Inhibition of CASK Expression by Virus-mediated RNA Interference in Medial Prefrontal Cortex Affects Social Behavior in the Adult Mouse

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Background : Calcium/calmodulin-dependent serine protein kinase (CASK) is a synaptic scaffolding protein and mutations in the *CASK* gene have been identified in various types of neurodevelopmental disorders. Deficit in social behavior is a common symptom of many neurodevelopmental disorders and also accompanies CASK related disorders. The deep layer of the medial prefrontal cortex (mPFC) has been suggested to be responsible for social behavior.

Methods : To study the effect of CASK deficiency on social behavior, we generated mPFC specific CASK knockdown mice by introducing CASK short-hairpin RNA using adeno-associated viral (AAV) injection. We studied the behaviors of CASK knockdown and enhanced green fluorescent protein (EGFP) expressing AAV injected control mice using open field and three-chamber apparatus.

Results : CASK knockdown mice showed normal locomotor activity, anxiety level, and repetitive behavior in the observation in the open field arena, but they showed deficit in reciprocal social interaction with a juvenile mouse. In the three-chamber test, CASK knockdown mice showed more interaction with a novel juvenile mouse than an empty cage at a similar level to control mice, suggesting that social recognition is intact. On the other hand, no significant difference was observed in the interaction between a novel target mouse and a pre-exposed mouse, suggesting that social memory ability was impaired in the CASK knockdown mice.

Conclusion : By using mPFC specific CASK knockdown mice, we found that CASK deficiency in this area affects the social memory ability. Our results provide insights not only into CASK related disorders, but also a wide range of neurodevelopmental disorders associated with social deficits. *Shinshu Med J 69 : 45—52, 2021*

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Key words : CASK, neurodevelopmental disorder, medial prefrontal cortex, short-hairpin RNA, adeno-associated virus

I Introduction

Calcium/calmodulin-dependent serine protein kinase (CASK) is a family of membrane associated guanylate kinase protein (MAGUK) fundamentally localized at the pre- and postsynaptic terminals. CASK protein consists of the CaMKII like domain, L27 domain, PDZ domain, SH3 domain, and guanylate

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kinase domain, each of which binds intracellular or transmembrane proteins that mediate signal transductions required for the development and maintenance of synapse[1]. Mutations, deletions, and copy number variations of the *CASK* gene have been identified in the patients with neurodevelopmental disorders[2]. The best known CASK-related disorder is microcephaly with cerebellar and pontine hypoplasia (MICPCH) syndrome[3]. MICPCH syndrome is an X-linked neurodevelopmental disorder characterized by a smaller brain, especially in the cerebellum, and facial malformation[4]. Social deficits, intellectual disability, and infantile epilepsy accompany many cases of the syndrome. Most of the patients with MICPCH syndrome are females, probably due to prenatal lethality in males. CASK knockout (KO) mice have been generated and studied. The male CASK KO and homozygote female mice die shortly after birth due to multiple organ failure[5]. We previously studied heterozygote CASK female KO mice as models of MICPCH syndrome. We identified that the X chromosome inactivation is involved in the pathophysiology of MICPCH syndrome and the balance between excitatory and inhibitory synapses projecting to the CASK-deficient neurons was disrupted in the model mice[6].

One major question for genetic neuropsychiatric disorder is the relevance between genes and the cause of behavioral symptoms. Social deficits are the principal behavioral phenotype in most of the neurodevelopmental disorders. Protocols for evaluating social behavior in mouse models have been well established. Open field and three-chamber tests are commonly used for the purpose, but the major limitation is that females cannot be used for those kinds of experiments. Pheromone exhaling from female mice affects the results of social behavioral studies. As mentioned, male CASK KO mice die shortly after birth. To study the social behavior in CASK-deficient mice, we established CASK knockdown male model mice by introducing short-hairpin RNA for CASK (shCASK) specifically in the medial prefrontal cortex (mPFC) where relevance to social behavior was suggested. We studied the general activities, social interaction, social recognition, and social learning and found that the CASK knockdown mice exhibited deficits in social interaction and social learning. Our results indicate that CASK deficiency in the mPFC is responsible for the social deficits observed in the patients with CASK mutation.

Ⅱ Methods

A Construction of adeno-associated viral (AAV) vectors

pAAV-H1-shCASK/U6-hSyn-EGFP-WPRE:

The shRNA target sequence for CASK, of which knockdown efficiency was verified previously[6][7], was 5'-ATCCATGAGCAGGGGCTGA-3'. shCASK oligos (KT14322 and KT14323) were annealed (95℃, 5min, then slowly cooled down to room temperature) and cloned into the XhoI/XbaI site of pAAV-H1/U6-hSyn-EGFP-WPRE.

