이학석사 학위논문

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2020 년 2 월

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Studies on social behavior of *Dscam* mutant mice generated by two *cre*-lines

Advisor. Professor Bong-Kiun Kaang

A dissertation submitted to the Graduate Faculty of Seoul National University in partial fulfillment of the requirement for the Degree of Master of Science

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Brain Science Major

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Abstract

Studies on social behavior of *Dscam* mutant mice generated by two *cre*-lines

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Interdisciplinary Program in Neuroscience

The Graduate School

Seoul National University

Down syndrome cell adhesion molecule (DSCAM) is one of the cell adhesion molecules that cause down syndrome. In the human study, overexpression of *Dscam* gene in the development of the nervous system leads to intellectual disability.

Recently, Autism spectrum disorder (ASD) patient who has N terminal truncated DSCAM mutation is uncovered. ASD is characterized by the decline of social interaction, memory impairment, and abnormal increase of repetitive behavior. Although

many genes regulating ASD are well known, the relevance between *Dscam* gene and ASD is poorly discriminated.

Here, I conducted several social behavior tasks using two conditional N-terminal *Dscam* knockout (KO) mice as an ASD mouse model. Using *cre* system, *Dscam* is conditionally KO only in *CaMKII*-positive cells or *Nestin*-positive cells. I found that *Nestin*-specific *Dscam* KO mice show fewer interactions with other mice compared to their littermate, though *CaMKII*-specific *Dscam* KO mice show more interactions. This study suggests that *Dscam* takes an important role to control sociability. Furthermore, this is the first study to reveal the social-behavioral phenotypes of *Dscam* mutant mice as ASD mouse model.

Keyword : Autism Spectrum Disorder (ASD), *Dscam* knockout mice, behavioral study, social interaction

Student Number : 2018-25927

Introduction

Down syndrome cell adhesion molecule (DSCAM) is a specific neuronal adhesion protein, and is well known as key factor causing Down syndrome (DiCarlo, Aguilar et al.) when it is overexpressed in human (Yamakawa, Huot et al. 1998). Previous researches have shown that various functions of DSCAM in different species. In the case of arthropods, several isoforms of DSCAM exist by alternative splicing (Schmucker, Clemens et al. 2000, Wojtowicz, Flanagan et al. 2004), and they function by recognizing different pathogens (Ng, Chiang et al. 2014, Armitage and Brites 2016). Specifically, in the fly nervous system, DSCAM is involved in determining neuronal wiring, axonal guidance, and is strictly restricted at axons and dendrites (Schmucker, Clemens et al. 2000, Celotto and Graveley 2001). In terms of mammals, there is a single isoform of DSCAM in the mammalian nervous system, and reduction of DSCAM causes ataxia or seizure in mouse study (Fuerst, Koizumi et al. 2008). Furthermore, DSCAM takes a role in neuronal differentiation and synaptic plasticity (Yamakawa, Huot et al. 1998).

Recently, the relevance between *Dscam* and Autism spectrum

disorders (ASD) is appeared (O'Roak, Stessman et al. 2014). ASD is one of the neurodevelopmental disorders defined defective social interactions, obesity, and rapid increase of repetitive behaviors (Yu, Chahrour et al. 2013, Murphy, Wilson et al. 2016, Zheng, Zhang et al. 2017). Besides, ASD patients show deficient cognitive function (Knutsen, Mandell et al. 2017) Various molecules such as DVL1, WNT2, RELN, UBE3A, SHANK, NRXN, NLGN, and MECP2 were uncovered as triggering agents of ASD (Lijam, Paylor et al. 1997, Fatemi, Stary et al. 2001, Sheng 2001, Wassink, Piven et al. 2001, Morrow, Yoo et al. 2008, Guang, Pang et al. 2018).

Since molecular mechanisms of ASD is too complex, and there is no clear medication to relieve ASD symptoms yet, studying the underlying mechanisms causing ASD is necessary. Instead of direct research of ASD patients, ASD mouse models are commonly used to study human diseases (Provenzano, Zunino et al. 2012, Bey and Jiang 2014, Kazdoba, Leach et al. 2016, Faraji, Karimi et al. 2018). For instance, cre-loxP system and short hairpin RNA (shRNA) are used to knock-in or knock-out target gene in specific tissues or cell types (Koornneef, Maczuga et al. 2011, Kim, Kim et al. 2018). Moreover, Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated 9 (CRISPR/Cas9) can be used to generate

genomic editing (Jinek, Chylinski et al. 2012, Cho, Kim et al. 2013).

Although various molecules affecting ASD have emerged, the linkage between DSCAM and ASD is poorly described. In this study, I generated ASD mouse model through *Dscam* exon 1 molecule knock out in different cell types by using cre-loxP system. ASD-related behavior battery was conducted with *Dscam* KO mice. This report firstly suggests a correlation between *Dscam* and ASD by using rodent model and phenotypes can be different according to specific cell types and specific period.

Materials and methods

Mice

Heterozygous *Dscam* exon 1 floxed mice were generated by crossing *Dscam* exon 1 floxed mice with CAG-cre mice from RIKEN BRC. *Dscam* exon 1 floxed mice were breed with homozygous *CaMKII*-cre or heterozygous *Nestin*-cre mice to conditionally knock-out.

Two *cre* mice used to make KO mice show different expression patterns in terms of cell types and period. Firstly, CaMKII protein is famous for its effects on long-term synaptic plasticity and synaptic maturation (Cornelia Koeberle, Tanaka et al. 2017). In the case of the *CaMKII*-alpha promoter is specifically expressed in cells located in the forebrain, such as pyramidal cells in the hippocampus, and it is highly active after the third week of the postnatal period (Tsien, Chen et al. 1996).

On the other hand, Nestin is well known as intermediate-filament protein (Chen, Kwon et al. 2009) or neuroepithelial stem cell protein

and is related to self-renewal, migration (Bernal and Arranz 2018). *Nestin* promoter is expressed in neuronal stem and progenitor cells, glial precursors (Giusti, Vercelli et al. 2014, Bernal and Arranz 2018) and is normally presented after embryonic day 11. By crossing heterozygous *Dscam* exon 1 floxed mice and respective cre-lines, *Dscam* exon 1 is heterozygously knocked-out in different cell types for the different period.

Genotypes of mice were identified by Polymerase Chain Reaction (PCR) with primers written in Table 1. DNA from mouse tail was homogenized by incubation at 60℃ for 1 day with a mixture of DirectPCR (FIAT) and proteinase K (Qiagen). A mixture of PCR includes Han taq buffer (Genemed), deoxynucleoside triphosphate (Roche), Han taq (Genenmed), forward / reverse primers, DNA extract, and distilled water. Cycling condition of PCR was 95℃ for 5 min, followed by 30 cycles of 95℃ for 60 sec, 60℃ for 60 sec, 72℃ for 60 sec, followed by 72℃ for 5 min. The PCR products were visualized on 2% agarose gel with safeview. WT band appeared on 499bp, and KO band was shown on 666bp. All behavior tests were examined with adult mice (8-19 weeks) exposed to 12 hr light / dark circadian cycles. Mice were provided enough food and water. Managing animals and all experiments complied with the instruction

of Institutional Animal Care and Use Committees (IACUC) of Seoul National University.

