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Research Article

Korean Red Ginseng and Rb1 restore altered social interaction, gene expressions in the medial prefrontal cortex, and gut metabolites under post-weaning social isolation in mice

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ABSTRACT

Background: Post-weaning social isolation (SI) reduces sociability, gene expressions including myelin genes in the medial prefrontal cortex (mPFC), and alters microbiome compositions in rodent models. Korean Red Ginseng (KRG) and its major ginsenoside Rb1 have been reported to affect myelin formation and gut metabolites. However, their effects under post-weaning SI have not been investigated. This study investigated the effects of KRG and Rb1 on sociability, gene expressions in the mPFC, and gut metabolites under post-weaning SI.
Methods: C57BL/6J mice were administered with water or KRG (150, 400 mg/kg) or Rb1 (0.1 mg/kg) under SI or regular environment (RE) for 2 weeks during the post-weaning period (P21–P35). After this period, mice underwent a sociability test, and then brains and ceca were collected for qPCR/immunohistochemistry and non-targeted metabolomics, respectively.
Results: SI reduced sociability compared to RE; however, KRG (400 mg/kg) and Rb1 significantly restored sociability under SI. In the mPFC, expressions of genes related to myelin, neurotransmitter, and oxidative stress were significantly reduced in mice under SI compared to RE conditions. Under SI, KRG and Rb1 recovered the altered expressions of several genes in the mPFC. In gut metabolomics, 313 metabolites were identified as significant among 3027 detected metabolites. Among the significantly changed metabolites in SI, some were recovered by KRG or Rb1, including metabolites related to stress axis, inflammation, and DNA damage.
Conclusion: Altered sociability, gene expression levels in the mPFC, and gut metabolites induced by two weeks of post-weaning SI were at least partially recovered by KRG and Rb1.

1. Introduction

Social isolation (SI) is strongly associated with mental health and cardiovascular health [1], and significantly related with cognitive decline in old age [2], increased risk of dementia [3] and mortality [4]. The rodent model implements a SI model that shows impaired social interaction by rearing one animal per cage. In a rodent model, SI induces changes in social behavior, myelin of prefrontal cortex (PFC), and gut environment.

Changes in SI-induced sociability are associated with the reduced myelin level in the PFC. Raising juvenile mice (Postnatal day (P) 21) socially isolated for two weeks reduces sociality and myelin thickness in the PFC compared to the mice raised in groups [5,6]. Moreover, since myelin levels were not recovered at P65 after SI of P21–P35 and post re-integration of P35-P65, two weeks of post-weaning are considered a critical period for social interaction required for proper myelination of

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PFC [6].

SI also changes the balance of gut microbiome. Post-weaning SI of rats increased *Actinobacteria* phylum and decreased *Clostridia* classes in gut bacteria [7]. When rats were isolated for 3 h daily during P4–19, *Lactobacillus* genus decreased and the activation of hypothalamic-pituitary-adrenal (HPA) axis increased, resulting in increment of the levels of corticosterone [8].

Alterations in gut microbiome affect gut metabolites, which can induce changes in social behavior and myelin level. When p-cresol was administered to mice for 4 weeks in water, social behavior deficits and altered microbiomes were observed [9]. Supplementation of short chain fatty acid butyrate, an intestinal metabolite, rescued behavioral and myelin-related disorders induced by antibiotics [10]. Similarly, oral butyrate administration inhibits demyelination and enhances remyelination in the multiple sclerosis mouse model [11]. These suggest that gut metabolites play an important role in the molecular mechanism of brain-gut axis and sociability.

Korean Red Ginseng (KRG) is steamed and dried ginseng (*Panax ginseng* Meyer, Araliaceae) [12]. The effects of KRG on health benefits have been studied, recent studies have shown that KRG changes social behavior and the environment of the brain and intestines. KRG rescued abnormal behaviors in the autism model [13], and showed a therapeutic effect on the food disorder induced by chronic social defeat stress [14].

Myelin structure, which is related to sociability [5,6], is formed by oligodendrocytes (OLs) in the brain. KRG-derived non-saponin fraction promoted OL precursor cell (OPC) proliferation. KRG-derived saponin fraction and Rb1, the ginsenoside with the highest content, promoted OPC differentiation and myelin membrane formation [15]. In addition, ginseng-derived gintonin increased OPC proliferation and helped cell survival [16]. KRG and Rb1 facilitated the remyelination process after demyelination induced by cuprizone diet [17].

KRG is known to change the gut environment. Administration of ginseng polysaccharides to mice increased the gut metabolite valeric acid and reduced L-kynurenine and Kyn/Trp ratio [18]. Rats drinking ginseng water for 34 weeks showed significant changes in gut metabolites compared to rats drinking general water, and levels of probiotic gut bacteria and anti-inflammatory cytokines were increased [19]. In addition, ginsenoside Rb1 reversed gut microbiota dysbiosis in high fat diet-induced type 2 diabetes mellitus (T2DM) model mice and changed fecal metabolite [20]. In a study of humans, adults (40–75 years old) who consumed 3g KRG every day for 24 weeks had significant changes in gut microbiome composition [21].

Childhood social interaction affects brain development and has a ripple effect on mental health throughout life. However, no research has been conducted on the effects of KRG and its major ginsenoside Rb1 on changes in social behavior, brain and gut during the juvenile period. To understand their impacts, we investigated social interaction, gene expressions in the medial PFC (mPFC), and gut metabolites in mice treated with KRG or Rb1, under social isolation from P21 to P35, a critical period of myelin development in the mPFC in mice.

2. Material and methods

2.1. Mice

All animal experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by the University of Brain Education's Animal Care and Use Committee (Approval number: 2021-AE-02Y). Mice were kept on a 12-h light-dark cycle with light period of 8:00–20:00 in a standard specific pathogen-free environment. Mice were provided with regular chow and water ad libitum. C57BL/6J mice were purchased from Central Lab Animal Inc (Seoul, Korea), mated in the animal facility and their offspring littermates of both sex were used at P21. For social isolation, mice were singly reared; one mouse per one cage during P21–P35. Other mice were reared in groups; 3–4 mice per one cage during the same period. During the experimental period (P21–P35), mice were oral-injected daily by using zonde with following reagents: distilled water (DW); 150 (=low) or 400 (=high) mg/kg KRG; 0.1 mg/kg Rb1 [15,17]. Body weights were daily measured when the injections were given. Total chow and water consumption were measured per cage during the experimental period. Blood glucose was measured from the tail on the day of the sacrifice (Accu-chek, Roche). For sociability test, quantitative real time PCR (qPCR), and immunohistochemistry (IH), mice were used by mixed gender. For metabolomic analysis, male mice were used.

2.2. Sociability test

Before the test, for adaptation, a mouse was allowed to explore freely in the open field arena (40 cm × 40 cm x 40 cm) containing two empty wire mesh containers for 5 min and removed. Then, one mouse of same sex and age was placed in one wire mesh container, while the other container was remained empty. The open field-adapted mouse was returned again to the arena to explore freely for another 5 min. The mouse movements were recorded by video tracking system ANY-Maze (Stoelting Co., USA). When the experiment mouse explored within 2.5 cm ('mouse zone') from the container containing a mouse, the time of the mouse head was in the zone, the distance travelled by the mouse head in the zone, and the number of entries of the mouse head to the zone were recorded. Same parameters were also recorded from the 'empty zone', which is within 2.5 cm zone from the empty container. The field was cleaned with 70% ethanol inbetween the mice.

2.3. Brain dissection, qPCR, immunohistochemistry

Fresh mouse brains were sliced in the coronal brain slicer for mouse. Brain sections of bregma 1.945–1.745 were collected and mPFCs were isolated under the microscope as indicated in Fig. 1A. RNA extraction, cDNA synthesis, qPCR, and IH were performed as described previously [15–17]. Primer information is given in Supplementary Table 1.

