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Therapeutic Potential of a Dietary Intervention with the Combination of Butyrate, Trehalose, Piceid, and Biochanin A on a Neuropsychiatric Disorder in Mice

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Abstract: Neuropsychiatric disorders are worldwide public-health concern. Schizophrenia is one of the well-known neuropsychiatric disorders, which may affect millions globally. Maternal immune activation might be one of the key factor for the development of neuropsychiatric disorders. Previously, a mouse model of neuropsychiatric disorders has originally been made, in which poly-I:C, sodium dextran sulfate (DSS) and κ-carrageenan (CGN) were used for the maternal immune activation of mice. In the neuropsychiatric disorder mice, a significant link between biochemical changes of p62 and/or GLAST in the mouse brain and the alteration of experimental behaviors. Therefore, potential therapeutic study has been achieved for the development of effective treatment against neuropsychiatric disorders with using butyric acid, trehalose, piceid, and biochanin A. As a result, some of the combination with these orally available molecules could be effective for the improvement of behavioral alterations as well as biochemical changes of p62 and/or GLAST in the mouse brain. Importantly, the correlation between behavioral score and p62/GLAST protein expression has been again recognized. The significant correlation between pathological behavior and the biochemical alterations would contribute to develop further innovative therapeutics for vaious psychiatric disorders.

Keywords: neuropsychiatric disorder; schizophrenia; behavioral test; autophagy; p62; GLAST; animal model

1. Introduction

Neuropsychiatric disorders are worldwide public-health anxiety [1]. Schizophrenia is one of the well-known neuropsychiatric disorders with the severe disabilities that may influence around 1% of worldwide people [2]. Schizophrenia is often clinically characterized by negative, positive, and cognitive symptoms [3]. Remarkably, schizophrenia is 1.4 times more often diagnosed in males than females [4]. Male patients may exhibit an earlier age of onset and exacerbated negative depressive symptoms as compared with same aged females [5]. These sex differences in biomolecular mechanisms remain mainly unidentified. A principal biomedical theory for the etiology of schizophrenia may be the dopaminergic hypothesis, which suggests that this neuropsychiatric disorder might be triggered by dopamine imbalance [6]. This hypothesis is further reinforced by the therapeutic effects of anti-dopaminergic drugs. The hypofunction hypothesis of N-methyl-d-aspartate (NMDA) receptor may also suggest a complementary explanation of the molecular pathogenesis of schizophrenia [7]. Patients with schizophrenia may exhibit a decreased expression of NMDA receptor in the region of prefrontal cortex, which might be related to the imbalance of dopamine levels [8,9].

It has been revealed that maternal immune activation is a latent risk factor for schizophrenia [10]. Therefore, maternal immune activation with the treatment of viral RNA imitator,

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polyriboinosinic-polyribocytidilic acid (poly I:C), during prenatal development could contribute to the development of behavioral alteration in animal models reminiscent of human schizophrenia [11]. Interestingly, behavioral neurological alterations in the poly I:C model could be avoided if some interventions are performed prior to the symptom appearance [12,13]. Several behavioral tests have been regarded as appropriate examination of animal model of schizophrenia for the disease evaluation [14]. Consequently, the poly I:C rodent model have been a neurodevelopmental paradigm of neuropsychiatric disorders, which may lead to a development of schizophrenia-like behaviors in model animals [15]. In addition, some behavioral examinations have recorded in the impairments of neuropsychiatric disorders [16,17], suggesting that patients with a neuropsychiatric disorder could exhibit changes in multisensory integration [18]. Prenatal inflammation has been considered as a risk factor for neuropsychiatric disorders [19]. Therefore, it may be of interest whether intake of some anti-oxidative and/or anti-inflammatory molecules could counteract the development of neuropsychiatric disorder-related consequences. For example, N-acetylcysteine (NAC) have been suggested as a supplement for high-risk pregnancies, as it may noticeably amend newborn consequences [20]. Taken together, the poly I:C model animal might be beneficial for studying the developmental factors of neuropsychiatric disorders [21].

At molecular levels in brain, it has been shown that autophagic dysregulation of p62 might potentially lead to cognitive impairment across brain conditions [22]. In addition, the p62 protein expression may be upregulated in cultured neurons isolated from materials with schizophrenia, and in brain samples from a mouse model of schizophrenia [23,24]. Therefore, controlling the p62 protein level could provide a potential target for therapeutic intervention at least against several symptoms of schizophrenia [22]. Interestingly, Di-(2-ethylhexyl) phthalate (DEHP) may concurrently enhance the number of autophagosomes and the amount of autophagy marker p62, which is known to impair tissue/organ functions [25]. The DEHP has the ability to traverse the blood-brain barriers (BBB), which could intensify the proliferation of astrocytes [26]. It has been also implied that the dysregulation of glutamate aspartate transporter (GLAST) may play a noteworthy role in the neuropathogenesis of various neurological disorders including autism, epilepsy, and/or schizophrenia [27]. It has been revealed that GLAST knockout (KO) mice could display exaggerated locomotor activity in response to the administration of NMDA antagonist [28,29]. In addition, enlarged incidence of a rare genetic variant in a human gene encoding the GLAST has been identified in schizophrenia patients [28,29]. Remarkably, GLAST KO could exhibit phenotypic abnormalities thought to positive symptoms of schizophrenia [30]. Abnormalities in regulatory components of the glutamate system might be significant risk factors for the development of schizophrenia.

