Mutation of the key residue for extraribosomal function of ribosomal protein S19 cause increased grooming behaviors in mice

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Abstract

Ribosomal protein S19 (RP S19) possesses ribosomal function as RP S19 monomer and extraribosomal function as cross-linked RP S19 oligomers which function as a ligand of the complement 5a (C5a) receptor (CD88). We have generated a Gln137Glu-RP S19 knock-in (KI) mouse, which is shown to possess the weakened extraribosomal function of RP S19. Because whether the extraribosomal function of RP S19 has a role in brain function had been unclear, we performed behavioral analysis on these mice and demonstrated that KI mice displayed an increased grooming behavior during open-field test and elevated plus maze test and an enhanced freezing behavior in contextual fear conditioning test. These results suggest an involvement of RP S19 oligomers in some anxiety-like behavior, especially grooming behavior.

Keywords:

Anxiety-like behavior, Complement system, Extraribosomal function, Fear memory, Grooming behavior, Ribosomal protein S19

Abbreviations:

ANOVA, analysis of variance

CNS, central nervous system

RP S19, ribosomal protein S19

KI, knock-in

WT, wild-type

Gln, glutamine

Glu, glutamic acid

C5a, complement 5a

1. Introduction

Ribosomal protein S19 (RP S19) is a component of the small ribosomal subunit, playing a role in ribosome biogenesis [1]. RP S19 is also present in blood plasma forming a complex with prothrombin [2]. RP S19 is oligomerized via the intermolecular crosslinking between Lys122 and Gln137 by a transglutaminase-catalyzed reaction and gains a ligand capacity to the complement 5a (C5a) receptor (CD88) as an extraribosomal function [2,3]. The RP S19 oligomer-C5a receptor system plays roles in apoptotic cell clearance, in blood coagulum resorption and in erythrocyte maturation with enucleation [4].

We recently generated the Gln137Glu-RP S19 knock-in (KI) mice to reveal further the extraribosomal roles of RP S19 oligomers *in vivo*. This mutation did not apparently affect the ribosomal function of RP S19 because homozygote mice with Gln137Glu-RP S19 were born alive and fertile [5]. Our previous *in vitro* study of a Gln137 mutation suggested at least a 70% reduction in the extraribosomal function of RP S19 in these KI mice [6]. Indeed, a significant delay of clearance of KI mouse-derived blood coagulum was observed in a hematoma resorption model [5].

In the central nervous system (CNS), several evidences gave a role of complement system in development, neuroprotection, neurogenesis and synaptic plasticity [7]. Expression of C5a receptor is revealed in astrocytes and microglia of the CNS [8] and most neurons in the cerebral cortex, hippocampus and cerebellum [9]. Therefore, complement system via C5a receptor is suggested to be involved in CNS function and neurodegenerative diseases [7,10]. In that case, the RP S19 oligomers could have a role in CNS function via binding to C5a receptor. Moreover, RP S19 mRNA expression was revealed in pyramidal neurons of hippocampus [11]. However, relevance of C5a

receptor and RP S19 extraribosomal function to *in vivo* function is largely unknown. Therefore we examined whether the Gln137Glu-RP S19 KI mice expressed some abnormalities in behavioral tests.

2. Materials and Methods

2.1. Animals

We prepared Gln137Glu-RP S19 KI heterozygous mice by knock-in technology via homologous recombination as described previously [5]. We purchased specific pathogen-free C57BL/6J mice from Charles River (Yokohama, Japan) and the heterozygous KI mice were backcrossed with C57BL/6J mice more than six times. Then heterozygous KI mice were intercrossed to obtain homozygous mice. The homozygous KI mice and the wild-type (WT) mice were bred and maintained, respectively, in the same way at the Center for Animal Resources and Development, Kumamoto University. Genotyping is performed on tail DNA via polymerase chain reaction using primers (Forward, TGGGCTGTACTCATCCAGGGT; Reverse, TGCTTCTTGTTGGCAGCTGCCAC) designed across the loxP site. Mice were maintained on a 12 h light/dark cycle (7 a.m. on/ 7 p.m. off) with laboratory chow and water available *ad libitum*. Male mice were used for all experiments and the behavioral tests were performed at light cycle. The present animal experiments were approved by the Ethical Committee for Animal Experiment, Kumamoto University (# A27-129).

