Journal of Pharmacological Sciences 146 (2021) 1–9

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full Paper

T-type Ca^{2+} channel enhancer SAK3 administration improves the BPSD-like behaviors in $App^{NL-G-F/NL-G-F}$ knock-in mice

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ARTICLE INFO

Article history:
Received 18 September 2020
Received in revised form
13 February 2021
Accepted 22 February 2021
Available online 26 February 2021

Keywords:
SAK3
T-type Ca^{2+} channel
BPSD
Alzheimer's disease

ABSTRACT

Alzheimer's disease (AD) accounts for the majority of dementia among the elderly. In addition to cognitive impairment, behavioral and psychological symptoms (BPSD) such as depression tendency and increased aggression impose a great burden on the patient. However, there is still no rational therapeutic drug for BPSD. Recently, we developed a novel AD therapeutic candidate, SAK3, and demonstrated that it improved cognitive dysfunction in AppNL-G-F/NL-G-F knock-in (NL-G-F) mice. In this study, we investigated whether acute SAK3 administration improved BPSD in addition to cognitive improvement. Acute SAK3 administration improved BPSD, including anxiolytic and depressive-like behaviors, and ameliorated aggressive behaviors. Furthermore, continuous SAK3 administration improved anxiolytic and depressive-like behaviors. Intriguingly, the anti-anxiolytic and cognitive improvement lasted two weeks after the withdrawal of SAK3, whereas the anti-depressive action did not. Taken together, SAK3 had comprehensive beneficial effects on BPSD behavior.

1. Introduction

Alzheimer's disease (AD) is characterized by the excessive accumulation of intracellular hyperphosphorylated tau protein and amyloid beta (Aβ) in the extracellular region of the brain. In addition to core symptoms such as cognitive impairment and memory impairment, AD presents behavioral and psychological symptoms (BPSD) such as hallucinations and auditory hallucinations, sleep disorders; impulsivity; depression tendency; and increased aggression.[1,2] These various symptoms place a heavy burden not only on the patient, but also on the caregiver. The number of patients is increasing rapidly along with an aging population that is over twenty-five million people worldwide as of 2016. Moreover, the number of patients is more than sixty million, including mild cognitive impairment (MCI), which is a pre-stage of AD, and there are concerns about adverse economic effects such as medical and nursing care costs accordingly.[3]

Currently, donepezil, rivastigmine, and galantamine as cholinesterase (ChE) inhibitors as well as memantine as an NMDA receptor antagonist, are used for AD therapy. However, these therapeutic regimens/treatments are symptomatic and cannot prevent the progression of the disease itself. Therefore, the development of new disease-modifying drugs targeting amyloid β and tau protein is an ongoing process. In addition, these drugs are not sufficient for the suppression of BPSD. For example, donepezil and memantine showed an improved effect on depression-like behavior in olfactory bulbectomy (OBX) mice only when administered in combination.[4] Currently, pharmacological treatments with antipsychotics and other psychotropic drugs are necessary, and antipsychotic drugs are the first choice to reduce BPSD.[5,6] However, antipsychotics cause severe extrapyramidal side effects by blocking striatal dopamine D2 receptors, and its risk increases or decreases depending on the drugs for concomitant use to treat core symptoms.[6] Therefore, in order to develop safe, effective, and comprehensive therapeutic agents for BPSD, it is necessary to develop novel therapeutics to ameliorate both core symptoms and BPSD with a single agent only.

We have focused on T-type voltage-gated calcium channels (T-VGCCs) as a new therapeutic target for AD therapy. The T-VGCCs are activated at a low threshold, have the characteristics of fast activation and slow inactivation, and single-channel conductance.[7] The T-VGCCs have subfamilies Cav 3.1/3.2/3.3.[8,9] The T-VGCCs are widely distributed in the brain and are involved in the generation of sleep spindles and the periodic firing that controls

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Peer review under responsibility of Japanese Pharmacological Society.

https://doi.org/10.1016/j.jphs.2021.02.006

sleep in thalamic relay neurons.[10] We recently clarified that the T-VGCCs play an essential role in improvement of memory and learning in AppNL-G-F/NL-G-F knock-in (NL-G-F) mice, an AD model mice.[11]

In 2007, we developed ST101 (ZSET1446: spiro [imidazole [1,2-a]pyridine-3,2-indan]-2(3H)-one), the world's first enhancer of T-VGCCs, jointly with Zenyaku Kogyo.[12] ST101 promoted ACh release by activating T-VGCCs in the mouse hippocampal CA1 region and activated choline acetyltransferase (ChAT), which is important for ACh synthesis. It also enhanced long-term potentiation (LTP) by increasing the autophosphorylation level of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which is critical for cognitive memory learning in rodents.[13] ST101 was tested in a phase IIa clinical trial in the United States. Although ST101 improved cognitive impairment in combination with donepezil, it was less effective when administered alone. In 2013, we developed SAK3 (ethyl-8-methyl-2,4-dioxo-2-(piperidin-1-yl)-2H-spiro [cyclopentane-1,3-imidazo [1,2-a]pyridin]-2-ene-3-carboxylate) (Fig. 1A), which has higher pharmacological activity than ST101. SAK3 significantly enhanced the Cav3.1 and Cav.3.3 currents at concentrations of 100 pM to 10 nM over ST101.[14] In addition, the effect of promotion of ACh release in the hippocampal CA1 region and improvement of cognitive dysfunction by acute oral administration in OBX mice was stronger than that of ST101.[14] NL-G-F mice are the next generation AD model mice and have been widely used to elucidate mechanism of BPSD. For example, NL-G-F mice exhibit BPSD-like behavioral abnormalities such as anxiety-related and depression-like behaviors after 6 months of age.[15,16]

We here tested whether acute and chronic oral SAK3 administration (0.5 mg/kg/day) improved BPSD-like behaviors including anxiolytic, depressive, and aggressive performances observed in NL-G-F mice. We also demonstrated that the ameliorating effects lasted after two weeks of withdrawal of chronic administration for anti-anxiolytic and cognitive improvement. These results suggest that SAK3 has great potential as a novel therapeutic agent for AD that can improve not only cognitive function but also BPSD.