Neuropsychiatric disorders are going to be a leading health concern in the near future, emphasizing an exceptional need for the development of novel effective therapeutics to treat them aimed at good quality of life (QOL). For example, the mainstream of studies concentrate on resveratrol with limited findings exploring other stilbenes such as pterostilbene, piceatannol, polydatin, tetrahydroxystilbene glucoside or synthetic resveratrol derivatives [31]. In addition, biochanin A treatment may improve learning and memory abilities and can alleviate Alzheimer's disease symptoms in a postmenopausal model of Alzheimer's disease [32]. A possible mechanism is suggested that biochanin A could rescue the imbalance of mitochondrial dynamics and abnormal mitophagy [32]. Interestingly, the modulation of autophagy/mitophagy with the alteration of the related signaling pathway may offer a novel approach to alleviating cognitive dysfunction [33]. In this meaning, trehalose and/or sodium butyrate may also be involved in the modulation of autophagy, which could also possess the potential of improvement [34]. In the present study, therefore, we challenged to confirm the link of neurobiochemical changes of p62 and GLAST in the poly I:C treated mice model underlying neuropsychiatric disorders to the alteration of several behaviors with the disorder, and also tested to evaluate the therapeutic potential of several candidate molecules. For this purpose, it has been struggled to look for the specific behavioral test which could represent the status of neuropsychiatric disorder levels. From our results of numerous preliminary experiments with various behavioral examinations and/or diverse scorings, we have utilized the original behavioral examinations presented previously. Here, a result of the study has demonstrated

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that there is a significant relationship between the test score and the biochemical GLAST protein expression level, and that treatment with the combination of butyrate, trehalose, piceid, and biochanin A could improve the pathology of a neuropsychiatric disorder in mice.

2. Materials and Methods

2.1. Mice

Male and Female ICR mice (4-week-old) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). At the age of 6 weeks after acclimation, female ICR mice were mated with male ICR mice, and the day when vaginal plugs were checked was described as the first day of the each pregnancy (GD1). Mother mice took DSS + CGN water which was dissolved in 0.5% (w/v) Sodium Dextran Sulfate 5000 (DSS) and 0.2% (w/v) κ-Carrageenan (CGN) in GD 8 to In addition, 5 mg/kg B.W. poly I: C was administered intraperitoneally on GDThe day mice were born was defined as PD1, and on day 22 (PD22), then mice were divided from their mothers and grouped in net cages distinctly for males and females. From PD 125, mice were provided 2 mg/L DEHP orally, and three types of behavioral tests: a descent step test, a modified three chambers test, and a light/dark room test were performed eight times. After the behavioral tests, some of the mice were dissected, and collected liver, kidney, and brain samples for western analysis. All mice were kept in an environmentally controlled room, at nearly 20°C and 60% humidity with a 12-h light/dark cycle (lights on at 07:00 and off at 19:00). The care and treatment of the experimental animals conformed along with the guidelines for the ethical treatment of laboratory animals established by Nara Women's University (Nara, Japan) (Approval No. 19-02).

2.2. Materials

Poly I: C, DEHP, and Dextran sulfate sodium (DSS, MW5000) were bought from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). κ-Carrageenan (CGN) was bought from Tokyo Chemical Industry Co., LTD. (Tokyo, Japan). Poly I: C was dissolved in saline. DEHP, DSS, and CGN were diluted with sterile water to drink for mice. Butyric acid, trehalose, biochanin A, and piceid were bought from Fujifilm Wako Pure Chemicals Corporation (Osaka, Japan). They were also dissolved in sterile water. The other reagents used in this study were also bought from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan).

2.3. Behavioral Tests

The score of behavioral tests were conducted as shown previously [35].

[1] Descent step test

A box (23 cm x 31 cm x 8.3 cm) was located in the center of a big plastic case (30 cm x 52 cm x 17 cm), and each mouse was placed on the box. After that, we measured whether or not the mouse descended from the box during or within one minute.

[2] Modified three chambers test

The field of the plastic case was divided into three compartments with boxes, and the central compartment was dark (27.5 cm x 21 cm). At first, each mouse was placed in a narrow light room (30 cm × 13 cm), and chambers were set as (I), (II), and (III) in order of nearness. The size of the dark entrance was 4 cm x 2.5 cm. The score was determined where the test mouse was placing 25 seconds after the start.