Table 1. Primers for genotyping PCR

Primer	Primer sequence (5' to 3')
Wild-type (499bp)	F TCT CTC CAG AGG GCA CTA A R CAT GTG GTA GAT TTC ACT CCA
Knockout (666bp)	F CAC ATC GCA TCA AAA CTG CT R CAT GTG GTA GAT TTC ACT CCA

Western blot

The Anterior cingulate cortex (Lijam, Paylor et al.) tissues from DSCAM KO mice and wild-type mice were homogenized with TEVP lysis buffer (10mM Tris (pH 7.4), 1mM EDTA, 1mM EGTA, 320mM sucrose, distilled water) containing protease inhibitor cocktail(PIC). After centrifuging at 1,000 g for 10 min, collect the supernatant. Then, centrifuge at 12,000 g for 20 min to extract pellet (crude synaptosomal fraction). Pellets were homogenized with RIPA

containing PIC. The protein extracted from crude synaptosomal membrane fraction is quantitatively analyzed with BCA (Thermo scientific) assay. Each sample was loaded as 10 μg on 4-12% SDS-PAGE gels (Invitrogen, USA). Gel with loaded proteins was transferred ECL membrane for 4℃ overnight. The membrane was blocked with 5% skim milk solution for 1h, and was treated followed primary antibodies: mouse anti-pan cadherin (1:5000, SantaCruz), rabbit anti-DSCAM (1:1000, LS Bio). Secondary antibody was treated as goat anti-mouse(1:5000, SantaCruz), and goat anti-rabbit (1:5000, SantaCruz).

Behavioral experiments

Before all behavioral tests, mice were habituated in the test room with dim light for at least 30 min. After tests, all apparatus were cleaned up with 70% alcohol and distilled water, then dry enough before the test of the next mouse.

Open field test.

Mice were located in the center of open field apparatus (40 cm x 40

cm square-shaped box) and freely moved for 30 min under dim light. The movement of each mouse was analyzed by Ethovision 3.1, Nodulus.

Marble burying test.

Mice could freely move around the cage with 20 marbles loaded on the beta chip (Orient). Each marble was located with even interval. For 30 min recording, manually count the number of buried marbles for every 5 min.

Repetitive behavior test.

Mice were placed a new cage with new bedding and were recorded for 20 min. For the last 10 min, time spent on the repetitive behaviors (self-grooming, rearing, and sniffing) were counted manually.

Reciprocal interaction test.

A pair of test mice were placed in the new cage with new bedding. Pairs of mice included three different types; wild-type (WT) mice and WT mice, WT mice and KO mice, KO mice and KO mice. Each pair was recorded for 10 min and was analyzed the whole recorded file. Time spent of interactions (sniffing, following, nose-to-nose)

was counted manually.

Three chamber test.

Before the test, test mice and stranger mice were habituated separately in the empty apparatus for two sequential days. Mice were located in the center chamber of the apparatus which has three identical chambers in a row. For the first 10 min, mice moved freely in the apparatus without any object or stranger mice. For the next 10 min, the test mouse moves around the whole apparatus with the object located on one side of the chamber, and a stranger mouse placed on the other side of the chamber (Sociability test). For the last 10 min, the test mouse gets around apparatus with a familiar mouse, located on one side of the chamber, and an unfamiliar mouse placed on the other side of the chamber (Social novelty test). Time spent exploring the object, the stranger was analyzed manually.

Ultrasonic vocalization test.

Before the experiment, test male mice were single-caged for 7 days. After test mice were habituated for 5 min in the new cage with new bedding, put the female on the estrous state in the cage. Ultrasonic vocalization between test mouse and female encountered mouse was

recorded with software (Avisoft). The total number of calls and latency to the first call were analyzed through Avisoft.

Statistical analysis

All data were analyzed by Prism 8 software. Unpaired t-test was conducted to compare two different groups. In all statistics, significance was shown with n.s ($p > 0.05$), * ($p < 0.05$), ** ($p<0.01$), and *** ($p<0.001$). Data is represented with a mean ± SEM.

Results

Generation and identification of *Dscam* KO mice.

Heterozygous *Dscam* exon 1 KO mice were generated by crossing *Dscam* floxed exon 1 mice and cre mice, *CaMKII*−cre mice or *Nestin*−cre mice (Fig. 1A, Fig. 1B). *Dscam* exon 1 knocked out only in *CaMKII* −positive cells or *Nestin*−positive cells, respectively. Genomic PCR and western blot were used to identify heterozygous *Dscam* KO. The genotype of each mice was confirmed with genomic PCR that showed only 499bp band in WT, and two bands, 499bp, 666bp, in heterozygous KO (Fig. 1C). Anterior cingulate cortex (Lijam, Paylor et al.) is one of the brain regions related to sociability (Zhou, Shi et al. 2016, Guo, Chen et al. 2019). I prepared ACC from two different mutant mice to verify DSCAM knockout. Knockout was also shown by western blot (Fig. 1E, Fig. 1G). The amount of DSCAM in heterozygous KO mice was half of that in WT mice on 220kDa (Fig 1D, Fig. 1F).

Nestin-specific *Dscam* KO mice showed decreased body weight.

In previous human study, intimate relevance between obesity and ASD was highlighted (Hill, Zuckerman et al. 2015, Zheng, Zhang et al. 2017), but tendency of body weight in ASD model mice has not been consistent (Portmann, Yang et al. 2014, Yang, Ahn et al. 2016, Zilkha, Kuperman et al. 2016, DiCarlo, Aguilar et al. 2019). I measured the body weight of WT mice, *CaMKII*-specific *Dscam* KO mice, and *Nestin*-specific *Dscam* KO mice (Fig. 3A, Fig. 3B). Only *Nestin*-specific *Dscam* KO mice showed significantly decreased body weight, which is different from the human case.

Both *Dscam* KO mice showed normal anxiety.

There were several human studies that ASD accompanies anxiety disorders (van Steensel, Bögels et al. 2011, Zheng, Zhang et al. 2016). To measure the anxiety of *Dscam* KO mice, open field test was conducted (Fig. 3C). Test mice placed in empty apparatus and recorded for 30 min. The more time the mouse located in the center zone, the more anxious mice were. I analyzed the percent of time

spent in the peripheral or central zone, and distance moved. Both mutant mice spent more time in the peripheral zone than the central zone. No significant difference between KO and WT littermates (Fig. 3D, Fig. 3F) was showed. Two mutant mice showed no difference in distance moved compared to the WT mice (Fig. 3E, Fig. 3G). I conclude that both mutant mice showed normal anxiety levels compared to the WT group.

Both *Dscam* KO mice showed normal repetitive behavior.

Increased level of repetitive behavior is one of the prototyped features of ASD (Ryan, Young et al. 2010). To examine the repetitive behavior, I examined the marble burying test and monitoring repetitive behaviors. For the marble burying test, 20 marbles were located with even interval at first, time goes by, mice buried marbles with bedding (Fig. 4A). I manually counted buried marbles for every 5 min. Both mutant mice showed no difference in the marble burying test (Fig. 4B, Fig. 4C).