2. Materials and methods

2.1. Animals

Twelve-month-old male NL-G-F mice were generated as described previously (Saito et al., 2014). The wild-type (WT) mice used as controls were of the same background (C57BL/6J). All animals were kept under constant temperature (23 ± 1 °C), humidity (55 ± 5%), and a regular light/dark cycle (light: 9:00–21:00, dark; 21:00–9:00) with free access to food and water. All experimental procedures using animals were approved by the Committee on Animal Experiments at Tohoku University (2019 PhA-024, 1st April 2019). We tried to reduce animal suffering and use the minimum number of mice.

2.2. Experimental design and drug administration

SAK3 (0.5 mg/kg/day) was dissolved in distilled water. The experimental schedule is shown in Fig. 1B. The dose of SAK3 was determined from a previous report in which the administered dose rescued the AD pathology in NL-F mice.[11]

To evaluate the effects of acute administration, we performed behavioral tests 30 min after administration according to the concentration peak of SAK3 in the brain, and tests were conducted at intervals considering the effects of drug accumulation.[11] In the chronic administration, mice were orally administered a vehicle or SAK3 once a day for two weeks. After two weeks of chronic administration, the animals were subjected to a series of behavioral tests. They were further subjected to the tests again after two weeks of withdrawal in the case that the chronic SAK3 administration affects their behavioral profiles.

2.3. Behavioral tasks

2.3.1. Novel object recognition tasks

The novel object recognition task was performed as described previously to evaluate object recognition memory.[11] We used

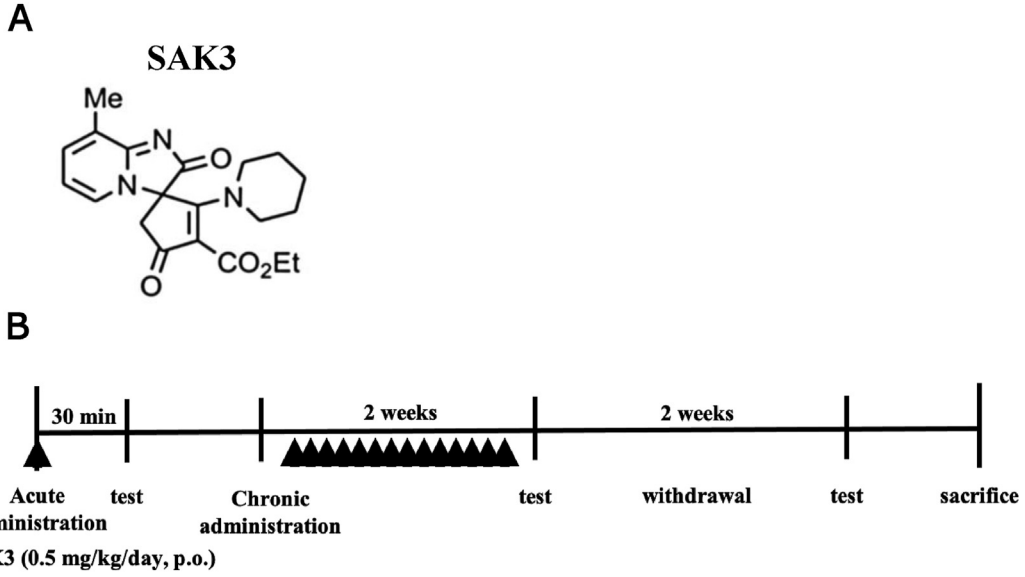

Fig. 1. Chemical structure of SAK3, and experimental schedules in the present study (A) SAK3 (ethyl-8′-methyl-2′,4-dioxo-2-(piperidin-1-yl)-2′H-spiro [cyclopentane-1,3′-imidazo [1,2-a]pyridin]-2-ene-3-carboxylate). (B) Animals were subjected to a series of behavioral tests 30 min after vehicle (distilled water) or SAK3 (0.5 mg/kg/day, p.o.) administration. After that, animals were administered with vehicle or SAK3 every day for two weeks. After two weeks of chronic administration, animals were subjected to a series of behavioral tests and they were subjected tests again after two weeks of withdrawal.

five–ten male mice for each group. In the training session, two objects consisting of a wooden block of the same shape were placed in the test box (21 × 32 × 15 cm), and the mice were allowed to explore for 10 min. Twenty-four hours later, one object was replaced with a novel object of a different shape than the previous one, and exploratory behavior was analyzed again for 5 min. After each session, the objects were thoroughly cleaned with 70% ethanol to prevent odor recognition. Exploration of an object was defined as rearing on, touching, and sniffing. A discrimination index was calculated as the ratio of exploratory contacts to familiar and novel objects accordingly.

2.3.2. Y-maze task

Short-term spatial reference memory was assessed using the Y-maze task.[11] We used five–ten male mice for each group. The apparatus consisted of three identical black Plexiglas arms (50 × 16 × 32 cm). Mice were placed at the end of one arm and allowed to move freely through the maze during a 7-min session. Three consecutive choices of arms were defined as one successful alternation. The maximum number of alternations was defined as the total number of arms entered minus two, and the percentage of alternations was calculated as actual alternations/maximum alternations × 100.

2.3.3. Elevated plus maze task

This test is a method of evaluating anxiety by placing a mouse at a high place. We used five–ten male mice for each group. The maze was approximately 70 cm above the floor and consisted of a central 6 × 6 cm platform and four arms (30 cm long, 6 cm wide) radiating from it in the shape of a cross. There were no walls for the two diagonal arms (open arms); only the other two arms had walls with a height of 15 cm (closed arms). Mice were placed in the center and then tracked using a tracking device and smart video tracking software (Panlab, Cornellà, Barcelona, Spain) for 10 min to measure the time spent on the open and closed arms and the central platform. As reported, more anxious mice stayed longer in the closed arms.[17]

2.3.4. Marble burying task

This task is based on the tendency of mice to exhibit increased digging behavior in high anxiety.[17] We used five–ten male mice for each group. The clear box (30 × 30 × 30 cm) was filled approximately 10 cm deep with wood chips lightly tamped down to make a flat surface. Twenty-five marbles were placed at equal intervals. After placing the mouse for 30 min, the number of marbles hidden in the bedding was counted and the anxiety state was accordingly evaluated.