[3] Light and dark room test

A dark space (27.5 cm × 21 cm) and a light space (30 cm × 31 cm) were both set up in a box within a field of a big plastic case, and the boundary between the light space and the dark space was operated as the starting point. The size of the dark entrance was 4 cm x 2.5 cm. Mice were allowed to explore freely for 2 minutes, and the total time spent in the light space was measured.

2.4. Western Blotting

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To extract protein for western analyses, the whole brain was homogenized with RIPA buffer. The homogenates were centrifuged to obtain supernatants (Tabletop micro-cooled centrifuge Model3500). The supernatants were mixed with sample buffer and adjusted 1mg/mL protein concentration. We used SDS-PAGE to separate proteins and transfer them to membranes (Immobilon-P, Merck KGaA, Darmstadt, Germany). These were blocked with 3% skim milk and then reacted with primary antibodies SQSTM1/p62 polyclonal antibody (Cosmobio) or GLAST polyclonal antibody (Cosmobio) for 1h and peroxidase-conjugated goat anti-rabbit secondary antibodies (Cell Signaling) for 1h. Proteins were detected by ImageQuant LAS500 (GE Healthcare Japan Com., Tokyo, Japan). Each detected bands were quantified by ImageJ, and the relative ratio of protein expression was analyzed using GAPDH (Glyceraldehyde 3-phosphate dehydrogenase, FUJIFILM Wako Pure Chemicals Co.) as an internal control protein. The intensities of the detected bands were also calculated using ImageJ software.

2.5. Statistical Analyses

All data are principally expressed as the mean ± standard error (SE), which were analyzed by Pearson's correlation analysis. $P < 0.05$ was considered a statistically significant difference. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

Consistent with the previous preprint report, we observed that acute poly I:C administered at PD10 plus DSS/CGN supply could elicit significant alteration of behaviors within our original tests for adult offspring mice [35]. In addition, certain behavioral test scores could indicate an exact pathological condition with biochemical alterations in the brain of disorder animals. This concept might boost the development of a good treatment without any burden to animals/patients against neuropsychiatric disorders that would significantly exhibit some biochemical alteration in the brain [35]. To accelerate the development of neuropathological disorder in the offspring, some modifications have been employed in addition to the basic poly I:C method for making model mice with schizophrenia-like behaviors [35]. Three behavioral tests had originally been designed for the study with several modifications from literatures [35]. The psychological behavior index (PBI) score was calculated with a sum of three behavioral tests scores for each of individual pups. Behavioral tests were conducted 10 times during the whole experiment period (2times before treatment and 8 times after the start of treatment) for an individual mouse. The average scores were employed for the analyses. Note that zero or low values of the PBI score were found in untreated and/or standard offspring (n>10). However, note that it was impossible to make all the mice to retain psychiatric behavior (data not shown, personal communication).

In preliminarily accomplished experiments, some behavioral tests suggested that butyric acid, trehalose, piceid, and biochanin-A could slightly improve the behavioral score of this psychiatry related disorder model mice (data not shown). In line with this, it has been shown that schizophrenia may be characterized by the reduction of butyrate-producing genera [36]. Therefore, we employed our neuropsychiatric model mice whether these molecules could contribute to the improvement of the behavioral symptoms. Anticipating favorable additional effects, in addition, we used two patterns of combination with butyrate and other molecule for the treatment of psychiatry related disorder model mice. Overview of this treatment study design is shown at **Figure 1.** In the present study-situation, there was no significant difference in water intake **(Figure 2A)**, food intake **(Figure 2B)**, body weight gain **(Figure 2C)**, and brain weight **(Figure 2D)** among three groups (SZ, SZ/BTP, SZ/BTB). No mice spontaneously died during the entire experiment. After documented PBI scores, all mice were sacrificed and examined for protein expressions on their whole brain. The protein expression levels of p62, GLAST, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in each mouse brain were shown **(Figure 3A, 3C)**. The p62 and GLAST protein expression of the SZ group were higher than those of SZ/BTP and SZ/BTB groups **(Figure 3B, 3D)**. The SZ/BTB group showed similar levels of GLAST protein expression to the SZ/BTP group **(Figure 3A, 3B)**, which was almost

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similar level to that of untreated normal mouse (data not shown, personal communication). However, the lower protein expression of p62 in SZ/BTB group rather than that of SZ/BTP group **(Figure 3C, 3D)**. Efficacy outcome was assessed in terms of the PBI score reduction. Becase, low score shows the healthy condition of mice. The most efficient combination was exhibited by SZ/BTB group **(Figure 4A)**. Afterward, we further examined the relationship between the behavioral test score and the p62/GLAST protein expression levels in this therapy experiments. Importantly, the correlation between PBI score and GLAST protein expression (P: 0.003) has been again identified **(Figure 4B).** However, the correlation between PBI score and p62 protein expression (P: 0.06) was not significant in this study **(Figure 4C).** The results presented here may again suggest that a behavioral test score might be associated to the protein expression levels of GLAST in the brain of mice with probable neuropsychiatric disorders.