KT14322: 5'-TCGACCCATCCATGAGCAGGGGCTGATTCAAGAGATCAGCCCCTGCTCATGGATTTTTTTTGGAAAT-3'

KT14323: 5'-CTAGATTTCCAAAAAAATCCATGAGCAGGGGCTGATCTCTTGAATCAGCCCCTGCTCATGGATGGG-3'

B AAV preparation

HEK293-AAV cells (10cm dish, 80 % confluent) were co-transfected with pAAV plasmid (5ug), pHelper plasmid (10ug) and pRC-DJ plasmid (5.5ug) using polyethyleneimine (PEI). 3 days after transfection, cells were harvested, disrupted by repeated freeze-and-thaw (Liq. nitrogen/37℃ water bath, 4 times), centrifuged and virus-containing supernatant was collected. The supernatant was treated with benzonase nuclease (0.125U/ul) at 37℃ for 30 min, then centrifuged and the supernatant was collected as the AAV solution. AAV solutions were titrated by quantitative PCR using primers KT18345 and KT99100 for EGFP.

KT18345: 5'-AAGGGCGAGGAGCTGTTCAC-3'

KT99100: 5'-TGCAGATGAACTTCAGGGTC-3'

C AAV injection

Wild-type C57B/6J males, deeply anesthetized with a ketamine and xylazine cocktail (100mg/kg B.W and 10mg/kg B.W., respectively, i.p.), were

injected with AAV into the mPFC at postnatal 19–or 20–days old on a stereotaxic device (Narshige). The coordination of viral injections was 1.75 mm (anterior from bregma), 0.3 mm (lateral from the midline) and 1.75 mm (depth from the brain surface). Approximately 0.5 μl of AAV was injected in the mPFC of each hemisphere of the mouse. The AAV–injected mice were examined in the following behavioral tests at the age of 2–4 months old. Correct injection was confirmed histologically by EGFP signals in the brain sections after the behavioral experiments.

D Behavioral tests

1 Open field test

Each mouse was placed in the center of the cylindrical open field apparatus (Φ 50 X 40 cm, OF–25M, Muromachi). Subject's behaviors in the apparatus were recorded from above the apparatus using a CCD camera (Canon, Japan). The video frame rate was 30 frames per second (fps). The test lasted 30 min. The travel distance, the time in the center, and the walking speed was analyzed with CompACT VAS ver. 3, a video tracking system (Muromachi, Japan). We manually measured the vertical activity (rearing and leaning), and grooming using the video.

2 Juvenile interaction tests

One day before the test, subject and stimulus mice were individually placed into a test arena (25×25×31 cm), and allowed to explore the arena for 10 min. Juvenile objects were 3–4 week old male wild–type C57BL/6J mice. On the test day, the test (AAV injected) mouse and a juvenile object mouse were placed together into the arena. Their behaviors were recorded for 10 min using a CCD camera. The time spent in social contact by the subjects were analyzed by a trained observer. Behaviors that were scored as social contact included the followings: sniffing, grooming, contact with nose, and following.

3 Sociability and social novelty preference tests

The apparatus was a rectangular, three–chambered box. The chamber was 20×40×25 cm and the dividing walls were made from clear Plexiglas, with small openings (5×3 cm) allowing access into each chamber. The mouse was habituated in the entire box for 10 min. The two side chambers contained an inverted empty small black wire cup. After the habituation, mice were placed back into the central chamber and the doors to the side chambers were closed. In the sociability test, an unfamiliar male mouse (stranger 1, S1), that had no prior contact with the subject mouse, was placed in one of the two cups, and then the doorways were unblocked. The location of S1 in the left or right side chambers was randomly alternated between trials. The test mouse's behaviors were recorded for 10 min using a CCD camera from the top. After the sociability test, the subject mouse was again confined in the central chamber. In the social novelty preference test, a second unfamiliar male mouse (stranger 2, S2) was enclosed in the cup, which was empty (E) in the sociability test, and the doorways were again unblocked. In both tests, the amount of time that the subject head was in each chamber was measured as "time in chamber" and within a 2–cm distance of the wire cup was measured as "contact time". The video was analyzed on idTracker and further analyzed on a custom program written in R.