2.3.5. Tail suspension task

This task is a method for evaluating depression based on behavioral decline observed when mice are hung in the air.[17] We used five–ten male mice for each group. A piece of vinyl tape was placed 1 cm from the tip of the mouse's tail, sandwiched with clips, and hung on a hook about 50 cm above the floor. The observation was carried out for 8 min, and the immobility time observed in 5 min, excluding the first minute and the last 2 min, was measured. After the experiment, the clip and vinyl tape were quickly removed to relieve the pain in the mice.

2.3.6. Forced swim task

This task is a method for evaluating depression based on behavioral decline observed when mice were placed in deep water.[17] We used five–ten male mice for each group. A beaker with a diameter of 15 cm was filled with water at 25 ± 1 °C to a depth of 20 cm. After placing the mouse in this equipment, an observation was carried out for 8 min, and the immobility time observed in 5 min, excluding the first minute and the last 2 min, was measured. After the experiment, the mice were gently wiped with a towel to relieve pain.

2.3.7. Resident-intruder paradigm

This test is a method for evaluating aggression based on the habit that male rodents establish territory when given ample living space.[18] In this test, we tested six month-old, four to six male mice. We placed one male mouse and one four-month-old female mouse in each cage (21 × 32 × 15 cm) to form a territory and kept them for a week without changing the bedding. On the test day, female mice were removed 1 h before the start of the experiment. Simultaneously, at the start of measurement, a two-month-old male WT mouse was put in as an intruder, and the time until the territory owner made an attack action and the total number of attacks was measured for 10 min. The actions considered aggressive behavior were as follows: scratching, biting, leaning, and relentless chasing.

2.4. Data analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significance was tested by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Pairs of means were compared using Student's *t*-test. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, CA, USA). Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Oral administration of SAK3 sustainably improved the cognitive impairment observed in NL-G-F mice

To examine the improvement effect of SAK3 (0.5 mg/kg) on the core symptoms of AD, we first evaluated the cognitive function of twelve-month-old WT mice and NL-G-F mice using the novel object recognition and Y-maze tests. In the novel object recognition task, the discriminative ability of NL-G-F mice was significantly reduced compared to WT mice (WT + veh: 60.1 ± 3.04%, $p < 0.01$ vs. familiar, NL-G-F + veh: 46.6 ± 2.58%, $p > 0.05$ vs. familiar). As a result of the oral administration of SAK3 in these mice, the discrimination ability improved to the same level as the WT regardless of acute or chronic administration (NL-G-F + SAK3 acute: 61.3 ± 3.22%, $p < 0.001$ vs. familiar, NL-G-F + SAK3 chronic: 57.2 ± 3.17%, $p < 0.01$ vs. familiar). Interestingly, this improvement effect continued even after two weeks of withdrawal (WDL) after chronic administration (NL-G-F + SAK3 withdrawal: 57.2 ± 2.18%, $p < 0.001$ vs. familiar) (Fig. 2A and B). In the Y-maze task, spatial working memory was significantly reduced in NL-G-F mice compared to WT mice (WT + veh: 71.5 ± 1.74%, NL-G-F + veh: 51.5 ± 4.34%, $p < 0.024$). As a result of oral administration of SAK3 to these mice, an improvement effect was observed both acutely and chronically (NL-G-F + SAK3 acute: 78.9 ± 3.85%, $p < 0.0001$ vs. NL-G-F + veh, NL-G-F + SAK3 chronic: 81.7 ± 3.43%, $p < 0.0001$ vs. NL-G-F + veh). Although no significant improvement was observed, this improvement effect tended to continue even after two weeks of withdrawal after chronic administration (NL-G-F + SAK3 withdrawal: 66.1 ± 3.36%, $p = 0.0933$ vs. NL-G-F + veh). In this condition, the total number of arm invasions was not significantly different in all groups (WT + veh: 18.8 ± 1.91 times, WT + SAK3 acute: 13.9 ± 0.97 times, WT + SAK3 chronic: 17.4 ± 1.29 times, NL-G-F + veh: 13.9 ± 1.32 times, NL-G-F + SAK3 acute: 14.2 ± 0.85 times, NL-G-F + SAK3 chronic: 15.4 ± 1.39 times, NL-G-F + SAK3 withdrawal: 15.6 ± 1.47 times, $p > 0.0332$ vs. each group) (Fig. 3A and B). From the above results, it was observed that oral