For the repetitive test, every mouse was placed on the new cage with new bedding and was recorded for 20 min (Fig. 4D). I analyzed

the time spent on repetitive behaviors such as self-grooming, digging, rearing. Both mutant mice showed no difference in time spent of repetitive behaviors compared to that of WT littermate, respectively (Fig. 3E, Fig. 3F). Interestingly, *CaMKII*-specific *Dscam* KO mice showed only self-grooming without any rearing and digging (Fig. 3E), whereas *Nestin*-specific *Dscam* KO mice showed all three phenotypes, self-grooming, rearing, digging (Fig. 3F).

Nestin-specific *Dscam* KO mice showed a defect of social interaction.

In previous studies, the sociability of ASD model mice is measured through reciprocal interaction test and three-chamber test (Grabrucker, Jannetti et al. 2013, Bales, Solomon et al. 2014, Buffington, Di Prisco et al. 2016).

Reciprocal interaction test is for analyzing the interaction between two different mice when they are freely moved. Normally, test mice encounter juvenile mice aging 24-26 postnatal days (Bales, Solomon et al. 2014). Instead of using juvenile stranger, I formed pair of test mice, and each pair includes KO and KO, KO and WT, WT and WT with matched sex to focus on the interaction between KO and WT

mice.

There are three types of interactions, such as following, nose-to-nose, sniffing (Fig. 5A). Each time spent of respective interactions was manually counted via python program. *CaMKII*-specific *Dscam* KO mice showed tendency of increased interaction time, but not significant except interaction time of nose-to-nose (Fig. 5B-5E). In contrast, *Nestin*-specific *Dscam* KO mice exhibited decreased interaction time compared to those littermates (Fig 5F-5I). Specifically, total interaction time and following time were significantly decreased (Fig 5F-5G). With this experiment, *CaMKII*-specific *Dscam* KO mice and *Nestin*-specific *Dscam* KO mice showed conflicting data set of interaction levels.

I tested sociability and social novelty preference with three-chamber test. First 10 min, test mice freely moved in empty three-chamber apparatus. Next 10 min, object and stranger 1 were located on each side of the chamber (Fig. 6A). Although WT mice show more interaction with the stranger than that with the object, ASD phenotypic mice spent less time to show interest on stranger than the object. Final 10 min, stranger 2 is newly encountered instead of object (Fig. 6A). In this time, normal mice show more interest in unfamiliar mouse, stranger 2 than familiar mouse, stranger 1. If test

mouse has a social memory deficit, mouse shows more interest on familiar mouse than unfamiliar mouse.

CaMKII-specific *Dscam* KO mice showed no deficit on sociability and social memory (Fig 6B-6C). In contrast, *Nestin*-specific *Dscam* KO mice showed same level of interest on both object and stranger 1 (Fig 6D), which means defect of social memory (Fig 6E). *Nestin*-specific *Dscam* KO mice were hard to discriminate object and stranger, and could not easily remember familiar mouse, stranger 1. Thus, Only *Nestin*-specific *Dscam* KO mice, not *CaMKII*-specific *Dscam* KO mice, show reduced sociability and social memory via reciprocal interaction test, and three-chamber test.

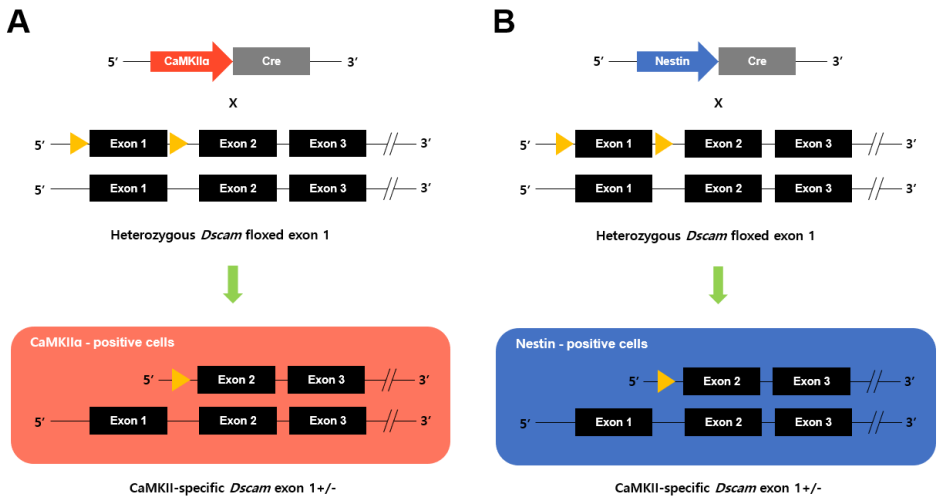
Both *Dscam* KO mice showed normal ultrasonic vocalization.

Ultrasonic vocalization (USV) task is another experiment to measure sociability, and it is also usually tested on ASD modeled mice (Grabrucker, Jannetti et al. 2013). USV test can be typed with three different categories, interaction with pups, with juveniles, and with adults (Wöhr 2014). Specifically, USV in adult mice usually occurs between male-female, female-female, except male-male. In

this study, USV between male test mouse and female stranger mouse was conducted (Fig. 7A). Male test mice were isolated for 7 days and met female in estrous state in test day. Among several analyzing methods that are used at studies (Yang, Loureiro et al. 2013), latency to first call and the number of calls were analyzed with Avisoft program.

CaMKII-specific *Dscam* KO mice showed faster first call than their littermate, but not significant (Fig. 7B). There was no difference in the number of calls (Fig. 7C). In the case of *Nestin*-specific *Dscam* KO mice showed slower first call compared to that of littermate, but not significant (Fig. 7D). The number of calls was showed same level between WT littermates and KO mice (Fig. 7E). In conclusion, both mutant mice showed no significant difference in USV with estrous females.

Figures


A
5′
CaMKIIα
Cre
3′
X
5′
Exon 1
Exon 2
Exon 3
3′
5′
Exon 1
Exon 2
Exon 3
3′
Heterozygous Dscam floxed exon 1
CaMKIIα - positive cells
5′
Exon 2
Exon 3
3′
5′
Exon 1
Exon 2
Exon 3
3′
CaMKII-specific Dscam exon 1+/-
B
5′
Nestin
Cre
3′
X
5′
Exon 1
Exon 2
Exon 3
3′
5′
Exon 1
Exon 2
Exon 3
3′
Heterozygous Dscam floxed exon 1
Nestin - positive cells
5′
Exon 2
Exon 3
3′
5′
Exon 1
Exon 2
Exon 3
3′
CaMKII-specific Dscam exon 1+/-