2.2 Monocyte chemotaxis assay on hippocampal extracts

Multiple hippocampi from WT and KI mice were pooled and weighed. The same weight of phosphate-buffered saline was added to tissues and homogenized by sonication. The

homogenates were centrifuged at $10,000\times g$ for 25 min at 4°C and the supernatants were recovered. The supernatants were immediately treated for 30 min at 56°C to deactivate the complement system and other proteases. This treatment does not lead to dissociation of RP S19 oligomers because of the covalent cross-link [6]. Then supernatants were centrifuged again at $10,000\times g$ for 25 min at 4°C and subjected to monocyte chemotaxis assay. Mononuclear cells were isolated from heparinized venous blood of healthy human donors according to Fernandez et al. [12] under approval of the Institutional Review Board of Kumamoto University (# 673). The mononuclear cells were suspended at 1×10^6 cells/ml in RPMI 1640 containing 10% fetal bovine serum. We evaluated the monocyte-attracting capacity of hippocampal extracts using a 48-well chemotaxis chamber with a polycarbonate filter ($5 \mu\text{m}$ pore size) according to Falk et al. [13]. After incubation for 90 min, each membrane was stained with Giemsa solution. The total number of monocytes that migrated beyond the lower surface of the membrane was counted in five microscopic high-power fields as described previously [14].

2.3. Behavioral analyses

Open-field test was performed on male mice at the age of 10 weeks. The test chamber were nearly cubic ($W45 \text{ cm} \times D45 \text{ cm} \times H40 \text{ cm}$), and illuminated at 100 lux [15]. The floor was divided into 81 equal squares, and the central 49 squares were defined as the center area. Each mouse was placed in a corner of the chamber, and behavior was recorded using a video recorder for 30 min. The distance traveled and time spent in the center areas at the initial 5 min were analyzed by the LimeLight software (Actimetrics, Wilmette, IL, USA).

One or two days later after the open-field test, elevated plus-maze test was performed according to Walf and Frye [16] with modifications. The maze consists of two

opposite-facing open arms ($30\text{ cm} \times 7\text{ cm}$), two opposite-facing closed arms ($30\text{ cm} \times 7\text{ cm} \times 20\text{ cm}$), and a central area measuring $6\text{ cm} \times 6\text{ cm}$, and was mounted on a base 37 cm above the floor. Each mouse was placed in the central area and a 10-min test session was initiated. Total distance traveled, the number of total entries, the percent of entries into the open arms and the time spent in the open arms were analyzed by the LimeLight software.

Ethological parameters are analyzed during open-field and elevated plus-maze tests [17]. Grooming includes all sequences of face-wiping, scratching/rubbing of head and ears and full-body grooming. Percent of time spent grooming was measured and grooming bouts was counted when lasted at least 3 sec. The number of rearing (vertical standing on two hind legs) was also counted. In the elevated plus-maze, the number of head-dips (downward movement of head toward the floor from the entire area of the open arms) was counted.

Five days later after the elevated plus maze test, contextual and cued fear conditioning test was performed according to Miyakawa et al. [18] with modifications. On conditioning day, mice were placed in a test chamber ($W26\text{ cm} \times D34\text{ cm} \times H29\text{ cm}$) and allowed to explore the conditioning context. After 2 min, 30 sec of white noise (90 dB) was presented and co-terminating with a 0.3 mA foot shock for 2 sec. This noise-shock pairing was repeated twice with an interval of 2 min. One day later, the conditioned mice were re-exposed to the conditioning chamber for 6 min without auditory cue and freezing were measured to test contextual fear memory. The mice were then tested in a novel chamber made of a triangle ceiling and white floor for 3 min, followed by the auditory cue for 3 min to test cue-induced freezing. All data were analyzed using FreezeFrame-3 Conditioned Fear System (Neuroscience, Tokyo, Japan).

