14	1.09 ± 0.21	1.93 ± 0.76 *	1.38 ± 0.15 *
Ntrk2	1.04 ± 0.29	1.02 ± 0.22	0.86 ± 0.15	1.12 ± 0.23	1.71 ± 0.54 *	1.27 ± 0.22
Motor proteins						
Kif21b	1.09 ± 0.31	1.03 ± 0.26	0.83 ± 0.13	1.18 ± 0.49	1.71 ± 0.71*	1.23 ± 0.22

Abbreviations: AGIQ, α-glycosyl isoquercitrin; ALA, α-lipoic acid; *Arc*, activity-regulated cytoskeleton-associated protein; *Bdnf*, brain-derived neurotrophic factor; *Chrm1*, cholinergic receptor, muscarinic 1; *Chrm2*, cholinergic receptor, muscarinic 2; *Chrna7*, cholinergic receptor nicotinic alpha 7 subunit; *Fos*, Fos proto-oncogene, AP-1 transcription factor subunit; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Gria1*, glutamate ionotropic receptor AMPA type subunit 1; *Gria2*, glutamate ionotropic receptor AMPA type subunit 2; *Grin2a*, glutamate ionotropic receptor NMDA type subunit 2A; *Grin2d,* glutamate ionotropic receptor NMDA type subunit 2D; *Hprt1*, hypoxanthine phosphoribosyltransferase 1; *Kif21b*, kinesin family member 21B; *Ntrk2*, neurotrophic receptor tyrosine kinase 2; *Slc17a7*, solute carrier family 17 member 7; *Slc17a6*, solute carrier family 17 member 6.

[a] Mean ± S.D.

* $P < 0.05$, significantly different from the untreated controls by Dunnett's test or Steel's test.

upregulated less number of genes than ALA. Among them, AGIQ upregulated *Grin2d* in the amygdala. In the prelimbic cortex, gene expression changes were rather minor and observed only with ALA. Summarizing these results, AGIQ-exposed offspring revealed enhancement of fear extinction learning, increase of FOS^+ GCL cells and transcript upregulation of *Fos* and *Kif21b* in the hippocampal dentate gyrus and *Grin2d* in the amygdala. However, ALA only showed *Fos* upregulation, among the parameters that AGIQ showed alteration. ALA showed expression change of other different genes in brain regions examined. These results may suggest that ALA and AGIQ have different targets of redox sensor signals in the brain.

In the present study, both of AGIQ and ALA did not show any significant changes in the alternation rate and total arm entries in the Y-maze test, suggesting that oral exposure to these antioxidants did not influence the short-term spatial memory. With regard to fear conditioning test, we found that the freezing time in the fear acquisition at 24 h after fear conditioning was unaltered between untreated controls and the AGIQ and ALA groups, suggesting that oral exposure to these antioxidants did not influence the fear acquisition. In the fear extinction tests, AGIQ resulted in a significantly lower freezing time in the 3rd trial of fear extinction, while the freezing time was unchanged at 1st and 2nd trials in this group. However, ALA did not alter the freezing time at all time points examined in the fear extinction. These results suggest that AGIQ facilitates fear extinction learning, while ALA did not alter it. Among bioactive substances, melatonin is known to be involved in the processes that contribute to learning and memory, and it is reported that exogenously administered melatonin facilitates extinction, but not acquisition or expression, of conditional cued fear in rats (Huang, Yang, Liu, & Li, 2014). Furthermore, decreased melatonin levels in patients with anxiety disorders, such as PTSD (Rothbaum & Davis, 2003), were reported in clinical studies (McFarlane, Barton, Briggs, & Kennaway, 2010). With regard to the effect of antioxidants on fear extinction learning, there are recently published studies showing facilitation of fear extinction learning memory with curcumin or flavonoids-enriched fraction in normal animals (de Oliveira et al., 2016; Monsey et al., 2015). Curcumin and flavonoids are polyphenolic antioxidants, similar to flavonol AGIQ. Recent studies have shown that polyphenolic antioxidants, such as flavonoids and stilbenoids, exert ameliorating effect on PTSD in animal models (Lee et al., 2018; Li et al., 2018; Sanz-García et al., 2016; Wu et al., 2017; Zhang et al., 2017). However, there are no such studies dealing with neurobehavioural effects by ALA, a nonphenolic antioxidant, in normal animals. Therefore, polyphenolic antioxidants may provide an available effective means of extinction-based exposure psychotherapy for the treatment of PTSD. Facilitating effects of AGIQ on fear extinction in the present study may suggest a similar possibility as a therapeutic or prophylactic agent. Further studies addressing amelioration using PTSD models are necessary to prove therapeutic or prophylactic effect of AGIQ.