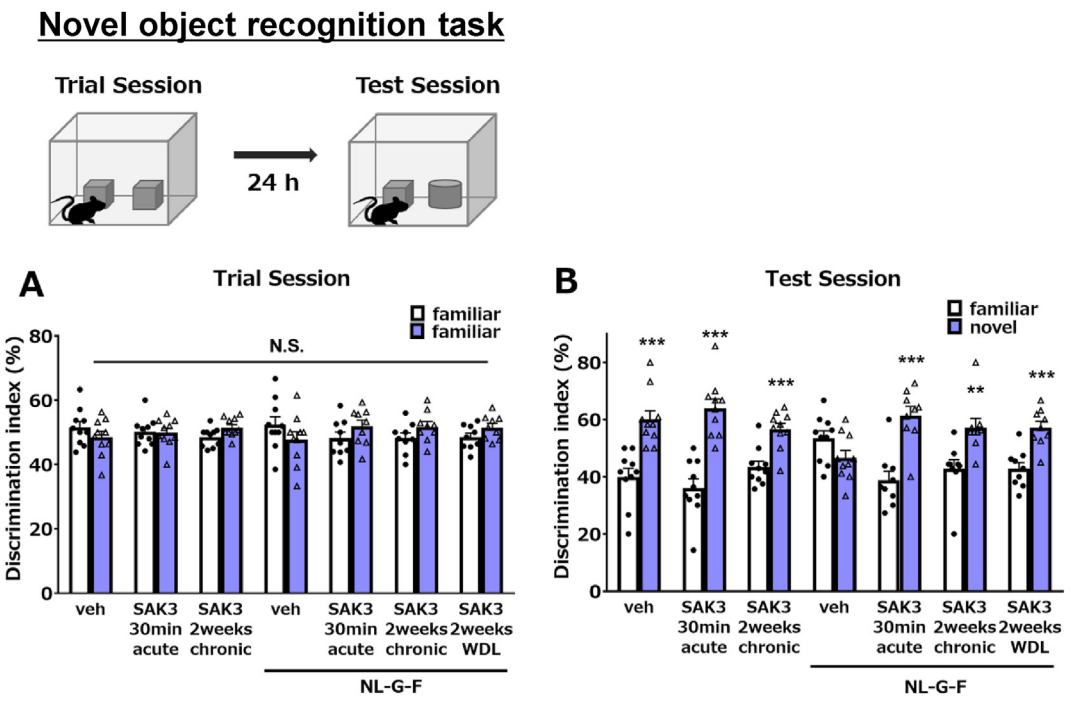

Fig. 2. SAK3 administration rescues impaired memory-related behaviors in NL-G-F mice via T-VGCC activation in novel object recognition task. (A) No differences were observed in the trial session between groups. (B) The impaired discrimination index in NL-G-F mice was improved by acute or chronic oral administration of SAK3 (0.5 mg/kg, p.o.). After two weeks of chronic administration, the improvement lasted for two weeks (n = 9–10 per group). Error bars represent SEM. **p < 0.01; ***p < 0.001 vs. familiar group.

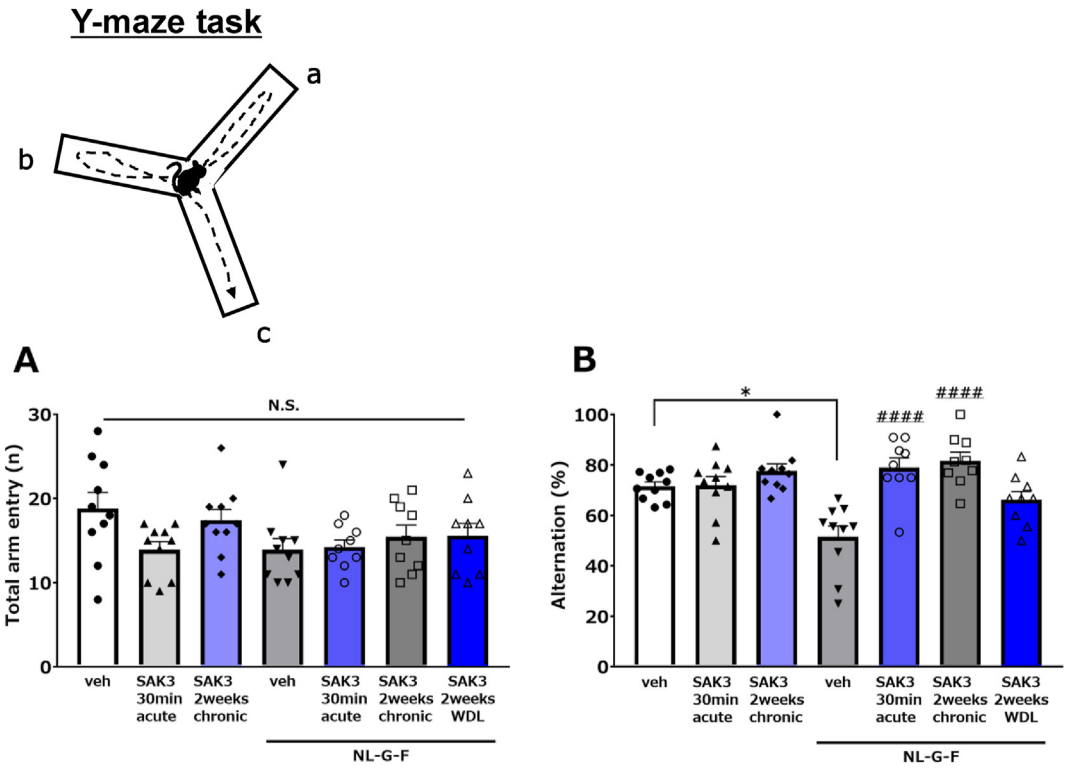

Fig. 3. SAK3 administration rescues impaired memory-related behaviors in NL-G-F mice via T-VGCC activation in Y-maze task. (A) No differences were observed in terms of the number of total arm entries between groups. (B) Decreased alternation behaviors in NL-G-F mice were rescued by acute or chronic SAK3 (0.5 mg/kg, p.o.) administration, and the SAK3 effect tended to last for two weeks after chronic administration (n = 9–10 per group). Error bars represent SEM. *p < 0.0332 vs. vehicle treated WT mice. ####p < 0.0001 vs. vehicle treated NL-G-F mice.

administration of SAK3 improved the cognitive function of NL-G-F mice, and in the novel object recognition task, its effect persisted two weeks after chronic administration. SAK3 did not affect cognitive function in WT mice (Novel object recognition task; WT + SAK3 acute: 64.0 ± 3.21%, $p > 0.05$ vs. WT + veh, WT + SAK3 chronic: 56.6 ± 2.07%, $p > 0.05$ vs. WT + veh, Y-maze task; WT + SAK3 acute: 71.9 ± 3.49%, $p > 0.0332$ vs. WT + veh, WT + SAK3 chronic: 77.6 ± 2.86%, $p > 0.0332$ vs. WT + veh).