Data were evaluated as a percent of freezing time periods in each minute.

Marble burying test was performed on male mice at the age of 12 months according to Gyertyan et al. [19] with modifications. The home cage was consist of the standard plastic cage (21 × 15 × 12 cm) with a 2 cm thick layer of wooden bedding, and used for the test. Six glass marbles (1.2 cm in diameter) were placed evenly on the bedding, and mice were then placed on its home cage. The number of buried ($>2/3$) marbles was analyzed 30 min later under blind to the genotype of the mice for an experimenter. After 5 days same experiment was performed again on both mice.

2.4. Statistical analyses

Data were analyzed by two-way repeated measures ANOVA with Bonferroni post-hoc test for fear conditioning test. The two-tailed unpaired Student's *t*-test was used for normally distributed samples in the open-field, elevated maze and marble burying test. Values in graphs are expressed as means ± SEM.

3. Results

3.1. Reduction in the monocyte chemotactic activity of the Gln137Glu-RP S19 KI mouse hippocampus

We have generated Gln137Glu-RP S19 knock-in (KI) mice via homologous recombination technology in mouse embryonic stem cells (Fig. 1a). We examined whether the chemotactic activity via RP S19 oligomers reduced in the hippocampus of KI mice. We initially measured monocyte chemoattraction capacities of extracts from hippocampi of WT and KI mice at 20-25 weeks of age. Figure 1b shows that each hippocampal extract possessed significant monocyte chemoattraction capacities that

produced dose-activity correlations. A comparison of the chemotactic capacities of the hippocampal extracts between WT and the KI mice according to the dose-activity correlation curves revealed that the capacity of KI mice was almost 33% of WT mice (Fig. 1b).

3.2. Enhanced grooming behavior in Gln137Glu-RP S19 KI mice

We performed behavioral analysis to assess phenotypes of locomotor activity, anxiety behavior and fear learning in KI mice. In the open-field test, the traveled course of each mouse (10 KI and 10 WT mice) was traced for 30 min and total moving distance and time spent in the center area at the initial 5 min were compared between the groups. There was no significant difference in the total moving distance and center time (Fig. 2a,b). In the elevated plus maze, there was no significant difference in the total distance travelled, the total entries into open and closed arm, percent of entries into open arms (Fig. 3a-c), while the time spent in open arms slightly decreased in KI mice ($p < 0.1$; Fig. 3d).

Grooming behavior is shown as an anxiety marker [20]. Therefore we measured grooming behavior during both tests. Grooming bout and time during open-field test slightly increased in KI mice but not significant (Fig. 2c,d), while rearing significantly increased in KI mice ($p < 0.01$; Fig. 2e). During elevated plus maze, KI mice showed remarkably increased both grooming bout and time ($p < 0.01$; Fig. 3e and f). Rearing during this test was not affected (Fig. 3g), but head-dipping was significantly reduced in KI mice ($p < 0.05$; Fig. 3h). These data suggest enhanced anxiety-like behaviors in Gln137Glu-RP S19 KI mice.

3.3. Enhanced fear memory in Gln137Glu-RP S19 KI mice

Next these mice were subjected to the fear conditioning test. Data in the contextual and cued fear response were evaluated as percent freezing times during each min. No difference in the duration of freezing was observed during the conditioning (Fig. 4a). However, KI mice exhibited significantly increased freezing behavior during contextual testing at the time point of 1, 2 and 6 min ($p<0.05$ or $p<0.01$; Fig. 4b). There was no difference between the two groups in freezing during cued testing which were tested in a novel context with same auditory cue (Fig. 4c). These results suggest that contextual fear memory was strengthened in KI mice.