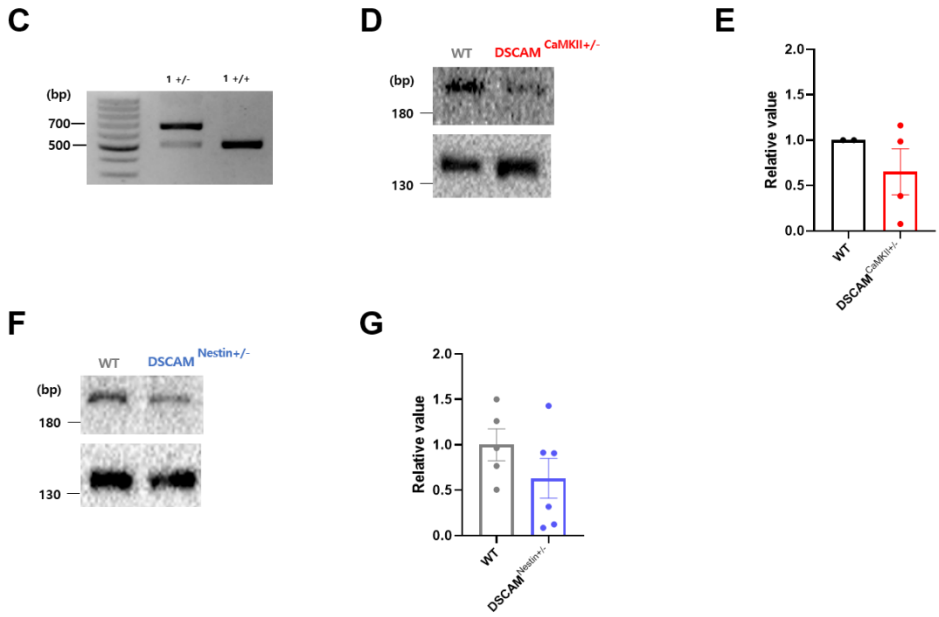
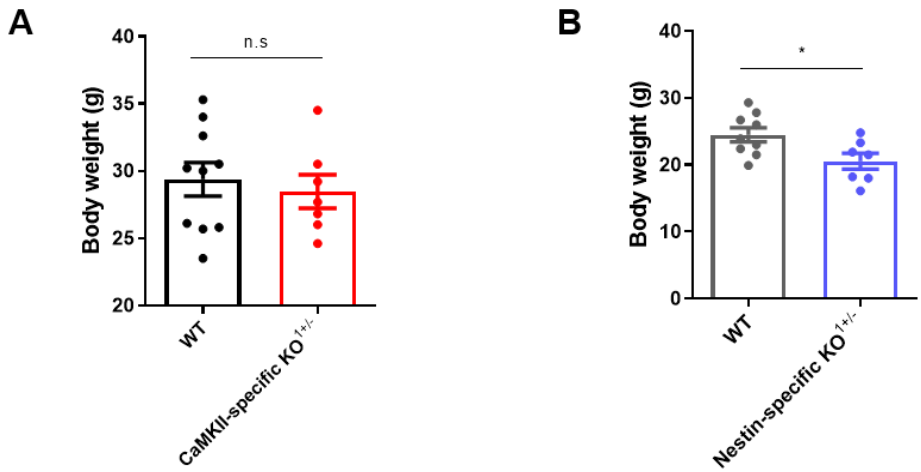

C
(bp)
700
500
1 +/-
1 +/+
D
WT
DSCAM CaMKII+/-
(bp)
180
130
E
Relative value
2.0
1.5
1.0
0.5
0.0
WT
DSCAM CaMKII+/-
F
WT
DSCAM Nestin+/-
(bp)
180
130
G
Relative value
2.0
1.5
1.0
0.5
0.0
WT
DSCAM Nestin+/-

Figure 1. Generation of *Dscam* KO mice.

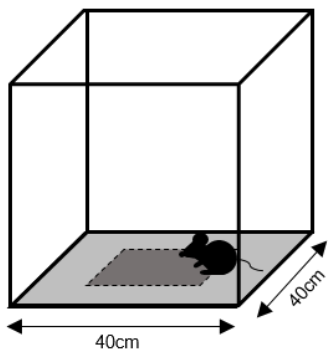
(A), (B) *Dscam* exon 1 heterozygous deleted with *Cre* – *LoxP* system. Heterozygous *Dscam* floxed exon 1 mice were crossed with *CaMKII*-cre mice or *Nestin*-cre mice, respectively. (C) Genomic PCR bands of WT and heterozygous KO mice. Upper band size is 666bp, and lower band size is 499bp. (D-G) Protein level of *Dscam* in ACC region of *CaMKII*-specific *Dscam* KO mice and *Nestin*-specific *Dscam* KO mice, respectively. (E, G) Protein level of DSCAM is normalized by pan-cadherin.

Open field test	Marble test	Repetitive test	Reciprocal interaction test	Three chamber test	Single caging	USV test
1 day	1 day	1 day	1 day	2 day	7 day	1 day

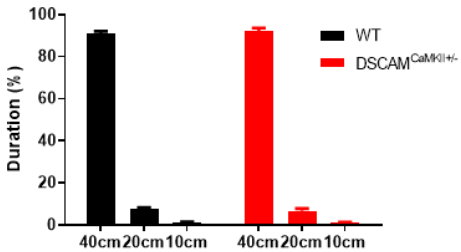
Figure 2. Behavioral scheme of *Dscam* KO mice.


A
n.s
Body weight (g)
40
35
30
25
20
WT
CaMKII-specific KO1+/-
B
*
Body weight (g)
40
30
20
10
0
WT
Nestin-specific KO1+/-

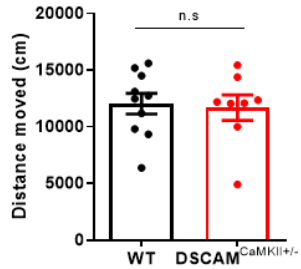
C


40cm
40cm

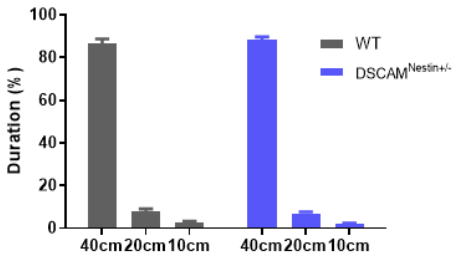
D


Duration (%)
100
80
60
40
20
0
WT
DSCAM CaMKII+/-
40cm 20cm 10cm
40cm 20cm 10cm

E


Distance moved (cm)
20000
15000
10000
5000
0
n.s
WT
DSCAM CaMKII+/-

F


Duration (%)
100
80
60
40
20
0
WT
DSCAM Nestin+/-
40cm 20cm 10cm
40cm 20cm 10cm

G

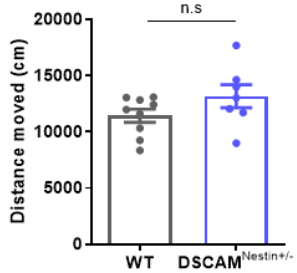
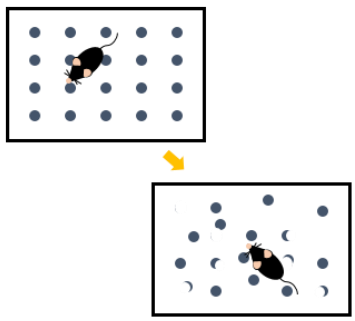

Distance moved (cm)
20000
15000
10000
5000
0
n.s
WT
DSCAM Nestin+/-

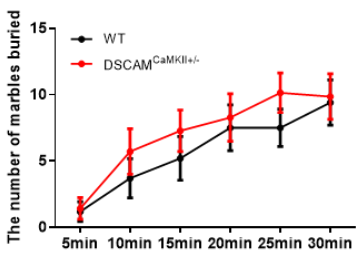
Figure 3. Only *Nestin*-specific *Dscam* KO mice exhibit reduced body weight, but normal anxiety level was presented in two types of KO mice.