E Statistical analysis

All values are represented by the average of independent experiments ± SEM. Statistical significance was determined by Student's t test. Statistical analysis was performed in R. Statistical significance ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) was indicated by asterisks.

Ⅲ Results

To investigate the effect of CASK deficiency in the social behavior, we generated CASK knockdown mice specifically in the mPFC by bilateral injection of AAV expressing shRNA targeting the *CASK* gene in adult mice **(Fig. 1)**. These mice were healthy without showing obvious physical abnormalities.

We first examined the general activities of these mice in the open field arena. CASK knockdown mice exhibited reduction of travel distance compared to the EGFP expressing AAV injected control mice, but other parameters, such as walking speed, time spent in the center, vertical activity, and grooming time

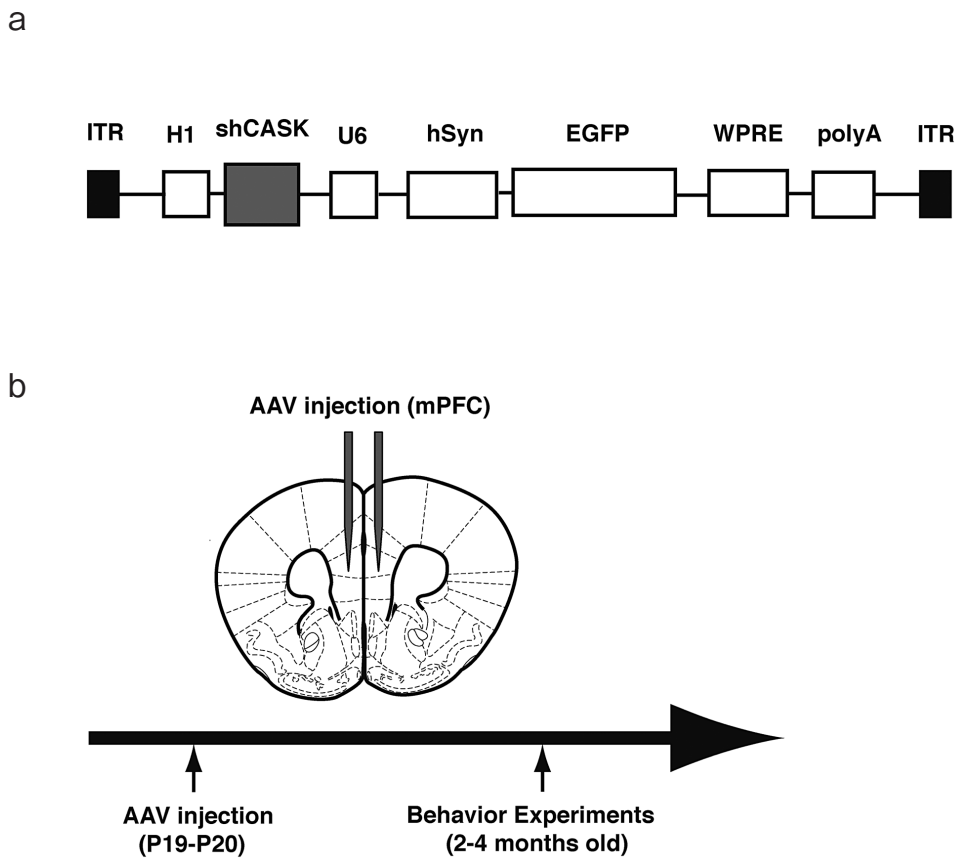

Fig. 1 CASK knockdown in the mPFC by AAV expressing shRNA
(a) shRNA targeting the *CASK* gene was introduced into the AAV (pAAV-H1-U6-hSyn-EGFP-WPRE) under control of the H1 promoter. (b) AAV was injected into mPFC of each hemisphere in postnatal 19- or 20-day old mice, and AAV-injected mice were subjected to behavioral tests at the age of 2-4 months old.

were unchanged **(Fig. 2)**, suggesting that the locomotor activity, anxiety, repetitive behavior were normal in CASK knockdown mice.

Next, we introduced juvenile target mice in the open field arena and examined the reciprocal social interaction during free moving conditions. Compared to the control mice, CASK knockdown mice showed less interaction with juvenile target mice **(Fig. 3)**, suggesting that the sociability was decreased in CASK knockdown mice.