3.4. Decreased marble burying in Gln137Glu-RP S19 KI mice

Given the results of enhanced grooming behavior, we performed the marble burying test as another index of repetitive behavior. However KI mice showed significantly decreased the number of buried marbles ($p<0.01$; Fig. 5). This result shows decreased repetitive behavior or decreased the interest to unfamiliar environment. Because the number of buried marbles was similar between WT and KI mice at another test 5 days after the first trial.

4. Discussion

In this study, we identified some abnormal phenotypes of behavior in KI mice. These mice showed slightly enhanced anxiety-like behavior in the open-field test and elevated plus maze and remarkably increased grooming behavior. Furthermore these mice showed enhanced contextual fear memory in fear conditioning test.

Grooming in rodents has been used as an indicator of anxiety and depression [20].

Severe grooming behavior appears in rodent model of autism spectrum disorders (ASD), obsessive-compulsive disorder (OCD) and Tourette's syndrome [21,22,23]. These are stress-based psychiatric disorders that show symptoms of tic-like compulsion and complex repetitive behavior [24]. KI mice seem to be more stressful in new environment as the open-field and elevated plus maze, thus self-grooming increased in these mice. Supporting this idea, KI mice had lower activity in the elevated plus maze as shown by lower value of total distance, number of total entries and % of entries into open arms in these mice (Fig. 3a-c), while grooming time increased instead. In the marble burying test KI mice showed decreased burying behavior. This test is thought as an index of repetitive behavior and reflects OCD-like behavior. Furthermore burying behavior also seems to reflect an interest of mice for novel marbles. Autistic patients have symptoms such as repetitive behavior and reduced interest for environment. KI mice seem to have autistic-like features supported by some reports that ASD model mice show the phenotype of increased self-grooming and decreased burying behavior [21,25]. At same test was performed 5 days after the initial trial, the number of buried marbles of WT and KI mice became equally (Fig. 5b), suggesting that KI mice might become familiar to the marbles and had equal interest for the marbles with WT mice.

Many brain regions have been implicated in grooming, such as the basal ganglia, striatum, hypothalamus, cerebellum and brain stem [24]. And over-grooming is observed in genetically mutated mice such as hyper-dopaminergic mutant mice and histidine decarboxylase knockout mice that caused by abnormalities of basal ganglia function [22,26]. The expression of functional C5a receptor is revealed in the hypothalamus and cerebellum [27,28], suggesting that signals from this receptor are involved in an increase of grooming behavior observed in our KI mice. Rearing in open

field test interact with anxiety in a complex manner [29]. Head-dipping in the elevated plus maze can be considered as a risk assessment and the increased number of this behavior is correlated with anxiolytic effect of drugs [30]. In KI mice, rearing increased in open field test and head-dipping decreased in elevated plus maze, showing enhanced anxiety-like behavior, respectively.

RP S19 is predominantly expressed in hematopoietic tissues and this gene mutation cause Diamond-Blackfan anemia [31]. However non-hematopoietic tissues like brain also transcribe RP S19 mRNA [31]. Moreover RP S19 mRNA expression was revealed in pyramidal neurons of hippocampus [11]. Thus neuronal RP S19 protein might has a role in some brain function, however, we cannot conclude which cell type causes behavioral changes observed in this study by Gln137Glu mutation such as neurons, astrocyte or oligodendrocyte.

In fear conditioning test, KI mice showed significantly enhanced contextual fear memory. Contextual fear memory is dependent on hippocampus [32], showing that acquisition of fear memory in the hippocampus is enhanced in KI mice. Similar phenotypes of increased anxiety and enhanced fear memory are also observed in interferon-gamma knockout mice [33] and in a posttraumatic stress disorder model mouse [34]. These reports suggest the involvement of hippocampal adult neurogenesis in these phenotypes [35,36]. The above-mentioned behavioral abnormalities may be attributed to the Gln137Glu mutation of RP S19 and a defect in the extra-ribosomal function of RP S19 oligomers. Adult neurogenesis modulates the hippocampus-dependent fear memory, and a reduced neurogenesis is accompanied by a prolonged hippocampus-dependent period of associative fear memory [35]. Therefore adult neurogenesis is involved in stress-regulated disorder, like anxiety and depression