(A-B) Body weight of *Dscam* mutant mice (WT, n=10; KO, n=7 for *CaMKII*-specific *Dscam* mice, WT, n=9; KO, n=7 for *Nestin*-specific *Dscam* mice). Unpaired t test was applied to body weight of each group; n.s (p=0.6300) for *CaMKII*-specific *Dscam* mice, * (p=0.0256) for *Nestin*-specific *Dscam* mice. (C) Scheme of apparatus for open field test. (D, F) Percent of time spent in 40 cm zone, 20 cm zone, 10 cm zone, respectively. (WT, n=10; KO, n=8 for *CaMKII*-specific *Dscam* mice, WT, n=9; KO, n=7 for *Nestin*-specific *Dscam* mice). Two way ANOVA was applied to each group; genotype x zone, n.s (p=0.6649); effect of genotype, n.s (p=0.1370) for *CaMKII*-specific *Dscam* mice, genotype x zone, n.s (p=0.5146); effect of genotype, n.s (p=0.8864) for *Nestin*-specific *Dscam* mice. (E, G) Distance moved of *Dscam* mutant mice (WT, n=10; KO, n=8 for *CaMKII*-specific *Dscam* mice, WT, n=9; KO, n=7 for *Nestin*-specific *Dscam* mice). Unpaired t test was applied; n.s (p=0.8088) for *CaMKII*-specific *Dscam* mice, n.s (p=0.1428) for *Nestin*-specific *Dscam* mice.

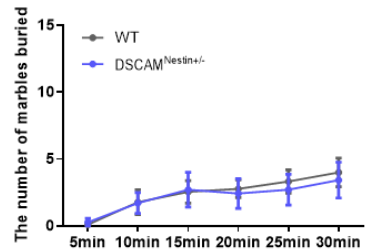
A



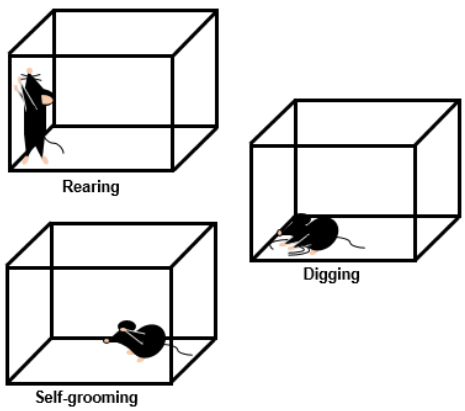
B


The number of marbles buried
15
10
5
0
WT
DSCAM CaMKII+/-
5min 10min 15min 20min 25min 30min

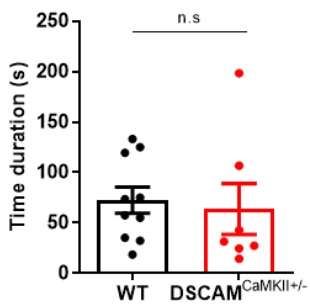
C


The number of marbles buried
15
10
5
0
WT
DSCAM Nestin+/-
5min 10min 15min 20min 25min 30min

D


Rearing
Digging
Self-grooming

E


n.s
Time duration (s)
250
200
150
100
50
0
WT
DSCAM CaMKII+/-

F

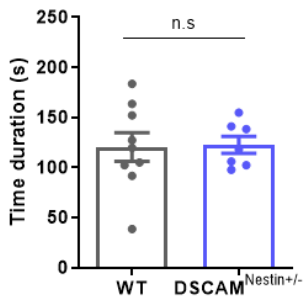
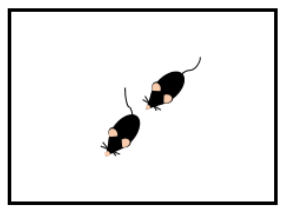

n.s
Time duration (s)
250
200
150
100
50
0
WT
DSCAM Nestin+/-

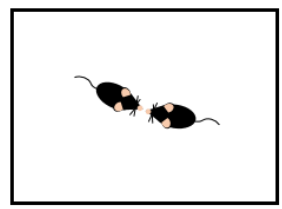
Figure 4. Both *Dscam* KO mice show normal repetitive behavior via marble test and repetitive behavior test.

(A) Scheme of marble burying test. (B, C) The number of buried marbles of *Dscam* mutant mice. Two way ANOVA was applied; genotype x time, n.s (p=9595); effect of genotype, n.s (p=0.1331) for *CaMKII*−specific *Dscam* mice (WT; n=10, KO; n=7), genotype x time, n.s (p=0.9961); effect of genotype, n.s (p=0.6932) for *Nestin*−specific *Dscam* mice (WT; n=9, KO; n=7). (D) Three different repetitive behaviors. (E, F) Total time duration of repetitive behaviors in *Dscam* mutant mice. Unpaired t test was applied; n.s (p=0.7386) for *CaMKII*−specific *Dscam* mice (WT; n=10, KO; n=7), n.s (p=0.9046) for *Nestin*−specific *Dscam* mice (WT; n=9, KO; n=7).