To dissect the type of the social deficits, we employed three-chamber tests. In the first round test, we examined the interaction of test mice (control or CASK knockdown mice) with a novel juvenile mouse (S1) and empty cage (E1). Both control and CASK knockdown mice interacted more with a novel juvenile mouse (S1) than the empty cage at a similar ratio **(Fig. 4 a-c)**, suggesting that the CASK knockdown mice have the normal ability to recognize mice as a social target. In the second round experiment, another novel juvenile mouse was confined in the empty cage of the three-chamber (S2). In this condition, previous S1 became a pre-exposed "familiar" mouse. Control mice showed more interest in the novel S2 mouse than the pre-exposed S1 mouse and spent more time around S2 than S1. In contrast, CASK knockdown mice spent similar time between S1 and S2, indicating the memory of the pre-exposed S1 mouse was impaired **(Fig. 4 d-f)**.

Ⅳ Discussion

In the present study, we generated mPFC-specific CASK knockdown mice to study the effect of CASK deficiency on the social deficits observed in CASK-related neurodevelopmental disorders. We found that the reciprocal interaction with juvenile mice and

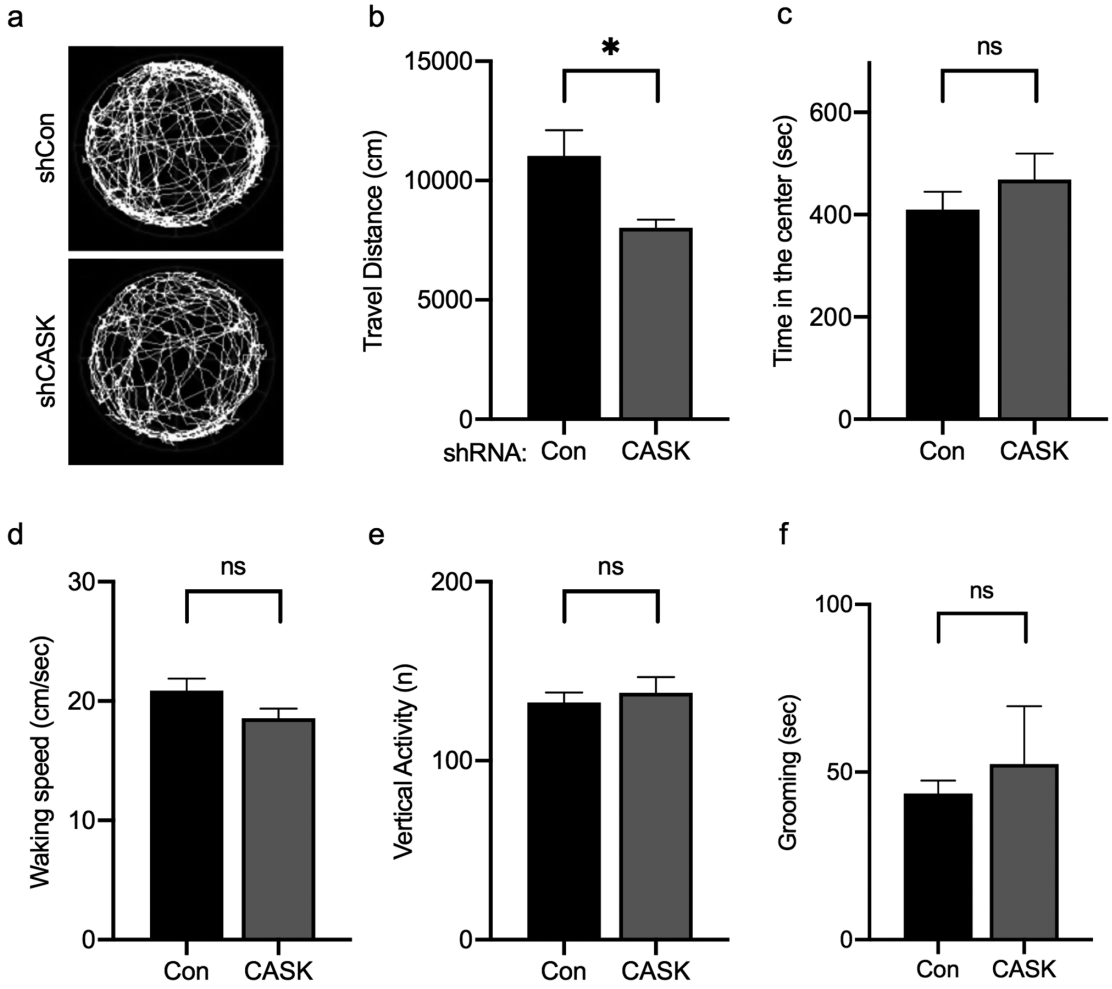

Fig. 2 Trajectories and graphs for open field tests

(a) Representative images for moving trajectories of control (shCon) and CASK knockdown (shCASK) mice. Summary graphs for the travel distance (b), time in the center (c), walking speed (d), vertical activity (e), and grooming time (f). $^{*}P<0.05$, ns : not significant. Control : 10 animals, CASK knockdown ; 9 animals.