2.4. Reagents

The KRG extract powder was provided by the Korean Society of Ginseng. Briefly, fresh ginseng roots were prepared and processed by steaming and drying in red ginseng manufacturing factory of Korea Ginseng Corporation (Buyeo, Chung-nam, Korea). Washed fresh ginseng roots were steamed for 4 h while slowly raising the temperature from 50 °C to 98 °C and then dried at 60 °C to 70 °C for 15 hours. Then, secondary drying processwas performed in a closed chamber at 50 °C for 5 days. Thereafter, the roots were sequentially extracted 7 times at 87 °C for 12 h with distilled water. The extracted water was filtered, concentrated, and spray-dried to produce KRG extract powder. The KRG production date is September 1st 2021 and the production number is H1312-1032 and item code is V15CB00066. According to the analysis of the KRG batch which was used in this study (Report Number 21–1818, Korea Ginseng Corporation R&D Head Office), the concentrations of constituent ginsenosides in the KRG are as follows: 0.41 mg/g Rg1, 0.46 mg/g Re, 1.50 mg/g Rf, 2.01 mg/g Rg2s, 6.24 mg/g Rb1, 2.32 mg/g Rc, 2.22 mg/g Rb2, 1.10 mg/g Rd, 4.03 mg/g Rg3s, 1.99 mg/g Rg3r, 1.55 mg/g Rh1. Rb1, one of the major components of KRG, was purchased from ChemFaces (Wuhan, China). For experiments, both KRG and Rb1 were dissolved in DW.

2.5. Statistics

For statistics, two-way ANOVA was performed by Sigmaplot 14.0 software.

2.6. Sample preparation and metabolomics analysis

After the sociability test, mouse ceca were collected and immediately

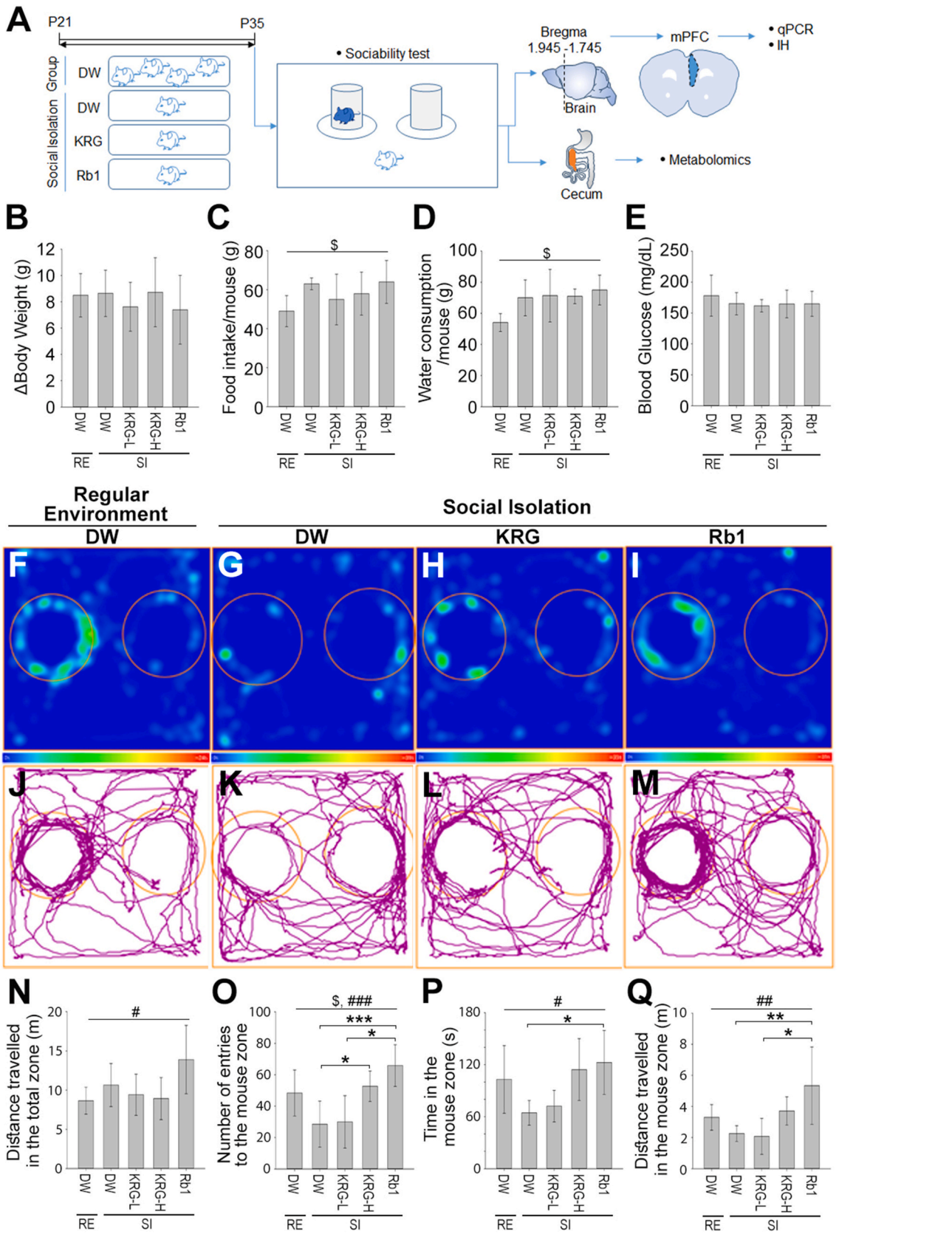

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Fig. 1. Sociability test on mice administered with DW, KRG or Rb1 during post-weaning social isolation. **(A)** Scheme of the experiment. **(B)** Change of body weight (g). **(C)** Food intake (g) per mouse. $, $P = 0.045$, $F = 4.592$, $DF = 1$. **(D)** Water consumption (g) per mouse. $, $P = 0.016$, $F = 6.982$, $DF = 1$. **(E)** Blood glucose (mg/dL). **(F–Q)** Sociability test. After two weeks of SI or RE (P21–P35) with the indicated treatments, mice were tested for social interaction. For the test, one stranger mouse with same sex and age was located in the left circular cage, while the right cage was empty. **(F–I)** Heat maps of mouse movements during the test. Colors of blue to red represent the minimum to maximum time spent for a mouse at the location. **(J–M)** Tracking lines of mouse movements during the test. **(N)** Distance which mice travelled in the total zone (m). #, $P = 0.032$, $F = 3.456$, $DF = 3$. **(O)** Number of entries of the animal's head to the mouse zone. $, $P = 0.022$, $F = 5.986$, $DF = 1$; ###, $P < 0.001$, $F = 8.059$, $DF = 3$; *, $P = 0.023$, $t = 3.021$ for DW vs KRG-H; ***, $P < 0.001$, $t = 4.665$ for DW vs Rb1; *, $P = 0.020$, $t = 3.186$ for KRG-L vs Rb1. **(P)** Time of the animal's head was in the mouse zone (s). #, $P = 0.037$, $F = 3.318$, $DF = 3$; *, $P = 0.046$, $t = 2.904$ for DW vs Rb1. **(Q)** Distance travelled by the animal's head in the mouse zone (m). ##, $P = 0.004$, $F = 5.792$, $DF = 3$; **, $P = 0.004$, $t = 3.867$ for DW vs Rb1; *, $P = 0.038$, $t = 2.910$ for KRG-L vs Rb1. Two-way ANOVA, general linear model: $, $P < 0.05$ for rearing condition; #, $P < 0.05$, ##, $P < 0.01$ for treatment; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ for post hoc analysis (Holm-Sidak method). N = 10–11, 4, 3–4, 6, 6–7 for RE_DW, SI_DW, SI_KRG-L, SI_KRG-H, SI_Rb1, respectively. Bars indicate mean ± standard deviation. KRG-L, 150 mg/kg; KRG, KRG-H, 400 mg/kg; Rb1, 0.1 mg/kg; RE, regular environment; SI, social isolation; DW, distilled water.

frozen at −80 °C and sent to Omics Research Center for metabolomics analysis. Briefly, for sample preparation, they were homogenized and protein concentration was adjusted to 200 μg/ml. The part of homogenate (50 μl) was added to a mixture (100 μl) containing acetonitrile (95 μl) and isotope-labeled standards which include [3-methyl-13C]-caffeine, [13C5, 15 N]-L-methionine, and [dimethyl-D6]-N, N-diethyl-*M*-toluamide. Then, it was vortexed for 5 min, followed by centrifugation at 15,000×*g* for 10 min at 4°C. The supernatant was subjected to LC separation and MS analysis. The data acquisition was performed using an Infinity 1290 UPLC system (Agilent, Santa Clara, CA, USA) connected to an Agilent 6550 iFunnel Q-TOF instrument with a separation using a stationary phase Hypersil GOLD aQ-C18 column (100 × 2.1 mm i.d. 1.9 particle size; Thermo Fisher Scientific, Waltham, MA, USA) and mobile phases comprising 0.1% formic acid (Sigma-Aldrich, St. Louis, MO, USA), HPLC-grade water (JT-Baker, Phillipsburg, NJ, USA) (solvent A), and HPLC-grade ACN (JT-Baker) (solvent B). The data was obtained using a mass to charge ratio (*m/z*) ranging from 50 to 1000 in positive ion mode, 1700 resolution, and electrospray ionization mode. For reliability and reproducibility, sample batches were randomized, and all samples were analyzed in triplicate. Data extraction was processed by apLCMS and xMSanalyzer and metabolic signatures were identified by xmsPANDA. The identified significant features were annotated by xMSannotator, which applies data from the Human Metabolome Database, Metlin, and Kyoto Encyclopedia of Genes and Genomes databases. Pathway analysis was performed by using Mummichog Pathway analysis [22].