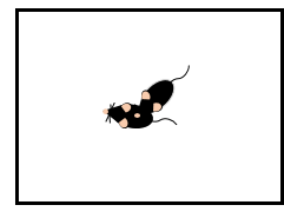
A



Following

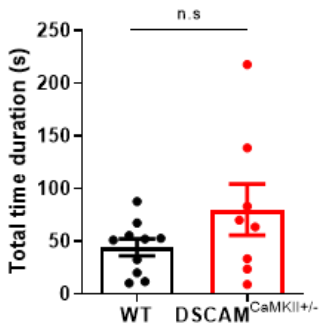


Nose-to-nose

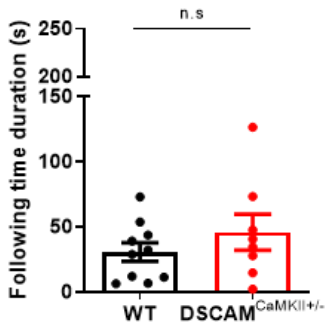


Sniffing

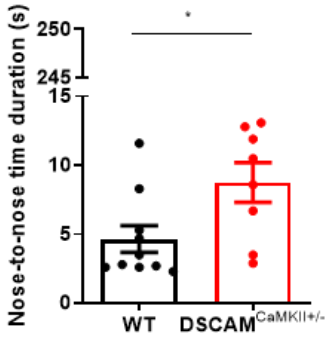
B


n.s
Total time duration (s)
250
200
150
100
50
0
WT
DSCAM CaMKII+/-

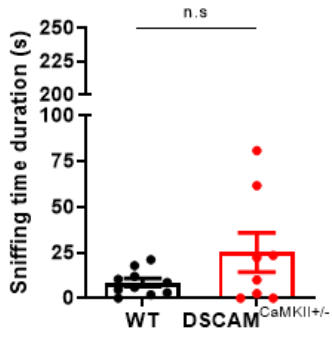
C


n.s
Following time duration (s)
250
200
150
100
50
0
WT
DSCAM CaMKII+/-

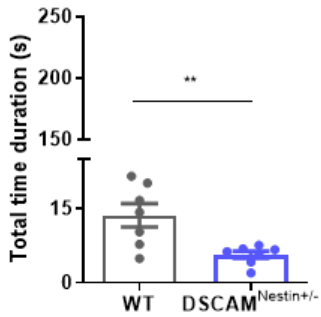
D


*
Nose-to-nose time duration (s)
250
245
15
10
5
0
WT
DSCAM CaMKII+/-

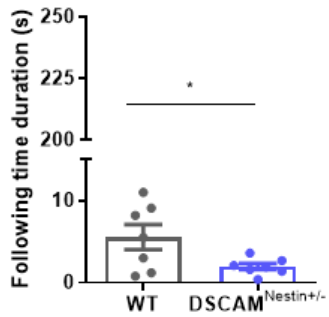
E


n.s
Sniffing time duration (s)
250
225
200
100
75
50
25
0
WT
DSCAM CaMKII+/-

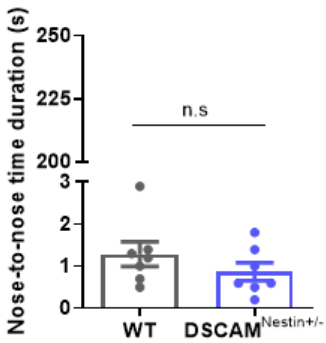
F


Total time duration (s)
250
200
150
15
0
**
WT
DSCAM Nestin+/-

G


Following time duration (s)
250
225
200
10
0
*
WT
DSCAM Nestin+/-

H


Nose-to-nose time duration (s)
250
225
200
3
2
1
0
n.s
WT
DSCAM Nestin+/-

I

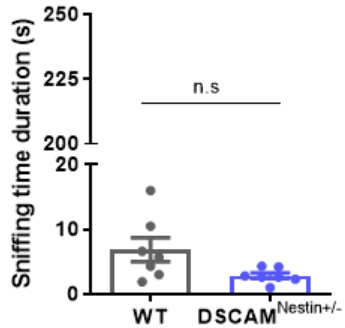
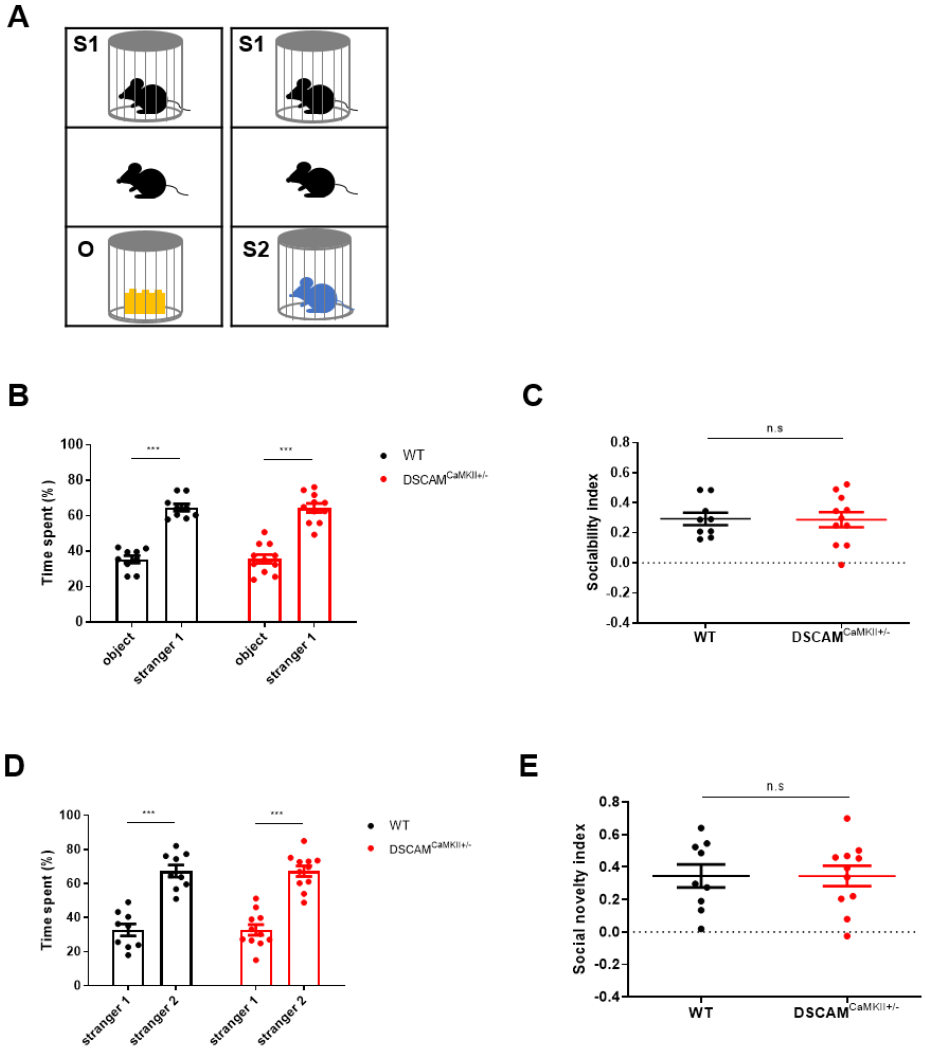

Sniffing time duration (s)
250
225
200
20
10
0
n.s
WT
DSCAM Nestin+/-

Figure 5. Only *Nestin*-specific *Dscam* KO mice show reduced social interaction through reciprocal interaction test.

(A) Three different interactions analyzed. (B) Total time duration of reciprocal interactions of *CaMKII*-specific *Dscam* mice (WT; n=10, KO; n=8). Unpaired t test was applied; n.s (p=0.1452). (C-E) Time duration of each interaction, following, nose-to-nose, and sniffing, respectively with *CaMKII*-specific *Dscam* mice (WT; n=10, KO; n=8). Each graph was analyzed with unpaired t test; n.s (p=0.3121) for following, * (p=0.0258) for nose-to-nose, n.s (p=0.1122) for sniffing. (F) Total time duration of reciprocal interactions of *Nestin*-specific *Dscam* mice (WT; n=7, KO; n=7). Unpaired t test was applied; ** (p=0.0073). (G-I) Time duration of each interaction, following, nose-to-nose, and sniffing, respectively with *Nestin*-specific *Dscam* mice (WT; n=7, KO; n=7). Each graph was analyzed with unpaired t test; * (p=0.0413) for following, n.s (p=0.2772) for nose-to-nose, n.s (p=0.0558) for sniffing.