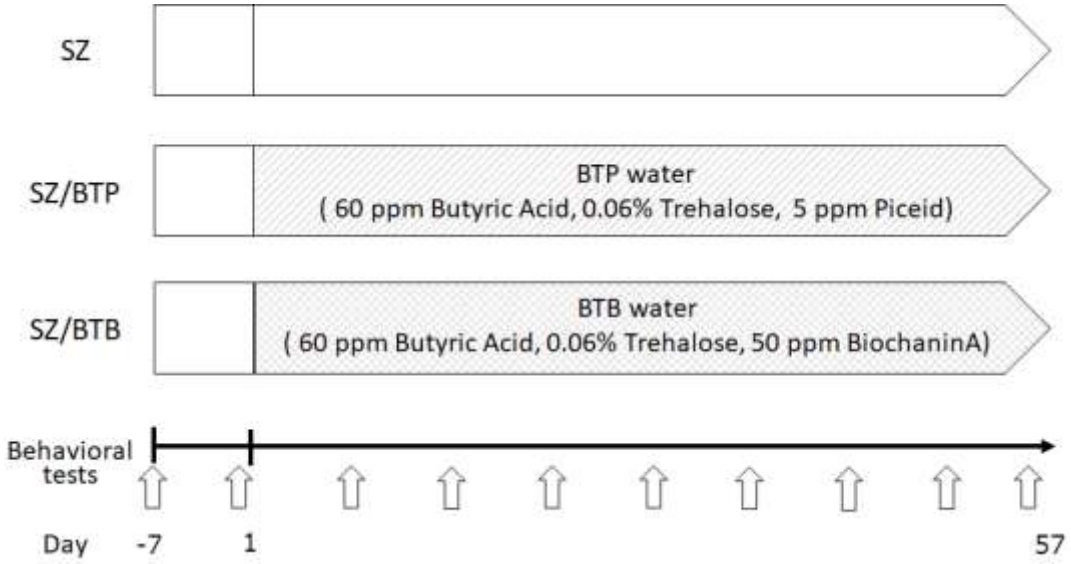

Figure 1. Study design: Psychiatric disorder model mice which were previously made were divided into three groups of SZ(water), SZ/BTP (60 ppm Butyric Acid, 0.06% Trehalose, 5 ppm Piceid), SZ/BTB (60 ppm Butyric Acid, 0.06% Trehalose, 50 ppm BiochaninA), and kept with the indicated freely available drinkable water (n = 10/group). All mice were conducted for behavioral tests on the arrowhead days and were sacrificed at day 57.

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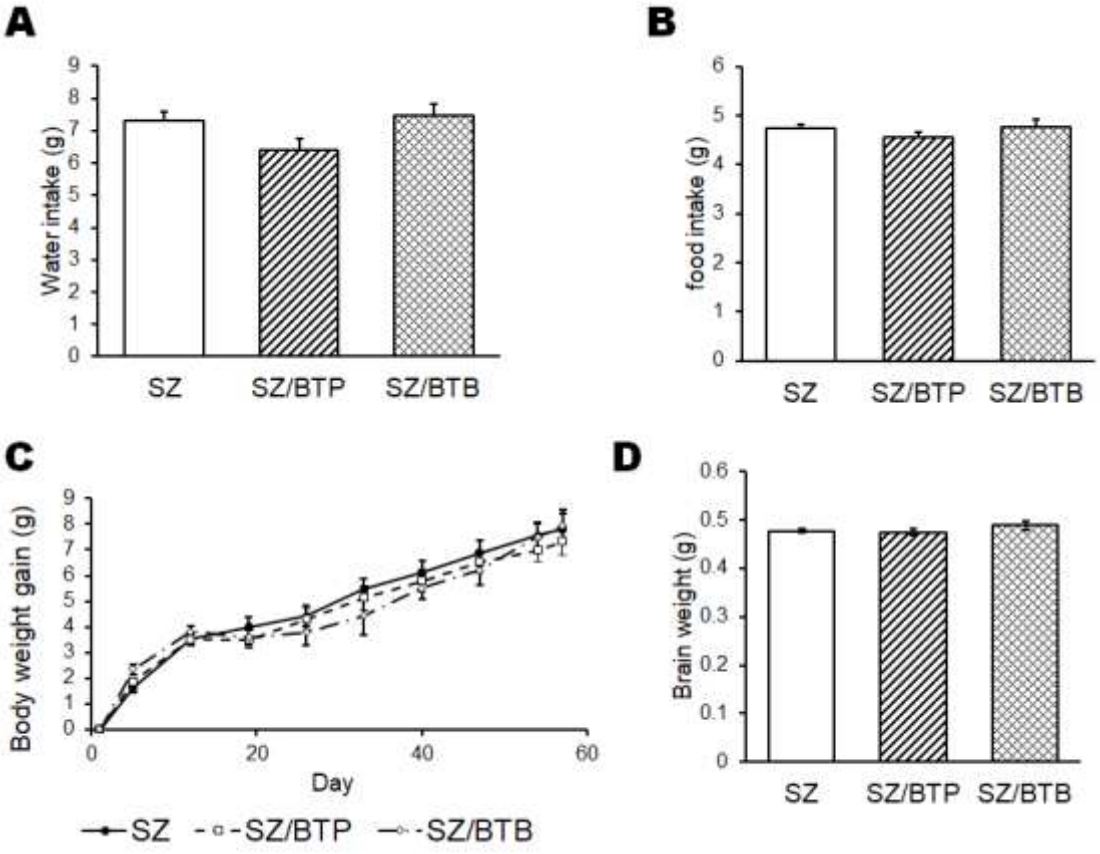