3. Results

3.1. KRG and Rb1 prevent sociability reduction induced by post-weaning social isolation

In order to investigate whether KRG and Rb1 restore the sociability decline induced by post-weaning SI, mice were raised in SI (1 mouse/cage) or regular environment (RE, 3–4 animals/cages) conditions for two weeks (P21–P35). While mice in SI conditions were administered with DW (SI_DW), low or high concentration KRG (150 mg/kg, SI_KRG-L; 400 mg/kg, SI_KRG-H), or Rb1 (0.1 mg/kg, SI_Rb1), mice in RE conditions were administered with DW (RE_DW), once a day as oral gavage by zonde during the period. Two weeks later, after conducting a sociability test, mPFC was isolated for qPCR and cecum was isolated for metabolomics. For IH, perfusion was performed after the sociability test (Fig. 1A). For mice which were used for the experiments, body weight change, food intake, water consumption, and blood glucose were investigated, and no significant changes were found by KRG-L, KRG-H, or Rb1 compared to DW administration under SI (Fig. 1B–E), although slight increases were observed in food and water consumption in SI compared to RE mice (Fig. 1C and D).

In sociability test, sociability was measured by the number of entries of the animal's head to the mouse zone (Fig. 1O), time of the animal's head was in the mouse zone (Fig. 1P), and the distance travelled by the animal's head in the mouse zone (Fig. 1Q), through analysis of heat map (Fig. 1F–I) and tracking lines (Fig. 1J-M). The total travelling distance in the entire field was significantly different in the treatment factor in the two-way ANOVA ($P = 0.032$), however no significant differences were found between group comparisons in post-hoc analysis (Fig. 1N). Socially isolated mice showed reductions in the number of access to the mouse zone, compared to RE mice ($P = 0.022$, Two-way ANOVA). Treatment variation showed a significant change ($P < 0.001$): KRG-H- or Rb1-treated mice showed higher access to the mouse zone than DW-treated mice under SI condition ($P = 0.023$, $P < 0.001$, respectively); Rb1-treated mice exhibited higher access to the mouse zone than KRG-L-treated mice under SI ($P = 0.020$) (Fig. 1O). For time of the animal's head was in the mouse zone, treatment variation showed a significant change ($P = 0.037$). Post-hoc multiple comparison procedures (Holm-Sidak method) showed one significant group difference: Rb1-treated mice spent significantly more time than DW-treated mice in the mouse zone after SI ($P = 0.046$) (Fig. 1P). In distance travelled by the animal's head in the mouse zone, there was a significant difference in treatment variation ($P = 0.004$). Post-hoc analysis showed two significant group differences: Rb1-treated mice moved significantly longer distance than DW- and KRG-L-treated mice in the mouse zone after SI ($P = 0.004$ and 0.038, respectively) (Fig. 1Q).

3.2. KRG and Rb1 partially recover gene expressions reduced by post-weaning social isolation in medial prefrontal cortex

The decrease in sociability seen in post-weaning SI is related to the degree of myelin in the mPFC [5,6]. In order to investigate whether social recovery by KRG or Rb1 is related to the degree of myelin-related gene expressions in the mPFC, gene expressions of *Myrf*, *MAG* and *MBP* were investigated in the mPFC (Fig. 2A–C). The expression of *Myrf*, a transcription factor that promotes the production of myelin proteins, was significantly reduced by SI, compared to RE ($P < 0.001$, Two-way ANOVA), and there was a significant difference in treatment variation for *Myrf* expression ($P = 0.006$): *Myrf* expression was higher in KRG-L-, KRG-H- and Rb1-treated mice, compared to DW-treated mice under SI ($P = 0.012$, 0.011, and 0.013, respectively, post-hoc analysis) (Fig. 2A).

The expression of myelin-associated glycoprotein (*MAG*), present in the innermost membrane of compact myelin in contact with axon, was significantly decreased in SI condition, compared to RE ($P < 0.001$, Two-way ANOVA), and the treatment variation for *MAG* expression exhibited a significant change ($P < 0.001$): *MAG* expression was higher in KRG-L-, KRG-H- and Rb1-treated mice, compared to DW-treated mice under SI ($P = 0.012$, <0.001, and 0.005, respectively); KRG-H-treated mice showed significantly higher expression of *MAG* in mPFC, compared to KRG-L ($P = 0.005$) and Rb1 ($P = 0.009$) (Fig. 2B). The expression of *MBP*, a major protein in myelin structure, was significantly reduced by SI, compared to RE ($P = 0.030$, Fig. 2C). KRG or Rb1 did not change *MBP* expression compared to DW under SI.

Myelination is affected by neural activity [23]. The expressions of both *choline acetyltransferase (Chat)* and *glutaminase* (*Gls)*, which promote the synthesis of neurotransmitter acetylcholine and glutamate, respectively, were reduced by SI in mPFC ($P = 0.010$ and 0.003, respectively) (Fig. 2D and E). Under SI, KRG-H-treated mice exhibited a significantly higher *Gls* expression level in mPFC compared to