A
S1
S1
O
S2
B
Time spent (%)
100
80
60
40
20
0
***
***
object
stranger 1
object
stranger 1
WT
DSCAMCaMKII+/-
C
n.s
Sociability index
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
WT
DSCAMCaMKII+/-
D
Time spent (%)
100
80
60
40
20
0
***
***
stranger 1
stranger 2
stranger 1
stranger 2
WT
DSCAMCaMKII+/-
E
n.s
Social novelty index
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
WT
DSCAMCaMKII+/-

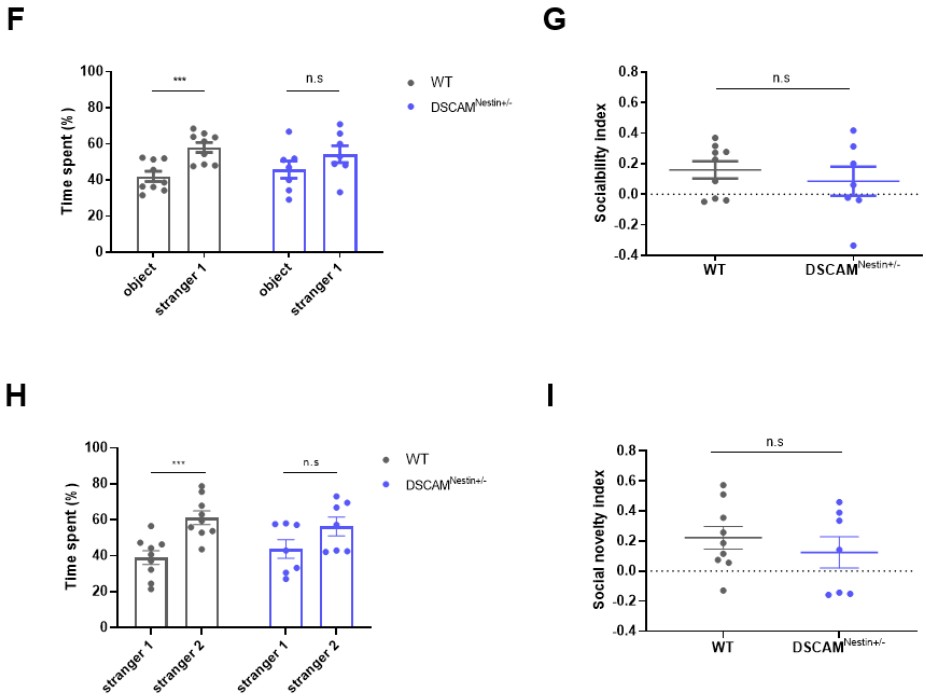

F
G
H
I
Time spent (%)
***
n.s
WT
DSCAM Nestin+/-
object
stranger 1
stranger 2
Socialbility index
Social novelty index
WT
DSCAM Nestin+/-

Figure 6. Only *Nestin*-specific *Dscam* mice show reduced sociability and social memory through the three-chamber test.

(A) Scheme of sociability test and social novelty test. (B, C) Percent of contact time spent and sociability index of *CaMKII*-specific *Dscam* KO mice on object and stranger 1. Unpaired t test was applied; *** ($p < 0.0001$) for contact time of *CaMKII*-specific *Dscam* WT mice (n=9), *** ($p < 0.0001$) for *CaMKII*-specific *Dscam* KO mice (n=11) and n.s ($p=0.9343$) for sociability index of *CaMKII*-specific *Dscam* mice. (D, E) Percent of contact time spent and social novelty index of *CaMKII*-specific *Dscam* KO mice on stranger 1 and stranger 2. Unpaired t test was applied; *** ($p < 0.0001$) for *CaMKII*-specific *Dscam* WT mice (n=9), *** ($p < 0.0001$) for *CaMKII*-specific *Dscam* KO mice (n=11) and n.s ($p=0.9925$) for social novelty index of *CaMKII*-specific *Dscam* mice. (F,G) Percent of contact time spent and sociability index of *Nestin*-specific *Dscam* KO mice on object and stranger 1. Unpaired t test was applied; *** ($p=0.0009$) for *Nestin*-specific *Dscam* WT mice (n=9), n.s ($p=0.2278$) for *Nestin*-specific *Dscam* KO mice (n=7) and n.s ($p=0.4910$) for sociability index of *Nestin*-specific *Dscam* mice. (H, I) Percent of contact time spent and social novelty index of *Nestin*-specific *Dscam* KO mice on

stranger 1 and stranger 2. Unpaired t test was applied; *** (p=0.0007) for *Nestin*-specific *Dscam* WT mice (n=9), n.s (p=0.1160) for *Nestin*-specific *Dscam* KO mice (n=7) and n.s (p=0.4501) for social novelty index of *Nestin*-specific *Dscam* mice.

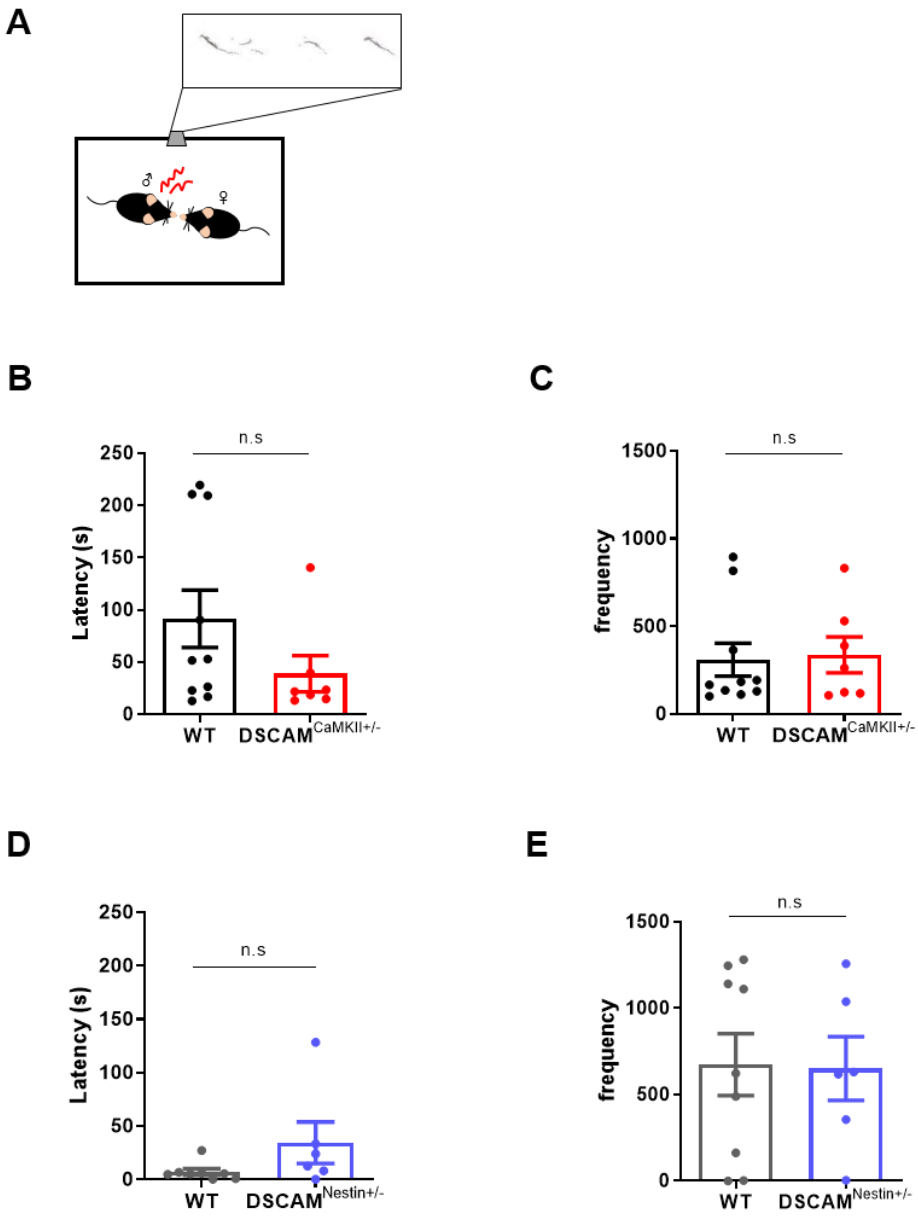

A
B
C
D
E
n.s
Latency (s)
frequency
WT
DSCAM CaMKII+/-
DSCAM Nestin+/-

Figure 7. Both *Dscam* KO mice show normal vocal interaction rate through ultrasonic vocalization test.