Fear extinction is a form of active learning (Bouton, Westbrook, Corcoran, & Maren, 2006), and the amygdala, hippocampus and infralimbic prefrontal cortex are involved in fear extinction (Bouton et al., 2006; Sotres-Bayon, Bush, & LeDoux, 2004). In the present study, by continuous AGIQ exposure, we found a significant increase in the number of FOS^+ mature granule cells along with transcript upregulation of *Fos* in the dentate gyrus cells, among synaptic plasticity-related molecules examined. While transcript upregulation of *Fos* was also observed in the dentate gyrus, ALA did not change the number of FOS^+ mature granule cells. In the hippocampal dentate gyrus, expression of the IEG product in granule cells suggested integration of these cells into neuronal networks (Stone et al., 2011). Increased expression of *Fos* in neurons in the hippocampal formation is regulated by neuronal activity associated with learning (Yochiy, Britto, & Hunziker, 2012) and

Table 6
Transcript expression changes in the prelimbic cortex.

	Control		AGIQ		ALA	
Normalization control	*Gapdh*	*Hprt1*	*Gapdh*	*Hprt1*	*Gapdh*	*Hprt1*
No. of animals examined	6	6	6	6	6	6
Cholinergic receptors						
Chrm1	1.11 ± 0.57[a]	1.09 ± 0.45	0.85 ± 0.48	1.09 ± 0.75	0.59 ± 0.21	1.01 ± 0.40
Chrm2	1.26 ± 0.65	1.08 ± 0.48	0.96 ± 0.36	1.04 ± 0.51	0.77 ± 0.45	0.99 ± 0.53
Chrna7	1.11 ± 0.57	1.03 ± 0.29	1.36 ± 0.67	1.32 ± 0.51	0.98 ± 0.54	1.18 ± 0.49
Glutamate receptors						
Gria1	1.14 ± 0.71	1.02 ± 0.26	1.20 ± 0.59	1.20 ± 0.54	0.98 ± 0.36	1.40 ± 0.84
Gria2	1.14 ± 0.63	1.04 ± 0.32	1.39 ± 0.83	1.29 ± 0.57	1.09 ± 0.47	1.50 ± 0.80
Grin2a	1.13 ± 0.61	1.06 ± 0.43	1.14 ± 0.70	1.06 ± 0.49	0.80 ± 0.35	1.05 ± 0.41
Grin2d	1.12 ± 0.58	1.03 ± 0.27	1.58 ± 0.75	1.51 ± 0.49	1.01 ± 0.35	1.56 ± 1.12
Glutamate transporters						
Slc17a7	1.16 ± 0.70	1.05 ± 0.39	1.21 ± 0.63	1.18 ± 0.48	1.00 ± 0.37	1.48 ± 0.96
Slc17a6	1.18 ± 0.55	1.02 ± 0.25	1.05 ± 0.37	1.18 ± 0.76	0.91 ± 0.56	1.04 ± 0.23
Synaptic plasticity-related						
Fos	1.13 ± 0.68	1.01 ± 0.20	0.80 ± 0.35	0.84 ± 0.43	0.56 ± 0.16*	0.84 ± 0.49
Arc	1.14 ± 0.69	1.06 ± 0.42	0.78 ± 0.60	0.72 ± 0.47	0.49 ± 0.22	0.86 ± 0.89
Neurotrophin-related						
Bdnf	1.09 ± 0.49	1.02 ± 0.24	1.34 ± 0.71	1.27 ± 0.45	0.82 ± 0.33	1.18 ± 0.71
Ntrk2	1.15 ± 0.71	1.01 ± 0.14	1.62 ± 0.83	1.50 ± 0.43	0.82 ± 0.29	1.20 ± 0.76**
Motor proteins						
Kif21b	1.17 ± 0.53	1.04 ± 0.35	1.03 ± 0.36	1.08 ± 0.47	0.99 ± 0.28	1.46 ± 0.79

Abbreviations: AGIQ, α-glycosyl isoquercitrin; ALA, α-lipoic acid; *Arc*, activity-regulated cytoskeleton-associated protein; *Bdnf*, brain-derived neurotrophic factor; *Chrm1*, cholinergic receptor, muscarinic 1; *Chrm2*, cholinergic receptor, muscarinic 2; *Chrna7*, cholinergic receptor nicotinic alpha 7 subunit; *Fos*, Fos proto-oncogene, AP-1 transcription factor subunit; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Gria1*, glutamate ionotropic receptor AMPA type subunit 1; *Gria2*, glutamate ionotropic receptor AMPA type subunit 2; *Grin2a*, glutamate ionotropic receptor NMDA type subunit 2A; *Grin2d,* glutamate ionotropic receptor NMDA type subunit 2D; *Hprt1*, hypoxanthine phosphoribosyltransferase 1; *Kif21b*, kinesin family member 21B; *Ntrk2*, neurotrophic receptor tyrosine kinase 2; *Slc17a7*, solute carrier family 17 member 7; *Slc17a6*, solute carrier family 17 member 6.

[a] Mean ± S.D.

* $P < 0.05$.

** $P < 0.01$, significantly different from the untreated controls by Dunnett's test or Steel's test.

involved in stabilizing the neural circuits that store experiences as long-term memory (Guzowski et al., 2005). Mutant mice null for *Fos* in the central nervous system show impairment in spatial and contextual long-term memory (Fleischmann et al., 2003). Therefore, increased expression of FOS in the hippocampal granule cells by continuous exposure to AGIQ may facilitate synaptic plasticity that may be involved in facilitation of fear extinction learning. On the other hand, we examined *Fos* expression and number of FOS^+ cells in animals that were not subjected to neurobehavioural testings. If the induction pattern of IEG products was examined in brain regions just after the last trial of fear extinction learning, some mechanistic relationship between brain regions involved in facilitation of fear extinction learning could be drawn. It is reasonable to consider that efficiency in gene induction response is dependent on the basal expression level.