Figure 2. Water intake, food intake, body weight gain, and the brain weight in mice: (A) Water intake were quantified once a week throughout the experiment. SZ group (white), SZ/BTP group (right upper diagonal), SZ/BTB group (mesh pattern). Values are expressed as the mean ± SE, n = 10/group. The data were tested by one-way ANOVA. (B) Food intake were quantified once a week throughout the experiment. SZ group (white), SZ/BTP group (right upper diagonal), SZ/BTB group (mesh pattern). Values are expressed as the mean ± SE, n = 10/group. The data were tested by one-way ANOVA. (C) Body weights were measured once a week throughout the experiment. Values are expressed as the mean ± SE, n = 10/group. SZ group (black circle), SZ/BTP group (white squared), and SZ/BTB group (white triangle). The data were tested by one-way ANOVA. (D) The brain weight was quantified after the sacrifice of the mouse. SZ/TB group (gray), SZ/PB group (right upper diagonal), SZ/MB group (mesh pattern). Values are expressed as the mean ± SE. The data were tested by one-way ANOVA.

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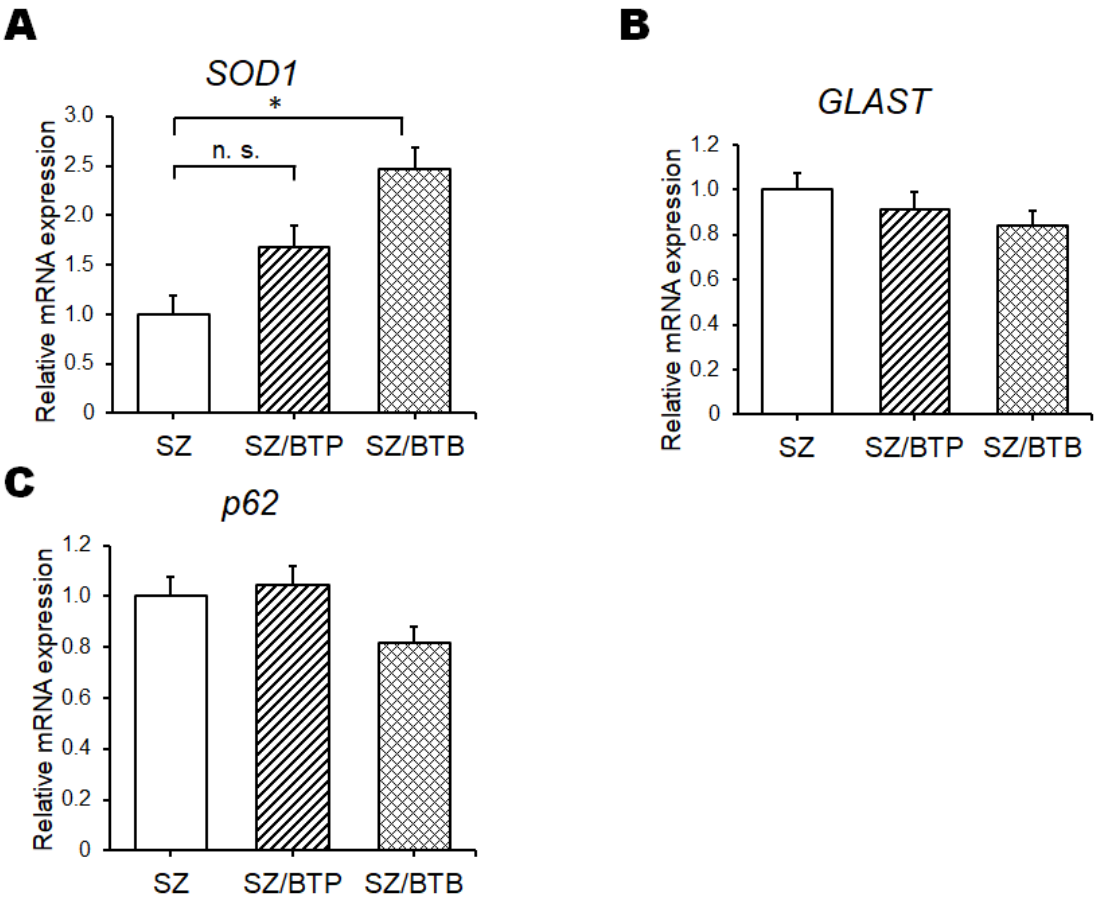

