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Short Communication

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Rho Guanine Nucleotide Exchange Factor 4 (Arhgef4) Deficiency Enhances Spatial and Object Recognition Memory

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Guanine nucleotide exchange factors (GEFs) play multiple functional roles in neurons. In a previous study, we reported that *Arhgef4* (Rho guanine nucleotide exchange factor 4) functioned as a negative regulator of the excitatory synaptic function by sequestering postsynaptic density protein 95 (PSD-95). However, the role of *Arhgef4* in behavior has not been examined. We performed comprehensive behavioral tests in knockout (KO) mice to investigate of the effects of *Arhgef4* deficiency. We found that the expressed PSD-95 particle size was significantly increased in hippocampal neuronal cultures from *Arhgef4* KO mice, which is consistent with the previous *in vitro* findings. *Arhgef4* KO mice exhibited general motor activity and anxiety-like behavior comparable to those of the wild type littermates. However, spatial memory and object recognition memory were significantly enhanced in the *Arhgef4* KO mice. Taken together, these data confirm the role of *Arhgef4* as a negative synaptic regulator at the behavioral level.

Key words: Arhgef4, PSD-95, Spatial memory, Recognition

INTRODUCTION

Rho guanine nucleotide exchange factors (GEFs) are involved in the activation of Rho family GTPases by accelerating the exchange of GDP to GTP. Moreover, due to their multiple domains, GEFs act as functional and structural regulators within the postsynaptic regions of neurons in response to external stimuli [1, 2]. Thus, GEFs play a crucial role in various behaviors, such as anxiety, learning, and memory in experimental animals and also in human pathological conditions [2]. For example, the genetic deletion of Kalirin7, a GEF of excitatory synapses for Rac1 and RhoG, shows normal object recognition but impaired passive avoidance fear memory in Kalirin7 knockout (KO) mice [3]. The lack of collybistin, a GEF of inhibitory synapses selectively activating the small GTPase Cdc42, results in a reduced capability of spatial learning and enhances anxiety-like behavior in collybistin-deficient mice [4].

Arhgef4, also known as Asef1, *Adenomatous polyposis coli* (APC)-stimulated GEF 1, was identified as a functional linker protein connecting the tumor suppressor APC and G-protein signaling, suggesting its role in colon cancer [5]. Although *Arhgef4* is highly expressed in the brain [6, 7], information on its function in the brain is limited. In our previous study, we suggested that *Arhgef4* functions as a negative regulator in excitatory synapses, reducing the level of postsynaptic density protein 95 (PSD-95, also known as synapse-associated protein 90), a major postsynaptic scaffolding protein [8]. PSD-95-deficiency causes memory impairment [9, 10]. The downregulation of *Arhgef4* increases PSD-95, but not Homer1, another postsynaptic density scaffolding protein. Conversely, the overexpression of *Arhgef4* decreases PSD-95. Correspondingly, downregulation of *Arhgef4* by RNA interference increases the frequency and amplitude of the miniature excitatory

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synaptic current (mEPSC) and its overexpression decreases them [11]. However, these cellular and molecular studies were not confirmed at the systemic or behavioral levels in experimental animals. Consequently, in this study, we examined the behavior of *Arhgef4* KO mice. Results showed that the ablation of *Arhgef4* improved spatial and recognition memories, again suggesting that *Arhgef4* functions as a synaptic negative regulator in the post-synaptic regions of excitatory synapses.

MATERIALS AND METHODS

Transgenic animals

Arhgef4 KO mice (*Arhgef4*$^{tm1a(KOMP)Wtsi}$, MGI Cat# 5782024, RRID:MGI:5782024) were purchased from the National Institutes of Health (NIH)-sponsored Knockout Mouse Program (KOMP repository, University of California at Davis, CA, USA) [12]. Heterozygotes (*Arhgef4*$^{+/tm1a(KOMP)Wtsi}$; *Arhgef4*$^{+/-}$) were crossed with heterozygotes to produce wild-type (WT, *Arhgef4*$^{+/+}$), heterozygous mutants (Hetero, *Arhgef4*$^{+/-}$), and homozygous mutants (Homo, *Arhgef4*$^{-/-}$). Genotyping was performed using the indicated primer sets according to the KOMP information. Further information on mouse generation and targeting strategies are available at the Mouse Genome Informatics[1] (MGI, http://www.informatics.jax.org) [13].