In addition, we found transcript upregulation of *Kif21b* in the dentate gyrus cells in AGIQ group. KIF21B is one of the kinesin superfamily proteins, that are microtubule-dependent molecular motors fundamental to various functions of the cells (Hirokawa, Niwa, & Tanaka, 2010). A recent study showed that KIF21B is abundantly expressed in the hippocampus and is responsible for a memory rewriting mechanism such as erasing fear memory (Morikawa, Tanaka, Cho, Yoshihara, & Hirokawa, 2018). That study proposed that KIF21B-mediated inactivation of Rac1, a small-molecular GTPase that is essential for *N*-methyl-D-aspartate receptor (NMDAR)-mediated long-term depression and cognitive flexibility, is a key molecular event in NMDAR-dependent long-term depression expression underlying cognitive flexibility in fear extinction. Therefore, upregulation of KIF21B by AGIQ in the dentate gyrus may link to its functions on extinction of fear memory directly. We recently confirmed facilitation of fear extinction learning by AGIQ exposure at the same dietary concentration with the present study, and we found upregulation of *Elmo1*, an interaction partner of *Kif21b* in relation with long-term depression (Morikawa et al., 2018), as with upregulation of *Kif21b* in the dentate gyrus (unpublished data).

We also found that AGIQ exposure from the developmental stage upregulated the transcript level of *Grin2d* in the amygdala. *Grin2d* encodes one of the NMDAR subunits, and is also known to function in enhancing synaptic plasticity associated with long-term memory (Harney, Jane, & Anwyl, 2008). The mechanism of fear memory storage in the amygdala is thought to be long-term potentiation (LTP) by glutamate nerve input (Pace-Schott, Germain, & Milad, 2015). Since NMDA type glutamate nerve input is essential for the generation of this LTP (Harney et al., 2008), it is possible that GRIN2D is involved in strengthening elimination memory via LTP. From these points of view, upregulation of *Grin2d* in the amygdala may be the signature of NMDAR activation in the amygdala that may be involved in facilitation of fear extinction learning.

In the present study, dams of ALA group showed lower body weight during lactation period and lower food and water consumption during the gestation and lactation period as compared with untreated controls. Offspring in this group also showed lower body weight, food and water consumption during the lactation period as compared with untreated controls. It was reported that mice fed with 1.0% ALA diet had a significant body weight loss that was related to decreased food consumption (Shen, Jones, Kalchayanand, Zhu, & Du, 2005). On the other hand, administration of ALA by intraperitoneal or intravenous injection also caused dose-dependent decreases of food intake, similar to the dietary ALA administration with the dose range of 0.25–1.00% (Kim et al., 2004). In that study, the effect on body weight loss was not due to systemic toxicity or to an illness caused by ALA intake which were proved by conditioned taste aversion test and plasma corticosterone

concentrations, an indicator of stress. Authors further found that ALA decreases hypothalamic AMP-activated protein kinase activity and causes profound body weight loss in rat by reducing food intake and enhancing energy expenditure. Thus, we think 0.1% dietary concentration of ALA was not toxic to both dams and offspring to influence the study results.

5. Conclusions

In conclusion, dietary exposure effect of AGIQ from GD 6 to PND 77 was examined in comparison with ALA in rats. AGIQ-exposed offspring revealed facilitation of fear extinction learning at the 3rd trial, and transcript upregulation of *Fos* and *Kif21b* in the hippocampal dentate gyrus and *Grin2d* in the amygdala. AGIQ also increased the number of FOS^+ hippocampal granule cells. While ALA showed transcript expression changes in brain regions related to fear extinction learning, it changed neither fear extinction learning nor hippocampal FOS^+ cellular numbers. The results suggest that the increases of FOS^+ cells and *Grin2d* transcripts by AGIQ are associated with enhancement of synaptic plasticity, leading to facilitation of fear extinction learning. In addition, AGIQ may also upregulate KIF21B to act on extinction of fear memory directly. Thus, AGIQ exposure may mitigate some stress disorders. Because this study was performed by setting only one dose, further study on dose-effect relationship of AGIQ on neurobehavioural changes and related mechanisms is necessary to be addressed.

Conflict of interest

Mihoko Koyanagi and Shim-mo Hayashi are employed by food additive manufacturer whose product lines include α-glycosyl isoquercitrin. The views and opinions expressed in this article are those of the authors and not necessarily those of their respective employers. Rena Okada, Yasunori Masubuchi, Takaharu Tanaka, Kota Nakajima, Sosuke Masuda, Kazuki Nakamura, Robert R. Maronpot, Toshinori Yoshida, and Makoto Shibutani declare that no conflicts of interest exist.

Ethics statement

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. All efforts were made to minimize animal suffering.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.02.024.

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