Figure 3. The mRNA expression in the brain: (A) The mRNA expression of SOD1 was measured and normalized to β-actin by RT-PCR. SZ group (white), SZ/BTP group (right upper diagonal), SZ/BTB group (mesh pattern). Values are expressed as the mean ± SE, n = 2 or 3/group. The data were tested by one-way ANOVA. (*$p < 0.05$) (B) The mRNA expression of GLAST was measured and normalized to β-actin by RT-PCR. SZ group (white), SZ/BTP group (right upper diagonal), SZ/BTB group (mesh pattern). Values are expressed as the mean ± SE, n = 2 or 3/group. The data were tested by one-way ANOVA. (C) The mRNA expression of p62 was measured and normalized to β-actin by RT-PCR. SZ group (white), SZ/BTP group (right upper diagonal), SZ/BTB group (mesh pattern). Values are expressed as the mean ± SE, n = 2 or 3/group. The data were tested by one-way ANOVA.

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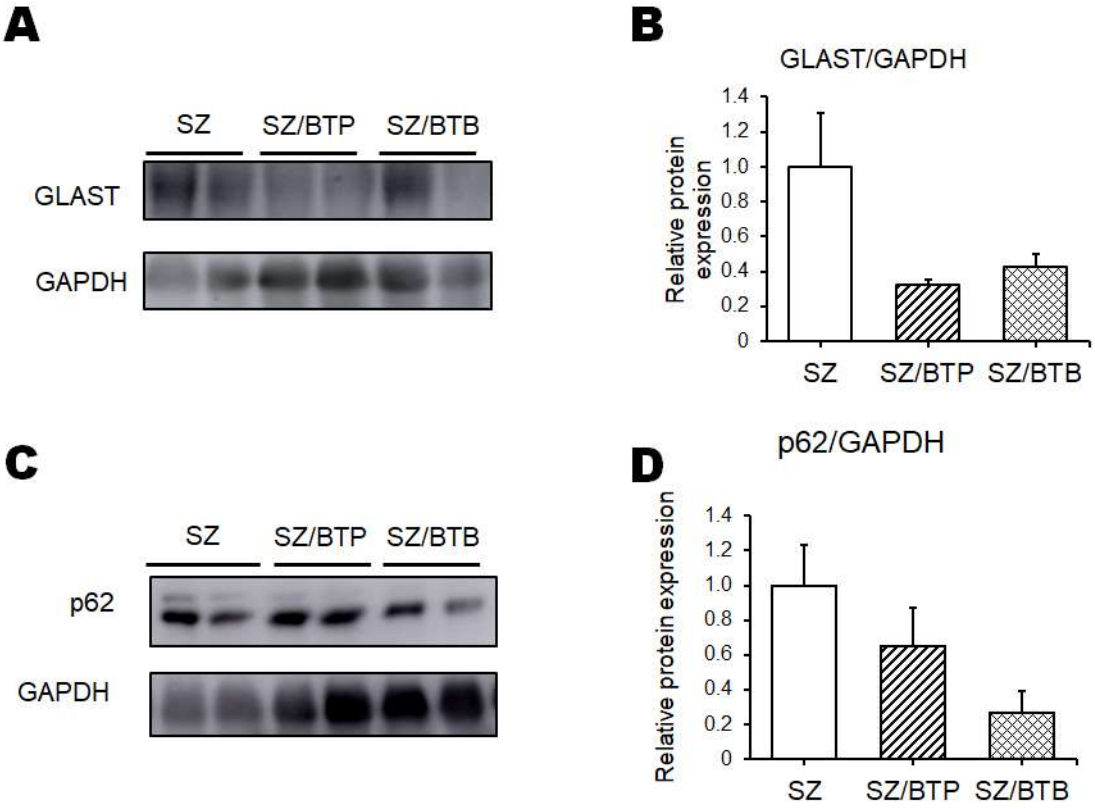

Figure 4. The protein expression of p62 and GLAST in the brain: (A) The image of GLAST and GAPDH expression by Western blot analysis. (B) The protein expression of GALST (98kDa) was quantified and normalized to that of GAPDH by Western blot. SZ group (white), SZ/BTP group (right upper diagonal), SZ/BTB group (mesh pattern). Values are expressed as the mean ± SE, n = 4 or 5/group. The data were tested by one-way ANOVA. (C) The image of p62 and GAPDH expression by Western blot. (D) The protein expression of p62 (60kDa) was quantified and normalized to that of GAPDH by Western blot. SZ group (white), SZ/BTP group (right upper diagonal), SZ/BTB group (mesh pattern). Values are expressed as the mean ± SE, n = 4 or 5/group. The data were tested by one-way ANOVA.