3.2. Oral administration of SAK3 continuously improved anxiolytic-like behavior in NL-G-F mice

Subsequently, in order to examine the effect of SAK3 on the BPSD of AD, the anxiety state of twelve-month-old WT mice and NL-G-F mice was evaluated by the elevated plus maze task and the marble burying task. In the elevated plus maze task, NL-G-F mice spent a significantly longer time in the open arms as compared to WT mice (WT + veh: 48.1 ± 11.9 s, NL-G-F + veh: 164.91 ± 21.8 s, $p < 0.0332$) (Fig. 4A). In the marble burying task, the number of marbles covered by NL-G-F mice was significantly smaller than that of WT mice (WT: 20.1 ± 1.35 marbles, NL-G-F: 8.40 ± 1.21 marbles, $p < 0.0001$) (Fig. 4B). As described above, unusual anxiolytic-like behavior was observed in NL-G-F mice. Oral administration of SAK3 to these mice resulted in improvement in both acute and chronic conditions, and this effect persisted even after two weeks of withdrawal after chronic administration (Elevated plus maze task; NL-G-F + SAK3 acute: 57.3 ± 8.49 s, $p < 0.0001$ vs. NL-G-F + veh, NL-G-F + SAK3 chronic: 48.6 ± 4.83 s, $p < 0.0001$ vs. NL-G-F + veh, NL-G-F + SAK3 withdrawal: 91.3 ± 21.0 s, $p < 0.0332$ vs. NL-G-F + veh, Marble burying task; NL-G-F + SAK3 acute: 21.1 ± 0.77 marbles, $p < 0.0001$ vs. NL-G-F + veh, NL-G-F + SAK3 chronic: 16.0 ± 0.76 marbles, $p < 0.0001$ vs. NL-G-F + veh, NL-G-F + SAK3 withdrawal: 16.1 ± 1.03 marbles, $p < 0.0001$ vs. NL-G-F + veh). SAK3 did not affect WT anxiety-related behaviors (Elevated plus maze task; WT + SAK3 acute: 34.3 ± 9.24 s, $p > 0.0332$ vs WT + veh, WT + SAK3 chronic: 39.0 ± 8.39 s, $p > 0.0332$ vs. WT + veh, Marble burying task; WT + SAK3 acute: 23.4 ± 0.50 marbles, $p > 0.0332$ vs. WT + veh, WT + SAK3 chronic: 22.4 ± 0.52 marbles, $p > 0.0332$ vs. WT + veh) (Fig. 4A and B).

3.3. Oral administration of SAK3 improved depression-like behavior in NL-G-F mice

To examine the further improvement effect of SAK3 on BPSD, we evaluated the depression status of twelve-month-old WT and NL-G-F mice by the tail suspension and the forced swimming tasks. In both tests, the immobility time significantly increased in NL-G-F mice as compared to WT mice (Tail suspension task; WT + veh: 167 ± 7.95 s, NL-G-F + veh: 235 ± 5.62 s, $p < 0.0002$, Forced swim task; WT + veh: 144 ± 5.16 s, NL-G-F + veh: 231 ± 21.9 s, $p < 0.0001$). Oral administration of SAK3 to these mice showed improvement in both acute and chronic conditions (Tail suspension task; NL-G-F + SAK3 acute: 192 ± 9.17 s, $p < 0.0332$ vs. NL-G-F + veh, NL-G-F + SAK3 chronic: 175 ± 10.6 s, $p < 0.002$ vs. NL-G-F + veh, Forced swim task; NL-G-F + SAK3 acute: 176 ± 30.2 s, $p < 0.0021$ vs. NL-G-F + veh, NL-G-F + SAK3 chronic: 164 ± 26.8 s, $p < 0.0001$ vs. NL-G-F + veh). However, there was no significant improvement after two weeks withdrawal of chronic administration (Tail suspension task; NL-G-F + SAK3 withdrawal: 229 ± 13.7 s, $p > 0.0332$ vs. NL-G-F + veh, Forced swim task; NL-G-F + SAK3