[36]. Expressions of C5a and C3a receptors were revealed in neural stem and progenitor cells derived from adult hippocampus [37]. One possible mechanism underlay the increased anxiety and enhanced fear memory in KI mice is some alterations in systems of hippocampal neurogenesis, while we cannot exclude the function of RP S19 oligomers in the hypothalamus, cerebellum or other brain regions. However the clear mechanism how the lack of extraribosomal function of RP S19 leads to these behavioral alterations is still unclear. Furthermore we cannot conclude the relation between the behavioral phenotypes seen in KI mice because the ages of animals at the behavioral test were not similar between tasks. Further experiments are required to obtain the final conclusion.

In summary, we show that the lack of the extra-ribosomal function of RP S19 leads the increased grooming behavior in mice. This behavioral abnormality is related to enhanced anxiety-like behavior. Our results suggest the implication of RP S19 protein and the signals via C5a receptor in stress-based behaviors.

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Figure Legends

Fig. 1. Monocyte chemotactic activity in the hippocampus of KI mice

(a) Schematic diagram of the endogenous *RPS19* locus and the targeting vector used to engineer embryonic stem (ES) cells by homologous recombination. This vector contains a point mutation in exon 5 that changes glutamine residue at position 137 to glutamic acid (Gln137Glu). neo, neomycin-resistant gene; pA, poly A. (b) Chemoattraction capacities of the hippocampal extracts for normal human peripheral monocytes were measured by the multiwell chamber assay at 3 logarithmic serial concentrations. The interpolated figure demonstrates concentration (logarithmic)-function (linear) relation curves to quantify the ratio of the monocyte chemotactic capacity in the KI mouse extract against that in the WT mouse extract.

Fig. 2. Open-field test on Gln137Glu-RP S19 KI mice.

(a,b) Open-field test. The total distance traveled (a) and time periods in the center areas (b) at the initial 5 min were analyzed using the video-based analytical software. (c-e) Grooming bouts (c), time (d) and rearing (e) was measured during test and compared between WT and KI mice. The values are shown as mean with S.E.M. (n=10). ** indicate $p < 0.01$ (two-tailed unpaired Student's *t*-test). WT, wild-type; KI, knock-in.

Fig. 3. Elevated plus maze test on Gln137Glu-RP S19 KI mice

(a-d) The total distance traveled (a), the number of total entries (b), the percent entry into open arms (c), and the time spent in the open arms (d) were analyzed during the elevated plus maze test. (e-h) Grooming bouts (e), time (f), rearing (g) and head-dipping

(h) was measured during test and compared between WT and KI mice. The values are shown as mean with S.E.M. (n=10). *, ** indicate $p<0.05$, $p<0.01$, respectively (two-tailed unpaired Student's *t*-test). WT, wild-type; KI, knock-in.

Fig. 4. Fear conditioning test on Gln137Glu-RP S19 KI mice.

(a-c) Behavior of each mouse in the test chamber was recorded and number and period of freezing time were analyzed using a video-based analytical system. Data were evaluated as a percent of freezing time periods in each minute at conditioning (a), context (b) and cued (c) test. The values are shown as mean with S.E.M. (n=10). *, ** indicate $p<0.05$, $p<0.01$, respectively (two-way repeated measures ANOVA with Bonferroni *post-hoc* test). WT, wild-type; KI, knock-in.

Fig. 5 Marble burying test on Gln137Glu-RP S19 KI mice.

(a) Each mouse was placed on the home cage consisting of 6 marbles. After 30 min the buried marbles were counted and compared between groups. (b) Same test was performed on same animals after 5 days of initial test. The values are shown as mean with S.E.M. (n=4). * indicate $p<0.05$ (two-tailed unpaired Student's *t*-test). WT, wild-type; KI, knock-in.

(a)

(b)

Figure 1

Figure 2

Figure 3

Figure 4

(a)

(b)