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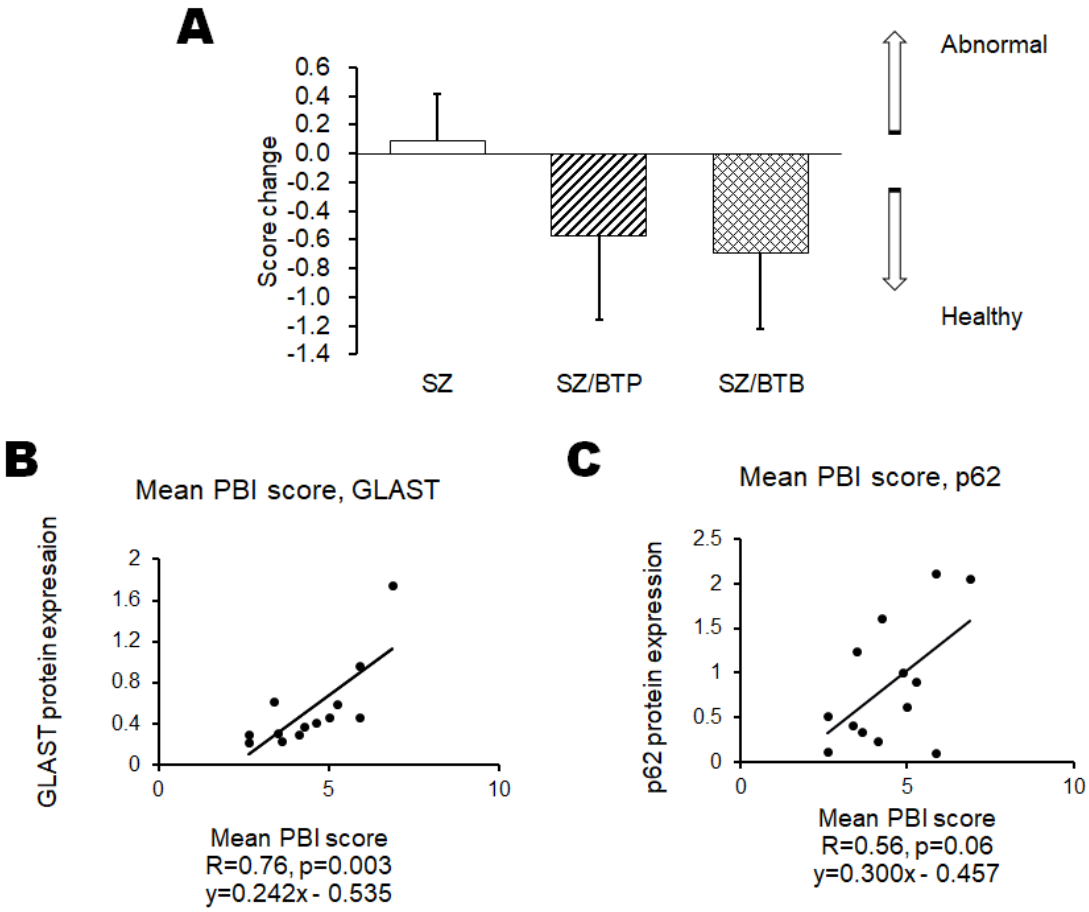

Figure 5. Improvement of the PBI score by the treatment and the correlation between the test score and the protein expression of p62/GLAST: (A) Alteration of the PBI score (Score change) was calculated by the following equation, Score change = (average of three times of PBI score before the treatment) – (average of at least five times of PBI score after the treatment). Value of the score change are expressed as the mean ± SE. SZ group (white), SZ/BTP group (right upper diagonal), SZ/BTB group (mesh pattern). (B) Significant correlation between the mean behavioral test score and GLAST protein expression. r=0.76, P=0.003, y=0.242x + 0.535. (C) Positive correlation between the mean behavioral test score and p62 protein expression. r=0.36, P=0.23, y=0.0376x + 0.1384.

4. Discussion

Mouse model of neuropsychiatric disorders have been prepared after exposure to maternal immune activation with poly I:C and DSS/CGN treatments [35]. In this regards, we believe that neuropsychiatric disorders may be characterized in immune related diseases [37], whose pathogenesis might be possibly grounded on the engram memory system [38–40]. The behavioral study have revealed an array of long-term alterations of behavior in the offspring. It has been reported that autophagy related materials such as metformin, butyrate, trehalose, and piceid may be beneficial for the treatment of schizophrenia [41–43]. As the efficient combination for the improvement of behaviors was exhibited by SZ/BTB and/or SZ/BTB groups, modification of autophagy might be one of the efficient therapy against neuropsychiatric disorders. Autophagy is a membrane trafficking mechanism responsible for degrading damaged lipids, proteins, and/or organelles such as mitochondria [44]. It has been shown that neuronal autophagy is related to the cognitive progressions via the regulation of synaptic components [45]. In addition, the PI3K/AKT signaling pathway might be involved in the autophagy [46], which might be involved in the development of schizophrenia [47]. In neurons derived from schizophrenia patients, the sensitivity to PI3K/AKT/GSK3 signaling might be changed [48], which may also be involved in the development