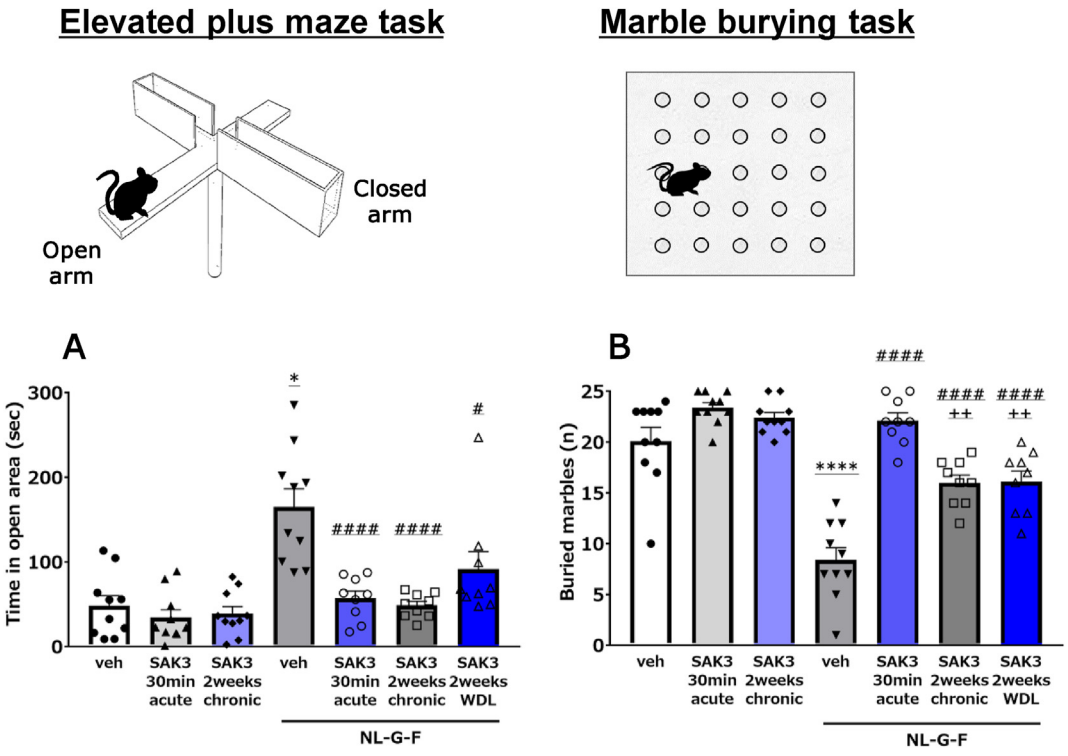

Fig. 4. SAK3 administration returned anxiolytic behaviors to the normal state in NL-G-F mice. (A) NL-G-F mice spent significantly more time exploring the open arms of the elevated plus maze, suggesting an anti-anxiety profile. It was improved by acute or chronic oral administration of SAK3 (0.5 mg/kg, p.o.). The improvement after two weeks of chronic administration lasted for two weeks (n = 9–10 per group). Error bars represent SEM. *$p < 0.0332$ vs. vehicle-treated WT mice. $^{\#}p < 0.0332$; $^{\#\#\#\#}p < 0.0001$ vs. vehicle-treated NL-G-F mice. (B) NL-G-F mice buried significantly fewer marbles in the marble-burying task, suggesting an anti-anxiety profile. It was improved by acute or chronic administration of SAK3 (0.5 mg/kg, p.o.), and this effect lasted for two weeks after chronic administration (n = 9–10 per group). Error bars represent SEM. ****$p < 0.0001$ vs. vehicle-treated WT mice. $^{\#\#\#\#}p < 0.0001$ vs. vehicle-treated NL-G-F mice. $^{++}p < 0.0021$ vs. SAK3 acutely treated NL-G-F mice.

withdrawal: 243 ± 11.2 s, $p > 0.0332$ vs. NL-G-F + veh). SAK3 did not affect WT depression-like behavior (Tail suspension task; WT + SAK3 acute: 153 ± 13.8 s, $p > 0.0332$ vs. WT + veh, WT + SAK3 chronic: 181 ± 16.7 s, $p > 0.0332$ vs. WT + veh, Forced swim task; WT + SAK3 acute: 142 ± 12.9 s, $p > 0.0332$ vs. WT + veh, WT + SAK3 chronic: 150 ± 9.37 s, $p > 0.0332$ vs. WT + veh) (Fig. 5).

3.4. Oral administration of SAK3 showed a tendency to improve aggressive behavior observed in NL-G-F mice

Furthermore, the aggression of six-month-old WT mice and NL-G-F mice was evaluated using the resident-intruder paradigm. Compared to WT mice, NL-G-F mice showed a decrease in the time to the first attack and an increase in the total number of attacks, indicating that the aggression significantly increased (first attacking time; WT + veh: 600 ± 0 s, NL-G-F + veh: 301 ± 26.7 s, $p < 0.0332$, the total number of attacks; WT + veh: 0 time, NL-G-F + veh: 6.33 ± 1.93 times, $p < 0.0332$). Although no significant improvement was observed, oral administration of SAK3 tended to decrease the aggressiveness of NL-G-F mice (first attacking time; NL-G-F + SAK3 acute: 473 ± 52.5 s, $p = 0.693$ vs. NL-G-F + veh, NL-G-F + SAK3 chronic: 426 ± 64.3 s, $p = 0.332$ vs. NL-G-F + veh, total number of attacks; NL-G-F + SAK3 acute: 3.2 ± 1.16 times, $p = 0.648$ vs. NL-G-F + veh, NL-G-F + SAK3 chronic: 1.67 ± 0.42 times, $p = 0.959$ vs. NL-G-F + veh) (Fig. 6).

4. Discussion

From the results of these behavioral tests, the AD model NL-G-F mice showed impaired memory and cognitive functions as core symptoms. They also showed anxiolytic-like and depression-like behaviors as BPSD, as previously reported,[15,19,20] and also showed aggressive behaviors. In this study, we discovered that these symptoms, except aggressiveness, were ameliorated by both acute and chronic administration of SAK3.

Regarding cognitive impairment, which is an AD core symptom, oral administration of SAK3 showed a marked improvement in both acute and chronic administration, and the effect was persistent in the novel object recognition task. Loss of ACh neurons is one of the pathological features of AD. A decrease in ChAT-positive neurons in the septal region of NL-G-F mice as used in the current study, has been already reported.[13] The ACh nerves in the septal area project to the hippocampus and are known to play an important role in cognitive memory learning.[13] This suggests that the cognitive impairment observed in NL-G-F mice is due to a decrease in ACh release in the hippocampus caused by impairment of projection of ACh nerves from the septal area to the hippocampus. SAK3 has been shown to enhance ACh release by activating T-VGCCs in the hippocampal CA1 region,[14] which suggests that SAK3 restored the cognitive impairment by enhancing ACh release in the hippocampus of NL-G-F mice. The effect of SAK3 on cognitive function was sustained in the novel object recognition task, while it was not sustained in the Y-maze task. The novel object recognition task and Y-maze task reflect the level of recognition memory and spatial working memory, respectively. The regions accounting for the memories are different. For example, the object recognition memory depends on the perirhinal cortex, while the spatial memory depends on the hippocampus.[21–23] However, the functions and relationships between brain regions and the memory formation process are still controversial. Therefore, we can only say that the pharmacological action for SAK3 is relatively higher in the perirhinal cortex than the hippocampus.