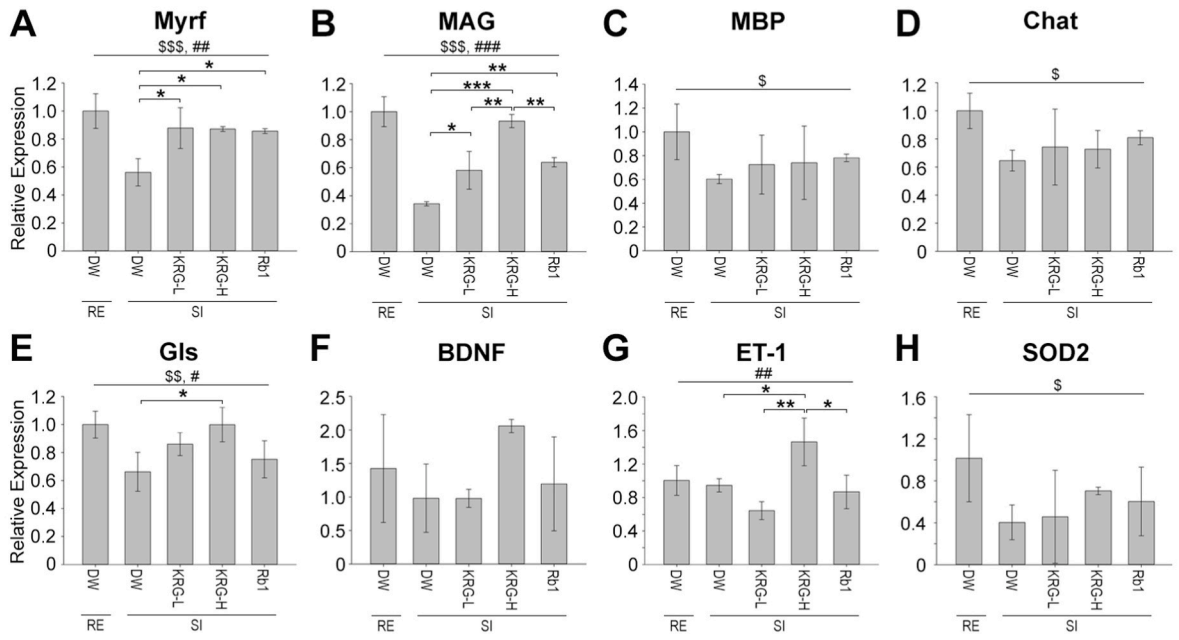


Fig. 2. Gene expression changes in mPFC by KRG or Rb1 under post-weaning social isolation. After two weeks of post-weaning SI or RE with the indicated treatments, mPFC was isolated from the mouse brains and gene expressions were investigated. **(A–C)** Myelin-related gene expressions. **(A)** *Myrf*, $$$, $P < 0.001$, $F = 33.351$, $DF = 1$; ##, $P = 0.006$, $F = 7.187$, $DF = 3$; *, $P = 0.012$, $t = 4.027$ for DW vs KRG-L; *, $P = 0.011$, $t = 3.945$ for DW vs KRG-H; *, $P = 0.013$, $t = 3.755$ for DW vs Rb1. **(B)** *MAG*, $$$, $P < 0.001$, $F = 105.373$, $DF = 1$; ###, $P < 0.001$, $F = 25.137$, $DF = 3$; *, $P = 0.012$, $t = 3.609$ for DW vs KRG-L; ***, $P < 0.001$, $t = 8.908$ for DW vs KRG-H; **, $P = 0.005$, $t = 4.478$ for DW vs Rb1; **, $P = 0.005$, $t = 4.279$ for KRG-L vs KRG-H; **, $P = 0.009$, $t = 3.577$ for KRG-H vs Rb1. **(C)** *MBP*, $, $P = 0.030$, $F = 6.194$, $DF = 1$. **(D–E)** Neuronal gene expressions. **(D)** *Chat*, $, $P = 0.010$, $F = 9.608$, $DF = 1$. **(E)** *Gls*, $$, $P = 0.003$, $F = 14.910$, $DF = 1$; #, $P = 0.022$, $F = 4.834$, $DF = 3$; *, $P = 0.020$, $t = 3.726$ for DW vs KRG-H. **(F)** *BDNF*. **(G)** *ET-1*. ##, $P = 0.001$, $F = 10.720$, $DF = 3$; *, $P = 0.017$, $t = 3.649$ for DW vs KRG-H; **, $P = 0.004$, $t = 4.625$ for KRG-L vs KRG-H; *, $P = 0.022$, $t = 3.364$ for KRG-H vs Rb1. **(H)** *SOD2*. $, $P = 0.028$, $F = 6.218$, $DF = 1$. Two-way ANOVA, general linear model: $, $P < 0.05$, $$, $P < 0.01$, $$$, $P < 0.001$ for rearing condition; #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$ for treatment; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ for post hoc analysis (Holm-Sidak method). N = 10–11, 4, 3–4, 6, 6–7 for RE_DW, SI_DW, SI_KRG-L, SI_KRG-H, SI_Rb1, respectively. Bars indicate mean ± standard deviation. KRG-L, 150 mg/kg; KRG-H, 400 mg/kg; Rb1, 0.1 mg/kg; RE, regular environment; SI, social isolation; DW, distilled water.

DW-treated mice ($P = 0.020$, Fig. 2E). *BDNF* expression was slightly reduced by SI and mildly recovered by KRG without significant differences (Fig. 2F).

The expression of *Endothelin-1 (ET-1)*, which is also associated with myelination, was not affected by SI, however there was a significant change in the treatment variation ($P = 0.001$) with significant differences in post-hoc analyses: *ET-1* expression was significantly increased by KRG-H compared to DW under SI ($P = 0.017$); *ET-1* expression was higher in KRG-H-treated mice compared to KRG-L- or Rb-treated mice ($P = 0.004$, 0.022, respectively) (Fig. 2G). The expression of *superoxide dismutase 2* (*SOD2*), a main antioxidant enzyme in the mitochondrial oxidative damage, was significantly reduced by SI ($P = 0.028$), and only mildly recovered by KRG-H without significant changes (Fig. 2H).

As the expressions of myelin-related genes and neurotransmitter system-related genes were changed in mPFC, we examined whether the numbers of OL-lineage cells, mature OLs and neurons have been changed or not in the mPFC by IH. The numbers of OL-lineage cells (Olig2+) (Fig. 3A–E), mature OLs (CC1+) (Fig. 3F–J), and neurons (NeuN+) (Fig. 3K-O) in the mPFC were not significantly changed by SI, as well as by treatment. Myelin staining exhibited a relatively reduced signal in SI compared to RE and mildly stronger intensities in KRG or Rb1 compared to DW under SI (Fig. 3P–S).

3.3. KRG and Rb1 administrations change gut metabolites during post-weaning social isolation

KRG, Rb1 have been reported to change the gut microbiome [19,20]. Therefore, in order to investigate how the gut environment changes by administration of KRG and Rb1 under SI, cecum metabolomics was performed on four groups of RE, SI, SI-KRG (400 mg/kg), and SI–Rb1 (0.1 mg/kg) after sociability test (male mouse, N = 6/each group, Figs. 4–6). These four groups were clearly divided into four different groups by partial component analysis (Fig. 4A) and partial last squares-discriminant analysis (Fig. 4B). In both analyses, SI-KRG and SI–Rb1 groups were located between RE and SI (Fig. 4A and B), suggesting changes in gut metabolites towards RE direction by KRG and Rb1 administration to mice under SI condition. Of the total 3027 gut metabolites detected, 313 metabolites were identified as significant (One way ANOVA, Fig. 4C). Among them, metabolites significantly changed by SI as well as significantly recovered by KRG or Rb1 were extracted (Figs. 5 and 6). Under SI, in order to recognize which biological pathways were changed in comparisons of KRG vs DW, Rb1 vs DW, and KRG vs Rb1, manhattan plot (Fig. 4D–F, J), two-way hierarchical cluster analysis (Fig. 4E–G, K), and pathway analysis (Fig. 4H, I, L) were conducted in order. As a result, KRG administration under SI significantly changed (1) fatty acid elongation and (2) valine, leucine and isoleucine biosynthesis compared to DW administration (Fig. 4H). Rb1 administration under SI did not significantly change biological pathway (Fig. 4I). There was one different pathway between KRG and Rb1 under SI: alpha-linoleic acid metabolism (Fig. 4L). Among metabolites which were significantly increased in SI compared to RE, some were recovered by both KRG and Rb1 (Fig. 5), while others were recovered only by KRG (Fig. 6A–C), or by Rb1 (Fig. 6D–I). On the other hand, among significantly reduced metabolites in SI compared to RE, some were recovered by both KRG and Rb1 (Fig. 6J–S), while others were recovered only by KRG (Fig. 6T). Predicted metabolites include the following metabolites whose functions are known: cortisol 21-mesylate (Fig. 5A), a stress hormone metabolite derivative; methyl pentadecanoic acid (Fig. 5B), a biomarker of rheumatoid arthritis; O6-benzyl-8-oxoguanine (Fig. 5C), a DNA repair protein.