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of schizophrenia [49]. Furthermore, the AKT activity has been shown to decrease in certain brain regions of patients with major depressive disorder and/or schizophrenia [50]. Interestingly, pregnenolone has been suggested to regulate schizophrenia-like behaviors via the modulation of AKT signaling [51]. As for the therapeutic studies presented here, some effective molecules for the improvement of psychiatric behavior might be butyrate, trehalose, piceid, and biochanin-A. These molecules have been reported conceivably to be involved in the modulation of autophagy [41–43]. In line with this, the intracellular autophagic activity could regulate the protein level of p62 [52]. It has been proposed that elevated p62 levels may have functional consequences on the neurotransmission, which might explain the behavioral changes relevant to schizophrenia [53]. Fortunately, it has been shown that protein expression levels of p62 seem to be slightly correlated, but not significantly, with the score of our behavioral tests **(Figure 4C).**

One explanation is that this combination treatment may not be sufficient for the adjustment of autophagy in the brain. Previous studies have elucidated the induction of autophagy by ionizing radiation [54]. Therefore, it would be probable innovatively to treat schizophrenia patients by appropriate whole-brain irradiation-therapy.

Optimizing radiation dose, dose rate, and combined treatment tactics might enhance the effect of this irradiation-therapy.

Aberrations in regulatory components of the glutamatergic system could be also important risk factors for schizophrenia, which might be regulated by a family of glutamate transporters including GLAST or excitatory amino-acid transporter Incidence of a genetic variant may be increased in the human gene encoding GLAST within schizophrenia patients [55]. Interestingly, some genetic variants may impair metabolic functions of astrocytes and might lead to cognitive dysfunction [56]. In addition, it has been revealed that the GLAST knockout (KO) mice could exhibit exaggerated locomotor activity [57], which may be a model for positive symptoms of schizophrenia. Therefore, roles of GLAST protein might be involved in the certain behavior relevant to the symptoms of schizophrenia [58]. Fortunately, expression levels of GLAST protein seem to be significantly correlated with the score of our behavioral tests **(Figure 4B).** The larger sample size of experiments might lead to more significant outcomes. These data provide the first demonstration of the relationship between behavioral score and the biochemical alterations of p62 and/or GLAST expression.

The symptom of neuropsychiatric disorders may be complicated. Although our results are limited to individual model animals, there are currently no animal model which entirely represent a human psychiatric disorder. Hence, the validity of the behavioral score should be re-evaluated with the each model animal and/or with the treatment of different inhibitors. Reaction to the administration of several antipsychotic drugs may be also compulsory. In addition, histopathological examination may be helpful for the elucidation of functional roles for the communication with separate brain areas. As for the precise function in brain, the elucidation of the communication between neurons and glial cells is also important. Forthcoming studies using an increased number of animals to address the above concerns would be informative. As the results of this study have been attained by using a mouse model, however, the generalization of our findings to humans should be cautiously assessed.

5. Conclusions

Taken together, the mouse model of neuropsychiatric disorders has been confirmed, in which poly-I:C, DSS, CGN and DEHP were used for maternal individual during the pregnancy. Treatment with the combination of BTP or BTB has efficiently improved the behavioral test scores as well as the GLAST protein expression in the brain. Modification of autophagy in the brain cells might be one of the probable therapeutic mechanism against neuropsychiatric disorders. The significant correlations between the behavioral test score and the protein expression levels of GLAST in the whole brain of offspring mice have been again identified. These findings suggests that certain behavioral tests could be effective for determining some of the brain neuropathological disorder as well as for the

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development of treatment tactics against neuropsychiatric disorders. However, it requires further investigation to fully understand the molecular mechanisms involved.

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Competing interests statement: The authors declare that they have no competing financial interests.

Data Availability Statement: Not applicable.

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Informed Consent Statement: Not applicable. As for ethics, the care and treatment of the experimental animals conformed along with the guidelines for the ethical treatment of laboratory animals established by Nara Women's University (Nara, Japan) (Approval No. 19-02).

Sample Availability: All compounds used in this study are commercially available.

Author Contributions: Conceptualization, MN and SM; original draft preparation and editing, MN and SM; visualization, MN and SM; experiment execution, MN, AF, SY, and SM; supervision, SM. Each author (MN, SY, AF and SM) has participated sufficiently in this work of drafting the article and/or revising the article for the important rational content. Then, all authors gave final approval of the version to be submitted. Finally, all authors have read and agreed to the published version of the manuscript.

Abbreviations

CGN: carrageenan
CNS: central nervous system
DEHP: 2-ethylhexyl phthalate
DSS: sodium dextran sulfate
FMT: fecal microbiota transplantation
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GLAST: glutamate aspartate transporter
KO: knockout
LPS: lipopolysaccharide
mRNA: messenger RNA
NAC: N-acetylcysteine
NMDA: N-methyl-d-aspartate
PBI: psychological behavior index
poly I:C: polyriboinosinic-polyribocytidilic acid
QOL: quality of life

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