With regard to BPSD, anxiolytic-like behavior and cognitive function were improved by both acute and chronic oral administration of SAK3, and the effect persisted even after chronic administration. On the other hand, administration of SAK3 to WT mice did not significantly alter anxiety-related behavior. Therefore, the decrease in anxiolytic-like behavior in NL-G-F mice induced by SAK3 treatment suggests that the treatment improves the impulsivity rather than simply causing anxiety. In addition, the depression-like behavior was significantly improved by acute and chronic oral administration of SAK3. It is considered that the improvement of these symptoms is mainly due to the promotion of the release of serotonin (5-HT) and dopamine (DA) by SAK3. Monoamines, including 5-HT and DA, mediate various central nervous system functions such as emotion, motivation, motor function, and cognition.[24,25]

For many years, the relationship between serotonin and depression in particular, had been the focus of attention for research. The administration of selective serotonin reuptake

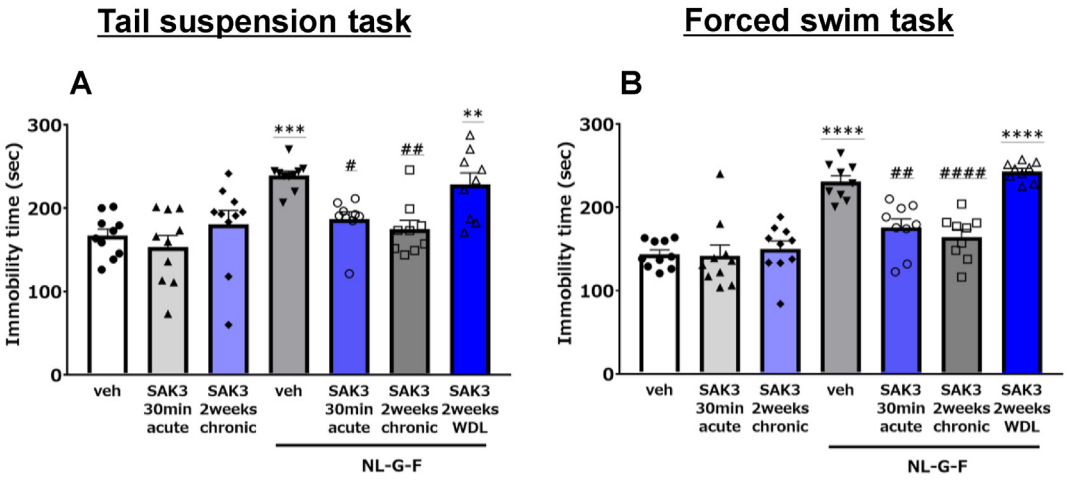

Fig. 5. SAK3 administration improved depressive-like behaviors in NL-G-F mice. (A) NL-G-F mice showed significantly long immobility time in the tail suspension test, suggesting depressive disorder. It was improved by acute or chronic oral administration of SAK3 (0.5 mg/kg, p.o.), but did not last for two weeks after chronic administration (n = 9–10 per group). Error bars represent SEM. $^{**}p < 0.0021$; $^{***}p < 0.0002$ vs. vehicle-treated WT mice. $^{\#}p < 0.0332$; $^{\#\#}p < 0.0021$ vs. SAK3 acutely treated NL-G-F mice. (B) NL-G-F mice showed significantly long immobility time in the forced swim test. It was improved by acute or chronic oral administration of SAK3 (0.5 mg/kg, p.o.), but did not last for two weeks after chronic administration (n = 9–10 per group). Error bars represent SEM. $^{****}p < 0.0001$ vs. vehicle-treated WT mice. $^{\#\#}p < 0.0021$; $^{\#\#\#\#}p < 0.0001$ vs. vehicle-treated NL-G-F mice.

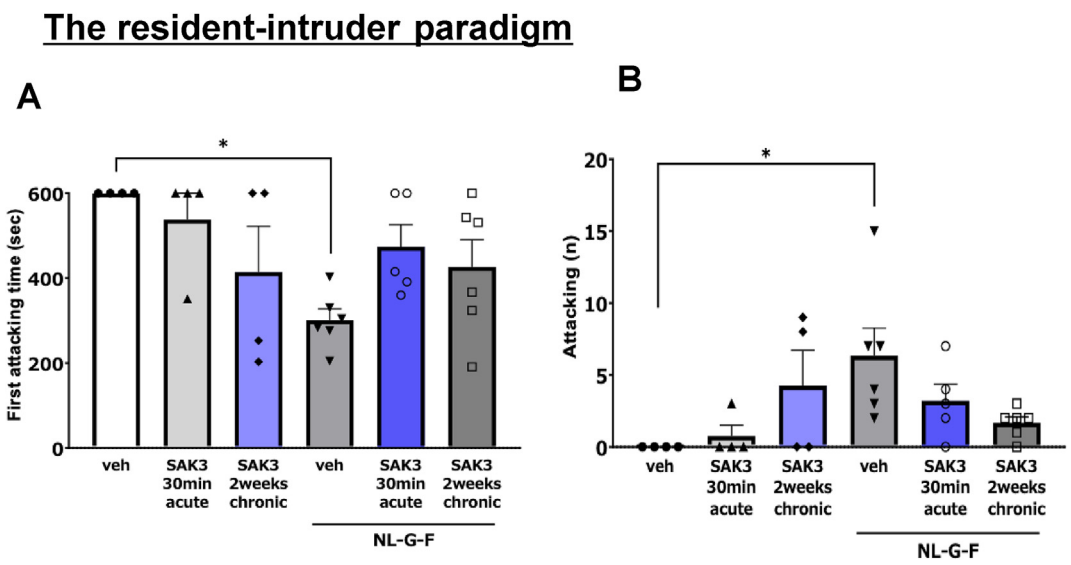

Fig. 6. SAK3 administration tended to improve aggressive behaviors in NL-G-F mice. (A) NL-G-F mice showed a significant decrease in the time to the first attack on the intruder mice. Acute or chronic oral administration of SAK3 (0.5 mg/kg, p.o.) tended to improve them, but it was not significant (n = 4–6 per group). Error bars represent SEM. *p < 0.0332 vs. vehicle-treated WT mice. (B) NL-G-F mice showed a significant increase in the total number of attacks to the intruder mice. Acute or chronic oral administration of SAK3 (0.5 mg/kg, p.o.) tended to improve them, but it was not significant (n = 4–6 per group). Error bars represent SEM. *p < 0.0332 vs. vehicle-treated WT mice.