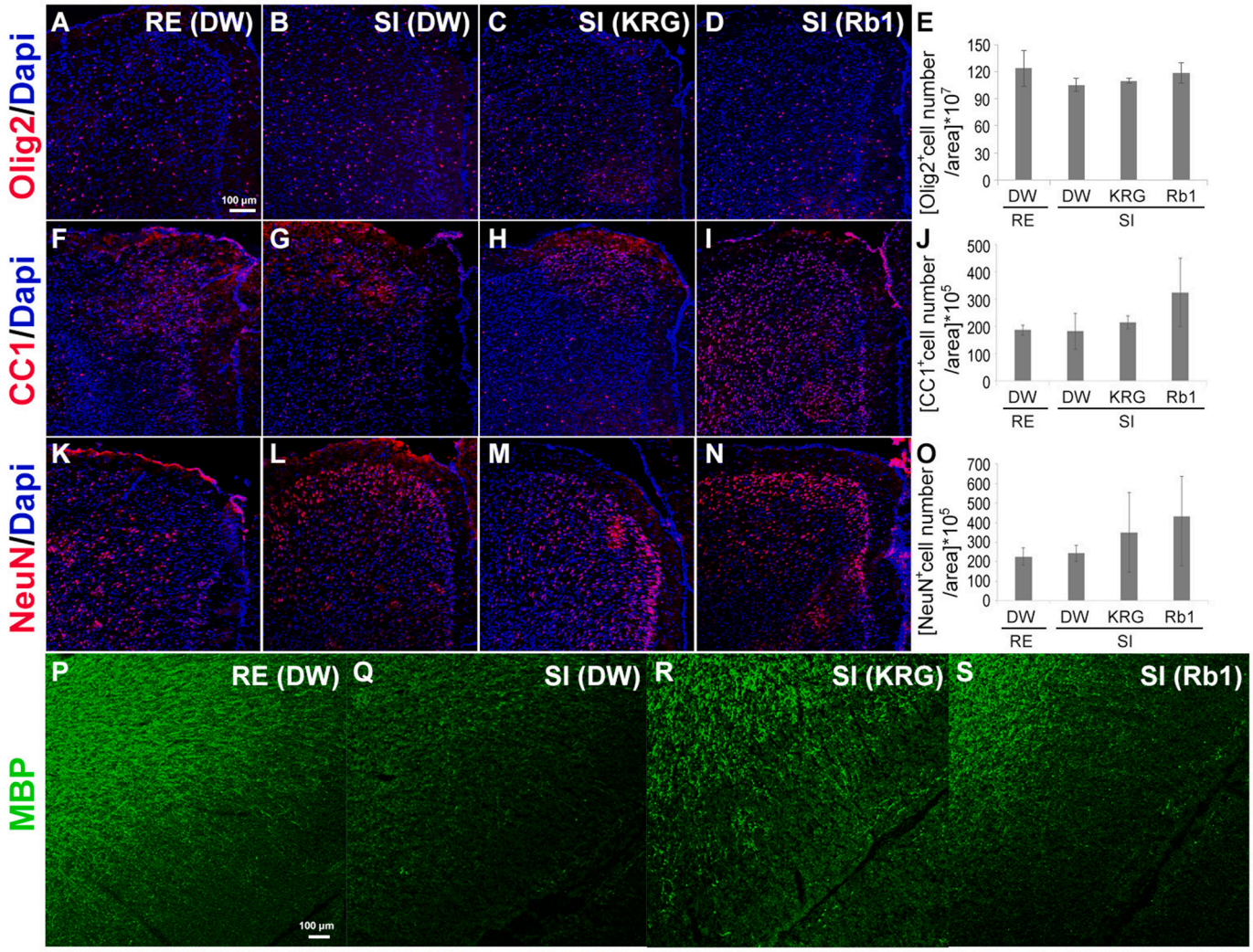
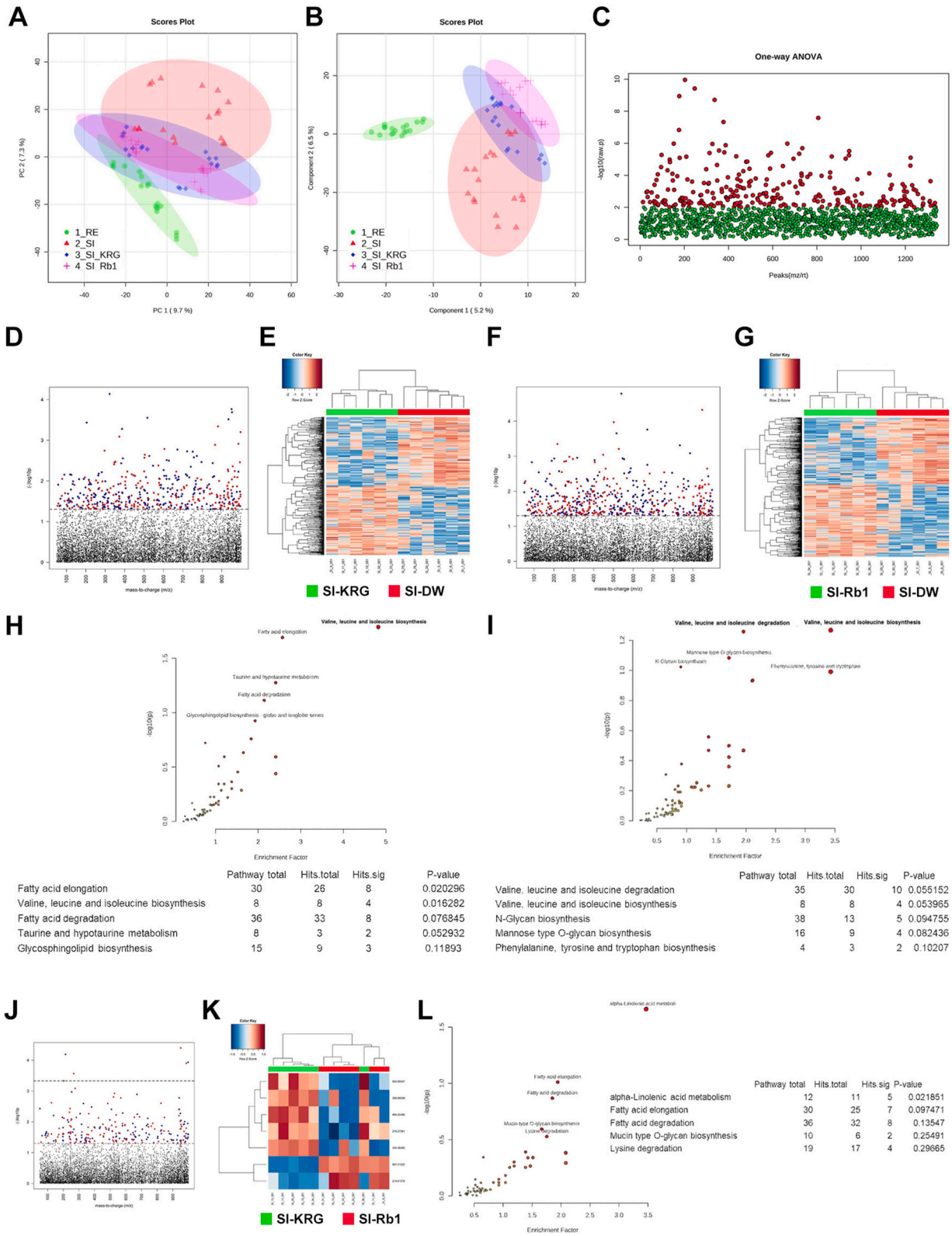


Fig. 3. Immunohistochemistry in the mPFC after post-weaning social isolation with DW, KRG or Rb1 administration. (A–D) Immunohistochemistry (IH) of Olig2 (red) and Dapi (blue). **(E)** Olig2-positive cell number in the field of view. **(F–I)** IH of CC1 (red) and Dapi (blue). **(J)** CC1-positive cell number in the field of view. **(K–N)** IH of NeuN (red) and Dapi (blue). **(O)** NeuN-positive cell number in the field of view. **(P–S)** IH of MBP. Scale bars, 100 μm. Bars indicate mean ± standard deviation. N = 3–4 mice/group. Concentrations for KRG and Rb1 were 400 mg/kg and 0.1 mg/kg, respectively. RE, regular environment; SI, social isolation; DW, distilled water.

4. Discussion

Post-weaning SI reduces myelin levels in the mPFC, which is related to decreased sociability [5,6,24], and myelination in the mPFC is influenced by gut metabolites [10,11]. Therefore, the connection between social behavior, mPFC, and gut metabolites is an interesting focus. KRG and Rb1 are known to promote myelin formation [15,17], and alter the gut microbiome and its metabolites [18–21]. However, the specific effects of KRG and Rb1 on social behavior, gene expressions in the mPFC and gut metabolites during post-weaning SI have not been elucidated, which we investigated in this study. Our findings reveal that administrations of KRG or Rb1 restored altered sociability, gene expressions in the mPFC, and gut metabolites, induced by two weeks of post-weaning SI (P21–P35), a critical period for sociability.