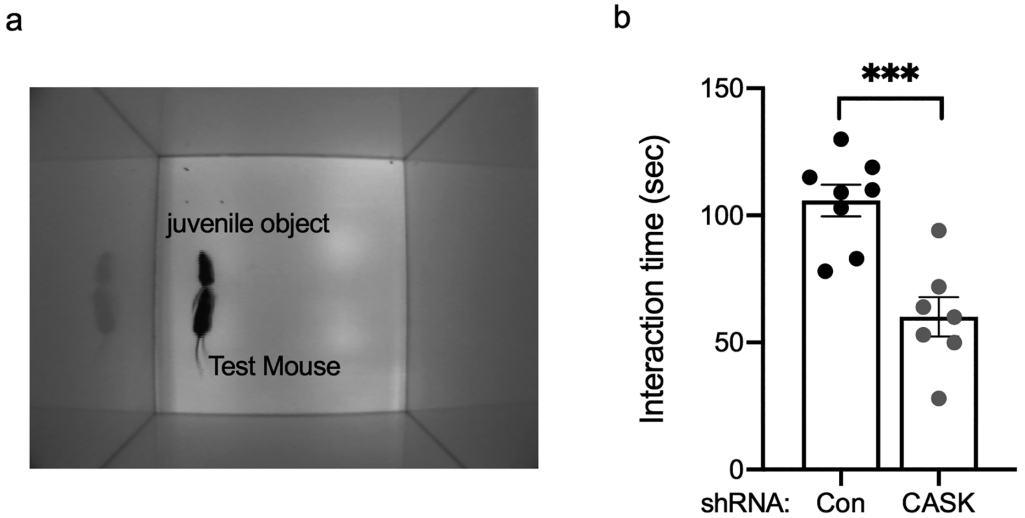

Fig. 3 Reciprocal interaction with juvenile mice

(a) Top view of the interaction with juvenile mice in the open field arena. (b) Summary graph for interaction time. $^{***}P<0.001$. Control : 8 animals, CASK knockdown : 7 animals.