inhibitors (SSRIs) improved depressive symptoms, and serotonin 1A receptor binding was significantly reduced in patients with depression.[26,27] These reports suggest that serotonin may play an important role in the development of depression and its treatment. In this study, the depression state of AD as BPSD was evaluated, but it was reported in other AD model mice that the relationship between depression-like behavior and serotonin levels. For example, in APP/PS1 mice that produce excess amyloid-β due to abnormal processing of APP, decreased serotonin levels in the brain and depression-like behavior was observed,[28] and intraperitoneal administration of citalopram, which is one of the SSRIs, ameliorated these accordingly.[29] In addition, in R406W Tg mice with mutations in the R406W human tau protein, a tendency toward a decrease in the amount of serotonin in the brain and depression-like behavior was also observed. Again, this improved with oral administration of fluvoxamine, which is one of the SSRIs.[30]

We previously reported that SAK3 improved the depression-like behavior observed in OBX mice and restored 5-HT and DA release in the hippocampal CA-1 region in NL-G-F mice.[31,32] In this experiment, depression-like behavior was indeed improved in NL-G-F mice. OBX mouse is a model of dementia that induces retrograde neuronal cell death by olfactory bulb removal. In contrast, NL-G-F mouse is a model closer to the actual pathological condition, in which time-dependent accumulation of amyloid causative protein causes various symptoms.[19] The improvement effects observed in this model further demonstrate the potent efficacy of SAK3 against BPSD in AD. Moreover, we have demonstrated that SAK3 upregulates proteasome activity.[33] Further, SAK3 is therapeutically effective not only for BPSD of AD but also for DLB and PD because clearance of accumulated causative proteins such as α-synuclein is vital in dementia with Lewy bodies (DLB) and Parkinson's disease (PD),[34–36] and down-regulation of proteasomal activity has been reported in Parkinson's disease.[37,38]

Unfortunately, the detailed pathogenic mechanism of mental disorders as BPSD of AD has not been completely clarified yet. It is complicated to interpret anxiety-related behaviors because AD model mice are reported to have inconsistent behavioral results. Another behavioral test method for assessing anxiety is the open field test. This test is a method to evaluate anxiety state in which as the anxiety of the mouse increases, it gets into the habit of staying closer to the wall of the field.[39] Latif-Hernandez et al. performed the same test on 6-month-old NL-G-F mice and discovered that they had an increased time to stay in the center of the field, that is, anxiolytic-like behavior.[15] On the other hand, Eleftheria et al. reported that 8-month-old NL-G-F mice showed an increase in search time near the wall, that is, anxiety-like behavior.[16] As described above, inconsistent results have been reported depending on the age of mice used in the same test method. This age-related behavioral change may be associated with lesion progression. However, this hypothesis does not always hold true. In the elevated plus maze test, we found that 12-month-old NL-G-F mice exhibited anxiolytic-like behavior. Despite the different ages, Latif-Hernandez et al. and Eleftheria et al. reported that 6-month-old and 8-month-old mice exhibited anxiolytic-like behavior similar to the results of this experiment.[15,16] Interestingly, although Eleftheria et al. evaluated the anxiety state in the same mice in the open field and the elevated plus maze test, they observed anxiety-like behaviors in the former and anxiolytic-like behaviors in the latter. Such contradictions were also observed in other mouse models. Tg2576 mice overexpressing APP695 with Swedish KM670/671NL mutation also showed anxiety-like behavior in the open field test and anxiolytic-like behavior in the elevated plus-maze test.[40] Further research is required to clarify the mechanism of anxiety-related behaviors as BPSD and a more detailed mechanism of the improvement effect of SAK3.

In this study, SAK3 showed improvement in cognitive function and anxiety-related behaviors after two weeks of withdrawal, but not in depression-like behaviors. This is possibly due to the fact that the acute drug effect, such as simply accelerating the release of monoamine, was not maintained long enough to prevent depression. The tail suspension task and forced swimming task to evaluate depression are commonly used methods, but the physical and mental burdens on mice are very high. In fact, it has been reported that repeating these processes increases the immobility time, which is an index of depression.[41] In the current study, the same mice were used to compare the improvement effects of SAK3 after acute administration, chronic administration, and drug withdrawal, thus proposing the possibility of increased loads. In addition, in

these tasks, a drug that clinically shows improvement only by chronic administration may have an acute effect.[42] Furthermore, SAK3 has anti-depressive action through the enhancement of neurogenesis in olfactory bulbectomized mice.[31] Unexpectedly, the effect was not prolonged in NL-G-F mice. We will further evaluate neurogenesis after two weeks of withdrawal in a future study. Depression is also associated with oxidative stress and brain inflammation in NL-G-F mice.[20] We should examine whether oxidative stress and brain inflammation in NL-G-F mice have recovered during the two weeks of withdrawal in the subsequent experiments.

Finally, with respect to the aggressiveness of NL-G-F mice, oral administration of SAK3 showed some improvement, but this was not significant. The improvement trend observed this time is considered to be due to the promotion of 5-HT release by SAK3. Although the detailed mechanism is still unknown, there have been many reports on the relationship between 5-HT and aggression; for example, reduction of 5-HT activity in the brain causes aggression[33,43] and 5-HT agonists have anti-aggressive effects.[44] In addition, although not significant, SAK3 administration to WT mice seemed to increase aggression. Administration to NL-G-F mice did not show a tendency to increase aggression; therefore, it is unlikely that SAK3 itself causes an increase in aggression. This issue observed in WT mice may be solved by increasing the number of cases to be measured.

In conclusion, this study showed the improvement effect of SAK3 on the core symptoms of AD and anxiolytic-like/depressive-like behavior of BPSD. In particular, for cognitive dysfunction and anxiolytic behavior, the effects were persistent even after chronic administration. These results indicate not only the potential of SAK3 as a lead compound for novel AD therapeutic agents, but also the efficacy of drug discovery targeting T-VGCCs.

Author contribution

T.D. and H.I. performed the experiments. T.D., I.K., Y.S., and K.F. designed the study. T.D., I.K., and K.F. wrote the manuscript. K.F. supervised the study.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgements

This research was partially supported by the Project of Translational and Clinical Research Core Center (B11) from AMED (to K.F.), Kakenhi (19H03406) and the Smoking Research Foundation (to K.F.).

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