The dosages of Rb1 and KRG in the current study were determined based on our previous studies and pre-tests for the current behavior test. In a previous study, we analyzed the dose effect of Rb1 on in vivo myelination [15]. Adult mice under environmental enrichment were orally administered with 0, 0.1, 1, 5 mg/kg Rb1 daily for 10 days and then sacrificed for qPCR and WB. Both mRNA and protein expressions of MBP were highest at 0.1 mg/kg [15]. Consistent with our previous result, in the current study, 0.1 mg/kg Rb1 significantly increased the expressions of myelin-related genes; *Myrf* and *MAG* (Fig. 2A and B). Furthermore, in another previous study of KRG [17], we tested three different concentrations for KRG (25, 250, 1000 mg/kg) and Rb1 (0.1, 1, 5 mg/kg) and found that even the lowest concentrations (25 mg/kg KRG and 0.1 mg/kg Rb1) exhibited accelerated recovery from cuprizone-induced demyelination during the remyelination phase. As the Rb1/KRG ratio is 6.24 mg/g, 25 mg/kg KRG corresponds to about 0.1 mg/kg Rb1, which was used in the current study. We kept the Rb1 concentration at 0.1 mg/kg, however we tested higher KRG concentrations (150, 400 mg/kg) in the current study, not 25 mg/kg, because the behavior test did not show a significant change at low KRG concentrations, whereas 0.1 mg/kg Rb1 still yielded a significant change in the behavior test (Fig. 1O–Q). The significant physiological effects of Rb1 at the low dosage were also reported by other groups [25–27]. The reason why the concentration of Rb1 did not have the same effect in KRG treatment as it did in Rb1 treatment alone does not rule out the possibility that various ginsenoside components contained in KRG extracts or their metabolites might cause unexpected inhibitory effects against



	Pathway total	Hits.total	Hits.sig	P-value
Fatty acid elongation	30	26	8	0.020296
Valine, leucine and isoleucine biosynthesis	8	8	4	0.016282
Fatty acid degradation	36	33	8	0.076845
Taurine and hypotaurine metabolism	8	3	2	0.052932
Glycosphingolipid biosynthesis	15	9	3	0.11893

	Pathway total	Hits.total	Hits.sig	P-value
Valine, leucine and isoleucine degradation	35	30	10	0.055152
Valine, leucine and isoleucine biosynthesis	8	8	4	0.053965
N-Glycan biosynthesis	38	13	5	0.094755
Mannose type O-glycan biosynthesis	16	9	4	0.082436
Phenylalanine, tyrosine and tryptophan biosynthesis	4	3	2	0.10207

	Pathway total	Hits.total	Hits.sig	P-value
alpha-Linolenic acid metabolism	12	11	5	0.021851
Fatty acid elongation	30	25	7	0.097471
Fatty acid degradation	36	32	8	0.13547
Mucin type O-glycan biosynthesis	10	6	2	0.25491
Lysine degradation	19	17	4	0.29665

Fig. 4. Gut metabolomics from mouse fed with DW, KRG or Rb1 under post-weaning social isolation condition or regular environment. **(A)** Principal component analysis. **(B)** Partial least squares-discriminant analysis. **(C)** One-way anova. Red circles indicate significantly identified 313 metabolites among total 3027 metabolites. **(D, E, H)** Gut metabolomics analysis for KRG vs DW under post-weaning SI. **(F, G, I)** Gut metabolomics analysis for Rb1 vs DW under post-weaning SI. **(J–L)** Gut metabolomics analysis for KRG vs Rb1 under post-weaning SI. **(D, F, J)** Manhattan plot. m/z features above the dashed horizontal line meet the selection criteria. Red dots indicate higher in KRG (D) or Rb1 (F) compared to DW, while higher in Rb1 compared to KRG (J). Dark blue dots indicate lower in KRG (D) or Rb1 (F) compared to DW, while lower in Rb1 compared to KRG (J). **(E, G, K)** Two-way hierarchical cluster analysis. Green indicates KRG (E, K) and Rb1 (G). Red indicates DW (E, G) and Rb1 (K). Color key indicates low to high expression by blue to red color. **(H, I, L)** Pathway and functional analysis. N = 6 male mice/group. Concentrations for KRG and Rb1 were 400 mg/kg and 0.1 mg/kg, respectively.

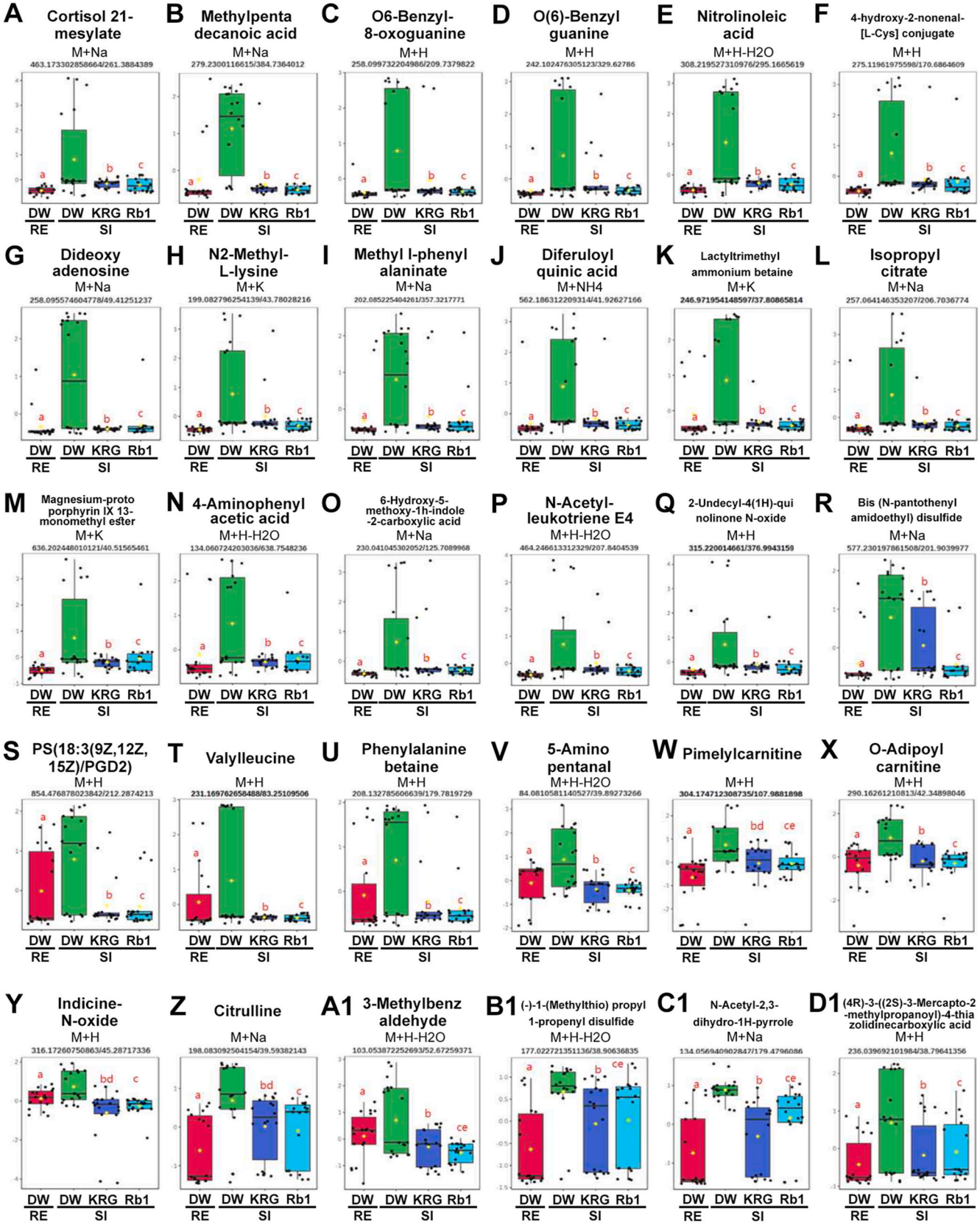


Fig. 5. Predicted gut metabolites which were significantly increased under social isolation compared to regular environment, however recovered by both KRG and Rb1 under social isolation condition. Name of predicted metabolite, ion form, mass to charge ratio (m/z), retention time are indicated above every box and whisker plot. All indicated metabolites are significantly altered in one way ANOVA. a, $P < 0.05$ in RE-DW vs SI-DW; b, $P < 0.05$ in SI-DW vs SI-KRG; c, $P < 0.05$ in SI-DW vs SI–Rb1; d, $P < 0.05$ in RE-DW vs SI-KRG; e, $P < 0.05$ in RE-DW vs SI–Rb1. N = 6 male mice/group. Concentrations for KRG and Rb1 were 400 mg/kg and 0.1 mg/kg, respectively.