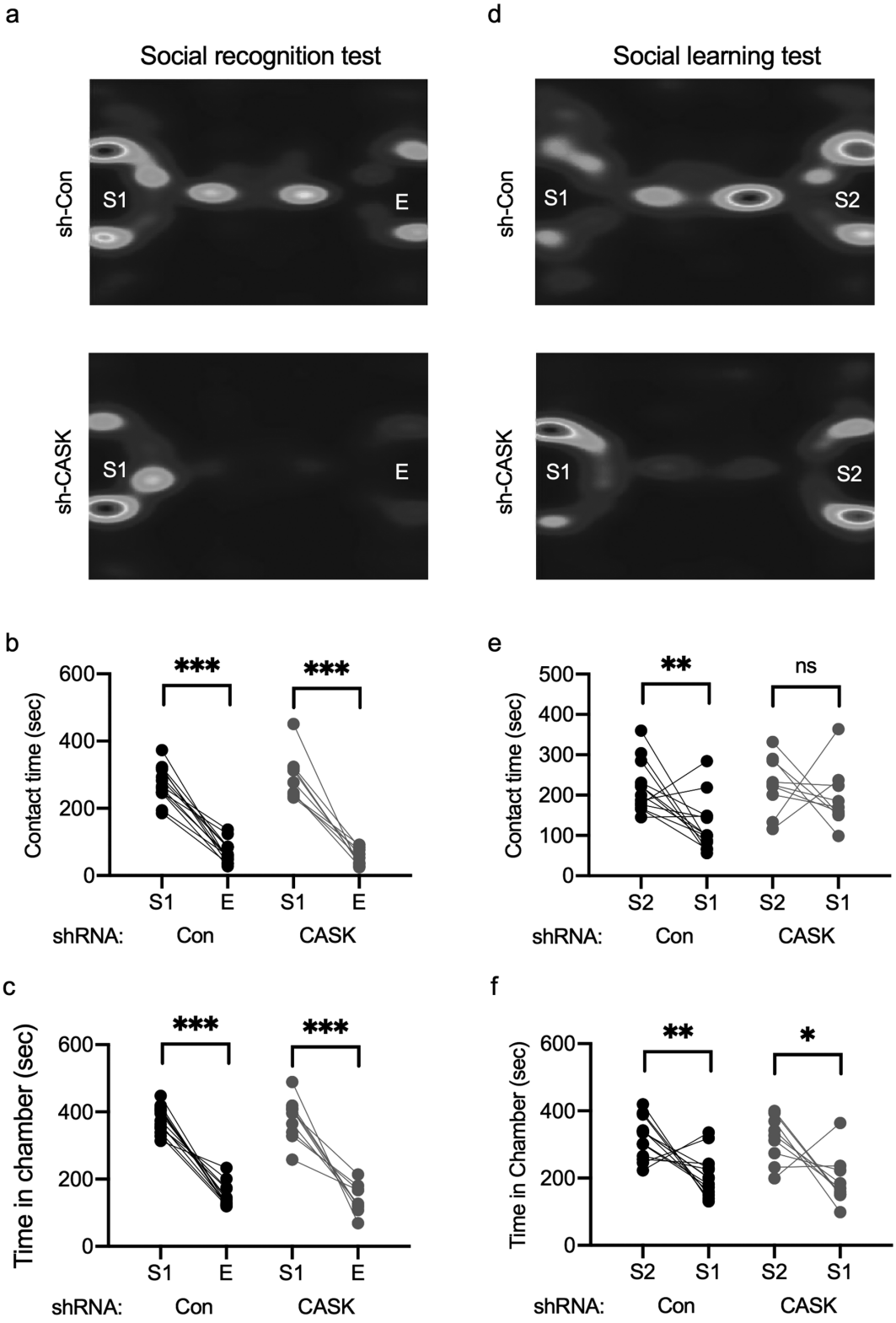

Fig. 4 Heatmap images and graphs for three-chamber tests

(a) Heatmap images (S1 vs E), contact time (b), and time in chamber (c) for the sociability test. (d) Heatmap images (S1 vs S2), contact time (e), and time in chamber (f) for social novelty test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, ns : not significant. Control : 13 animals, CASK knockdown : 9 animals.

social memory were significantly impaired in CASK knockdown mice. Travel distance was decreased, whereas waking speed, vertical activity, time in the center, and grooming were unchanged in the CASK knockdown mice.

While fully deleted CASK mutations are found only in females, hypomorphic mutations are still found in males[8]. Pathogenicity of CASK mutation between male and female patients is likely to be different because of the X chromosome inactivation effect. In female patients, CASK-intact and CASK-deficient neurons are distributed randomly in a mosaic pattern in the brain[6]. In males, genomic conditions of the *CASK* gene should be consistent in all neurons. Infection efficiency of AAV is known to be enormous and empirically almost all neurons within the injection area are expected to be infected[9]. Since CASK male KO is lethal, our study using an AAV-mediated CASK knockdown model is the first observation of CASK effects on behavior.

Vertical activity and grooming are known to represent repetitive behavior observed in autistic cases[10]. Neurocircuits in the deep layer of the mPFC have been shown to be one of the responsive areas for the behavior[11]. We failed to detect these changes in our mice, suggesting that CASK is not involved in the mPFC-dependent repetitive behavior. We also did not detect changes in the time in the center in the open field test. This parameter is used to assess anxiety level. Reduction of GABAergic synaptic function is suggested to be the major cause of anxiety. GABAergic function may not be decreased by CASK knockdown, or other brain regions may be more important for anxiety. Electrophysiological study is required to verify these possibilities.

A wide range of social behavioral abnormalities are observed in the CASK knockdown model. Many studies suggest that the deep layer of the mPFC is the center of dictating this behavior[11]. Although distinct neuronal pathophysiology is undefined, altered balance between excitatory and inhibitory synaptic function is reported in many animal models exhibiting social behavioral deficits[12]. Further electrophysiological study in this area is necessary to examine this possibility.

CASK has been shown to interact with various proteins through its functional domains[13]. The best studied interaction molecule is the presynaptic cell adhesion protein neurexin[1]. CASK binds neurexin through the PDZ domain and participates in the trams-synaptic complex including neuroligins and PSD-95. These molecules are shown to be strongly linked to autism[14]. To address whether the neurexin/neuroligin pathway is involved in the pathophysiology of the social deficits in CASK knockdown mice will open the window to dissect molecular mechanisms underlying the core symptoms of CASK-related neurodevelopmental disorders.

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