(A) Scheme of Ultrasonic vocalization (USV) test. (B, D) Latency to first call of two mutant mice, respectively. Unpaired t test was applied; n.s (p=0.1673) for *CaMKII*-specific *Dscam* mice, n.s (p=0.1313) for *Nestin*-specific *Dscam* mice. (C, E) The number of calls from two mutant mice. Unpaired t test was applied; n.s (p=0.8497) for *CaMKII*-specific *Dscam* mice, n.s (p=0.9344) for *Nestin*-specific *Dscam* mice.

Behaviors	CaMKII-specific *Dscam* KO $^{1+/-}$	Nestin-specific *Dscam* KO $^{1+/-}$
Body weight	n.s	**Decreased**
Open field	n.s	n.s
Marble test	n.s	n.s
Repetitive test	n.s	n.s
Reciprocal interaction test	**Tendency to increased**	**Impaired**
Three chamber test	n.s	**Impaired**
Ultrasonic vocalization test	n.s	n.s

Table 2. Summary of ASD-related behavioral phenotypes from two *Dscam* KO mice.

CaMKII-specific Dscam KO mice showed no significant difference compared to their littermates in terms of body weight, open field test, marble test, repetitive behavior test, reciprocal interaction test, three chamber test, and ultrasonic vocalization test. Nestin-specific Dscam KO mice showed only significant decrease in body weight, and sociability test such as reciprocal interaction test, three chamber test compared to their littermate. They showed normal level in open field test, marble test, repetitive test, ultrasonic vocalization test.

Discussion

This study firstly highlighted the connection between ASD and conditional *Dscam* KO in different cell types. *Nestin*-specific *Dscam* KO mice showed decreased body weight compared to their littermate (Fig. 3B). Two different *Dscam* mutant mice showed normal anxiety through open field test (Fig. 3D-3G). Both mutant group also showed no difference in repetitive behaviors compared to each WT group via marble burying test, and repetitive behavioral test (Fig. 4B-4C, 4E-4F).

In terms of social interaction, *CaMKII*-specific *Dscam* KO mice and *Nestin*-specific *Dscam* KO mice show conflicting phenotypes through reciprocal interaction test. *CaMKII*-specific *Dscam* KO mice were more interactive than their littermate, but the difference was not significant (Fig. 5B-5E). In contrast, *Nestin*-specific *Dscam* KO mice were significantly less interactive than their littermate (Fig. 5F-5I). ASD patients normally have difficulty with the recognition of others' faces. However, *Nestin-Dscam* KO mice showed no significant decrease in nose-poking behavior, one of the reciprocal interaction test. I discussed that mice use various sensory stimuli

such as olfactory sense, to recognize others, whereas humans highly depend on visual stimuli to recognize others.

Decreased sociability of *Nestin*-specific *Dscam* KO mice also was also reappeared through three-chamber test. Although *CaMKII*-specific *Dscam* KO mice showed normal sociability and social memory (Fig. 6B-6E), *Nestin*-specific *Dscam* KO mice showed decreased sociability and impairment of social memory (Fig. 6F-6I). *Nestin*-specific *Dscam* KO mice could show other memory deficits as well as social memory impairment.

In USV test, both mutant mice showed a similar level of the number of calls compared to their littermate (Fig. 7B, 7D). However, in terms of latency to first call, *CaMKII*-specific *Dscam* KO mice showed the faster tendency to the first call, though *Nestin*-specific *Dscam* KO mice showed the slower tendency to the first call (Fig. 7C, 7E).

To conclude results, if *Dscam* knocked out in CaMKII-positive cells, which are normally excitatory cells (Dittgen, Nimmerjahn et al. 2004, Nathanson, Yanagawa et al. 2009, Egashira, Mori et al. 2018), mice tended to show increased sociability. In contrast, if Dscam knocked out in Nestin-positive cells, which are total neuronal cells (Schmucker, Clemens et al. 2000, Bernal and Arranz 2018), mice showed decreased sociability. In other words, DSCAM takes a

different role in different cell types.

Sociability difference between *CaMKII*-specific *Dscam* KO mice and *Nestin*-specific *Dscam* KO mice can be explained with expressing period and expressed cell types by *CaMKII* and *Nestin* promoter. As previously mentioned, *CaMKII* is normally expressed on forebrain-neurons after third week of postnatal period (Burgin, Waxham et al. 1990, Benson, Isackson et al. 1992, Dragatsis and Zeitlin 2000, Trotter, Lee et al. 2013). However, *Nestin* is expressed in neural stem cells and glial precursor cells and starts to express from embryonic day 11 in rodent (Dahlstrand, Lardelli et al. 1995).

Based on the difference between two *cre*-lines, *CaMKII*-specific *Dscam* KO was not sufficient to induce impairment of social interaction. Moreover, *Dscam* KO in glial cells in may take a critical role to regulate sociability. Else, *Dscam* KO in developmental stage, before mice learn social skills, could be other explanation of phenotypic difference.

There are remaining parts to study further. In previous studies regarding DSCAM, molecular basis or intracellular signaling pathway of DSCAM related to the ASD is poorly explained. So, studies about molecular downstream mechanisms will be needed. Besides, research to find electrophysiological properties or downstream

mechanisms by using DSCAM N-terminal truncated mutation in induced pluripotent stem cells can be a good further study. I hope this study will be a challenging gate for further study about new molecules causing ASD.

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국 문 초 록

DSCAM 단백질은 세포 간 접합에 관여하는 분자로서, 다운 증후군을 유발한다고 알려져 있다. Human 연구에서는 신경계 발달 시기에 DSCAM 유전자가 과 발현 되었을 때, 지능이 떨어짐을 확인했다. 최근에는 DSCAM 단백질의 N 말단이 손상된 돌연변이를 가진 ASD 환자가 보고된 바 있다. ASD 환자의 특징으로는 사회성의 감소, 기억력 감퇴, 반복 행동의 증가 등을 제시할 수 있다. ASD에 관여하는 많은 유전자들이 연구되어 있지만, DSCAM 유전자와 ASD간의 관련성은 알려진 바가 없다.

본 연구에서는 두 가지 *Dscam* KO 마우스의 사회적 특성을 연구하였다. *Cre* 시스템을 이용하여, CaMKII를 발현하는 세포, 혹은 Nestin을 발현하는 세포 특이적으로 *Dscam* 유전자를 이형적으로 KO 시켰다. CaMKII 세포 특이적으로 *Dscam* KO 된 마우스는 사회성이 증가하는 경향성을 보이는 반면, Nestin 세포 특이적으로 *Dscam* KO 된 마우스는 WT 마우스에 비해 낮은 사회성을 보였다. 본 연구는 1) *Dscam* 유전자가 마우스의 사회성에 관련있고, 2) *Dscam* 돌연변이 마우스가 sociability가 감소하는 특징을 보이므로서, ASD 마우스 모델로의 가능성을 보여주었다.

주요어 : 자폐 범주성 장애, *Dscam* 유전자 변형 마우스, 행동 연구, 사회성

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