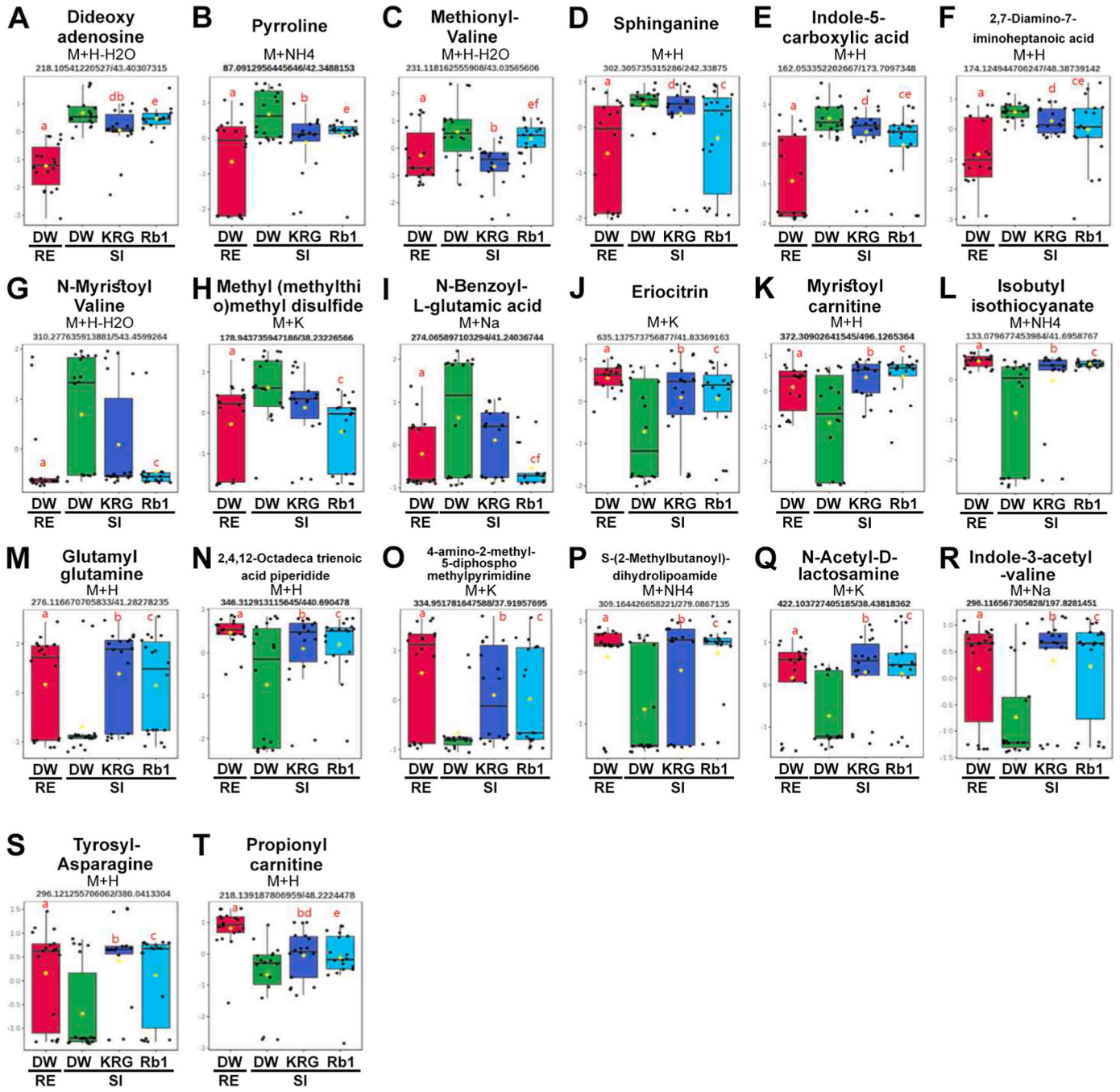


Fig. 6. Predicted gut metabolites which were significantly altered under social isolation compared to regular environment, however recovered by either KRG or Rb1 under social isolation condition. Name of predicted metabolite, ion form, mass to charge ratio (*m/z*), retention time are indicated above every box and whisker plot. All indicated metabolites are significantly altered in one way ANOVA. a, $P < 0.05$ in RE-DW vs SI-DW; b, $P < 0.05$ in SI-DW vs SI-KRG; c, $P < 0.05$ in SI-DW vs SI–Rb1; d, $P < 0.05$ in RE-DW vs SI-KRG; e, $P < 0.05$ in RE-DW vs SI–Rb1. N = 6 male mice/group. Concentrations for KRG and Rb1 were 400 mg/kg and 0.1 mg/kg, respectively.

Rb1-mediated signaling.

In our study, we measured sociability for behavior output induced by SI. In the rodent model, post-weaning SI has been well known to reduce sociability [5,6,24]. In human, the reduction of sociability is one among earliest signs of psychiatric disorders [28] and it is a common feature of bipolar disorder [29], major depressive disorder [30], schizophrenia [31], Alzheimer's disease [32]. Similarly, as rodents are highly social animals, their social behaviors can be phenotypes which underpin psychiatric disorders [33]. Therefore, in this study, although there are various psychiatric symptoms which may be induced by post-weaning SI, we specifically focused on social behavior (Fig. 1).

Our current results showed that KRG and Rb1 can restore the reduction in sociability induced by post-weaning SI (Fig. 1). Consistent with our findings, several studies have demonstrated a positive association of ginseng and its ginsenosides with social behaviors or related mechanisms. R2, a major saponin of Vietnamese ginseng (*Panax vietnamensis* HA *et* GRUSHV., Araliaceae), exhibited a protective effect against oxidative stress induced by protracted SI (6–8 weeks) in male ICR mice [34]. However, this study did not include sociability test. Abnormal behaviors related to autism, including sociability, induced in male offspring mice by *in utero* valproic acid (VPA) exposure to female pregnant ICR mice, were mitigated by KRG administration (100, 200

mg/kg) for one week (P21–P28) [13]. Furthermore, administering KRG (100, 200 mg/kg) to pregnant rats exposed to VPA also provided protections from VPA-induced abnormal sociability in male offspring rats at P30 [35]. Additionally, chronic social defeat stress (CSDS)-induced social avoidance was rescued by 20 mg/kg KRG administration on adult C57BL/6J mice [14]. These collectively suggest that components of ginseng are bioactive in mechanisms related to sociability.

In our current study, we focused on a specific brain region mPFC (Fig. 2), because it predominantly engages in advanced processing of social behaviors by establishing robust connections with cortical and subcortical regions, encompassing key areas associated with social behavior, such as the amygdala, subregions of the hypothalamus, the hippocampus, and the nucleus accumbens [36]. In the mPFC, we investigated multiple gene expressions including ones related with myelin, neurotransmitter systems, and oxidative stress (Fig. 2), as they have been known to be affected by SI [6,37–42]. A litter mate play behavior is one of the important stimulants for brain development during post-weaning period (P21–P35) [43]. Therefore, SI which lacks this social interaction resulted in reduction in neurotransmitter-related gene expressions (Fig. 2D and E) as well as neural activity-induced myelin-gene expressions (Fig. 2A–C). The *Gls* mRNA reduction (Fig. 2E), which may result in reduced glutamate level, can affect myelin-related gene expressions, as glutamate can regulate OPC proliferation, differentiation and myelination [44]. In mice of late adolescent isolation stress combined with genetic risk of schizophrenia, glutamatergic neurons exhibited abnormal features in the PFC including decreased level of glutamate [40]. The reduction of *Myrf* (Fig. 2A) indicates disruptions in OL differentiation and myelination by SI as it is a transcription factor for myelin genes [45], while the reduction of *MAG* by SI (Fig. 2B) presents decreased initiation of myelin as MAG localizes in the innermost membrane of compact myelin in contact with axon [46]. The reduction of *MBP* by SI (Fig. 2C) indicates decreased whole myelin structure as MBP presents in between lipid bilayers of compact myelin [47]. Both KRG-L and KRG-H significantly recovered the expressions of *Myrf* and *MAG* but not *MBP* under SI (Fig. 2A–C). These may reflect the contribution of KRG on OL maturation (i.e., upregulated *Myrf* expression) and initiation of myelin wrapping (i.e., upregulated *MAG* expression), however, it might not be enough for recovery of compact myelin structure (i.e., *MBP* expression) within the critical two week periods (P21–P35). The SI-induced reductions in MAG and MBP mRNA expressions in the mPFC (Fig. 2B and C) are consistent with previous report studied in male mice [6]. Oxidative stress is one of the underlying mechanisms of SI-related pathological phenotypes [41]. SOD2 is an antioxidative enzyme that reduces harmful reactive oxygen species. Decreased SOD2 activity can lead to various pathological phenotypes especially within the central nervous system [42]. *SOD2* mRNA expression was significantly reduced in the mPFC after SI (Fig. 2H), suggesting SI facilitates oxidative stress-related damage in the mPFC.

The SI-induced reduction of myelin gene-related expressions (*Myrf* and *MAG*) in the mPFC was significantly recovered by KRG (150 and 400 mg/kg) and Rb1 (0.1 mg/kg) (Fig. 2A and B). In our previous studies, Rb1 facilitated the production of myelin sheath the most among the major 11 ginsenosides of KRG in vitro and it also facilitated myelin levels in vivo [15,17]. After the cuprizone diet, KRG administration accelerated myelination in the corpus callosum during the remyelination phase, although the final myelin level reached an equivalent level after 3 weeks of remyelination [17]. However, KRG or Rb1 administration did not change the numbers of OL-lineage cells, mature OLs or neurons (Fig. 3A–O). These accumulated results indicate that KRG and Rb1 may facilitate myelin production in pathological conditions in vivo, but without changing OL numbers in SI.

The mRNA expression level of *Gls*, an enzyme which facilitates glutamate synthesis, was significantly increased by KRG under SI (Fig. 2E). In another study, KRG administration recovered N-methyl-D-aspartate receptor levels induced by chronic social defeat stress in the hippocampus [14]. These results suggest an association of KRG with the modulation of the glutamatergic system.

KRG-H-administered mice exhibited a significant increase in the expression of *ET-1*, compared to other groups, under SI (Fig. 2G). ET-1 increases OL maturation, contributing myelin formation, under pathological conditions: in vivo preterm white matter injury and in vitro oxygen-glucose deprivation conditions [48]. Therefore, the increase in *ET-1* expression level by KRG-H supports the upregulation of myelin-gene expression in our study.

In rodent models of SI, reductions in the expression of BDNF mRNA and protein in the hippocampus have been known as underlying mechanisms associated with depression [49]. However, in post-weaning SI experiments, both no changes [50] and increases [51] in BDNF expression in the hippocampus have been also reported. Moreover, in rodent post-weaning SI models, there have been reports of both no changes [50,52] and increases [51,53–55] in BDNF expression in the mPFC. In the current study, BDNF mRNA expression was not changed by SI, and administrations of KRG or Rb1 did not significantly alter the level (Fig. 2F). In other studies, the intraperitoneal injections of 100 mg/kg KRG to male adult rats for 14 days after single prolonged stress, a post-traumatic stress disorder model, improved the expression of BDNF mRNA in the hippocampus [56], while oral administration of 20 mg/kg Rb1 to adult male mice for 21 days under chronic unpredictable mild stress also increased BDNF protein levels in the hippocampal CA3 region and PFC [57]. The different results regarding the effects of KRG and Rb1 on BDNF expression may stem from rodent strain, age, brain region, and type of stressor.

In this study, among the significantly recovered metabolites by administrations of either KRG or Rb1 under SI, cortisol 21-mesylate is an alkylating derivative of cortisol, a stress hormone (Fig. 5A) [58]. The detection of its derivative may reflect changes in the HPA axis due to SI and recovery by KRG and Rb1. In both human and animal studies, SI can activate HPA axis, increasing cortisol secretion [59]. As an end product of the stress response, corticosterone is known as a main corticosteroid hormone in rodents, but cortisol is also significantly detected in stress environments of rodents [60,61]. In rodents, stress inhibits the proliferation of OL precursor cells in vivo [62]. Similarly, in humans, under stress, the level of myelin is reduced in patients with major depressive disorder [63,64]. Not only glucocorticoid directly reduces the formation of myelin in vitro [65], but also it can affect white matter integrity indirectly through inflammatory cytokines [66,67]. The administration of KRG or Rb1 under SI reduced cortisol derivatives (Fig. 5A), suggesting that their administration may have reduced stress-induced HPA axis activation. KRG has been known to alleviate stress response by controlling HPA axis [68], exhibiting a similar effect to the antidepressant fluoxetin in the depression model [69], and an anti-stress effect in vivo study [70,71]. Rb1 has also been reported to reduce neuronal toxicity associated with glucocorticoid activity [72]. Therefore, it is estimated that KRG and Rb1 not only directly promote myelin formation as our previous reports [15,17], but also reduce cortisol secretion by lowering the HPA axis activation under post-weaning SI, thereby preventing a potential myelin reduction by cortisol under stress. Moreover, cortisol/corticosterone not only affects myelin level in the brain but also social behaviors. Two weeks of corticosterone oral administration disrupted social interaction in adult C57BL/6 N female mice [73]. Mandarin voles with low sociability showed higher levels of corticosterone in serum than voles with high sociability [74]. Human individuals with higher cortisol levels exhibited a reduced social interaction [75]. These suggest that the recovery of SI-induced increase of cortisol 21-mesylate

by KRG or Rb1 (Fig. 5A) is potentially associated with improved sociability, which indeed we observed in sociability test (Fig. 1O-Q).

In human, SI and loneliness have been linked to systemic inflammation [76]. Social behavior has been known to be associated with inflammation in both animal and human researches [77]. In human and animal models, chronic social stress induces glucocorticoid resistance, upregulates inflammatory gene expressions, and increases cytokine production by immune cells, in turn strengthening glucocorticoid resistance. Consequently, inflammatory conditions are increased by SI [78]. In our results, methylpentadecanoic acid, a rheumatoid arthritis marker, increased (Fig. 5B), reflecting an increase in inflammatory conditions caused by SI. Moreover, the restorations by KRG and Rb1 suggest that their anti-inflammatory effects [79,80] may be effective in SI and potentially contributing to the recovery of sociability under SI.

Conditions induced by SI - cytokines, stress hormones, and increased ROS - [78] are known to cause damage to DNA [81]. In the central nervous system, OLs are very vulnerable to oxidative DNA damage, and the failures in repairments disrupt myelin gene transcription, and OL differentiation [82]. In addition, major psychiatric diseases which exhibit abnormal social behaviors show substantially high levels of DNA damages in the brain [83]. KRG protects DNA from damage in poor environments [84,85]. In our results, SI increases O6-benzylguanine (BG) and O6-benzyl-8-oxoBG, which strongly inactivate DNA repair protein, compared to RE, while administration of KRG or Rb1 under SI restored them to their original levels (Fig. 5C and D). This suggests the protective function of KRG and Rb1 against DNA damage under SI. Moreover, the recovery of sociability (Fig. 1O-Q) as well as myelin-related gene expression by KRG and Rb1 in the mPFC (Fig. 2A and B) may also have partially been contributed by the DNA damage protection function of KRG and Rb1, which is assumed by upregulation of DNA repair-related gut metabolites (Fig. 5C and D).

The stress induced by two weeks of post-weaning SI significantly changed the gut metabolites, including ones associated with HPA axis activity, inflammation, DNA damage, and changed gene expression in the mPFC, and sociality. During the post-weaning SI, the intake of KRG or Rb1 restored the aforementioned behavior, brain and gut phenotypes, suggesting they can partially correct the abnormality of the brain and gut, induced by SI. Both the direct effects of KRG and Rb1 on gene expressions in the mPFC and the indirect contribution through gut metabolite changes to brain may have contributed to the recovery. Post-weaning SI in rodents can be used as a model for schizophrenia [86] as well as developmental disorders such as attention deficit hyperactivity disorder and autism spectrum disorder [87]. In our study, restoration of sociability by KRG or Rb1 and its related molecular mechanisms suggest that they can affect the mechanisms of these mental disorders and contribute to symptom relief, therefore warranting further studies.

Author contributions

Data curation, Formal analysis, Visualization, Investigation Methodology: Oh Wook Kwon, Youngja Hwang Park, Dalnim Kim, Hyog Young Kwon; Funding acquisition: Hyun-Jeong Yang, Youngja Hwang Park; Conceptualization, Project administration, Supervision, Visualization, Investigation Methodology, Roles/Writing -original draft, and Writing - review & editing: Hyun-Jeong Yang.

Declaration of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2024.03.005.

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