RT-PCR and western blotting

Arhgef4 deficiency in *Arhgef4* KO mice was examined by RT-PCR and western blotting. For RT-PCR, 5 μg of the total RNAs purified from the brains of WT, Hetero, and Homo were subjected to reverse transcription using the oligo dT primer for 3'UTR of *Arhgef4* mRNA, or a gene specific primer (*Arhgef4*-RT: 5'-gggcctgatggtataggcc-3) and SuperScript III (Cat# 18080-051, Invitrogen, Carlsbad, CA) and subsequently amplified by PCR (primers for *Arhgef4* mRNA 3'-untranslated regions, *Arhgef4*-S: 5'-tccctggttccaggttagtg-3', *Arhgef4*-A: 5'-gcagccaggtcacttttcat-3'; β-actin mRNA, β-actin-S: 5'-gcgcaagtactctgtgtgga-3', β-actin-A: 5'-agcgccaaaacaaaacaaaa-3'; *Arhgef4* mRNA coding regions: *Arhgef4*-1500-S: 5'-ggaagccagaacagaagcag-3', Arhggef4-coding-A: 5'-ggttgtctgatggatg-3'). *Arhgef4* expression was examined on western blots containing 40 μg each of brain lysates from WT mice, Homo mice, and rats (Sprague Dawley, Samtaco Bio Korea, Osan, Korea), and 10 μg of human embryonic kidney (HEK) cells. Synaptic protein expression was detected on western blots containing 3 μg (for synaptophysin and α-tubulin) or 25 μg (for PSD-95, Homer-1, GluA1, and GluN1) of brain lysate from each genotyped mouse. All protein were separated on 8% acrylamide gels and transferred to a PVDF membranes (Millipore, Burlington, MA, USA). The antibodies used in western blotting included rabbit polyclonal anti-*Arhgef4* antibodies (raised by *Arhgef4* N-terminal regions of 105 amino acids including APC-binding regions and SH domain, Accession# EDL14459; AbClone, Seoul, Korea), polyclonal anti-PSD-95 antibodies (Cell Signaling Technology, Danvers, MA, USA), monoclonal anti-Homer 1 antibody (Synaptic Systems, Göttingen, Germany), polyclonal anti-GluA1 antibody (Millipore), monoclonal anti-GluN1 antibody (Millipore), polyclonal anti-synaptophysin antibody (Millipore), and monoclonal anti-α-tubulin antibodies (Clone B-5-1-2, Sigma-Aldrich, St. Louis, MO, USA). The protein levels of lysates were quantified by bicinchoninic acid assay (Pierce™ BCA assay, Thermo Fisher Scientific, Rockford, IL, USA). All RT-PCR and western blot analyses were performed more than twice to confirm reproducibility of data.

Quantitative real-time PCR

The 0.5 μg of total RNAs from the brain of WT, Hetero, or Homo was synthetized to cDNAs, and subsequently used to PCR containing SYBR Green ready mix (TOPreal™ One-step RT qPCR Kit, Enzynomics, Daejeon, Korea) and primers (identical sets used for RT-PCR analysis) by real-time PCR system (CFX96 Touch Real-Time PCR Detection System, Bio-Rad, Laboratory, Hercules, CA, USA). The relative change of *Arhgef4* expression in Homo or Hetero mouse to that in WT mouse was analyzed by $2^{-\Delta\Delta Ct}$ method [14, 15]. Total RNAs from two animals per genotype were subjected to qRT-PCR analysis and the reactions were repeated.

Neuronal culture, immunostaining, and image analysis

Hippocampi were isolated from the brain of postnatal day one (P1) animals and used for culture as previously described [16]. After twelve days, the cultures were infected with Sindbis virus encoding green fluorescent protein (GFP) [11] for 12 h, followed by immunostaining with monoclonal anti-PSD-95 antibody (Clone 6G6-1C9, Thermo Fisher Scientific) and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA, USA). Fluorescent images were acquired with confocal microscopy (Zeiss LSM 800 Airyscan, Carl Zeiss Microscopy GmbH, Jena, Germany), and the acquired images were analyzed with the ImageJ program (ver 1.46r, NIH, Bethesda, MA, USA). Image acquisition and analysis were performed in blinded experiments and image analysis was performed as previously described [16]. The data are presented as mean±standard error of the mean (SEM). The Student's unpaired *t*-test was applied to reveal statistical differences between the two groups. Statistical analyses were performed using

[1] http://www.informatics.jax.org/mgihome/nomen/IKMC_schematics.shtml (J:148605, J:173534)

GraphPad Prism (ver 5.0, GraphPad Software, La Jolla, CA, USA).

Behavioral tests

All experiments using mice were performed in accordance with the approved animal protocols and the guidelines of the Institutional Animal Care and Use Committee of the Chungbuk National University (CBNUA-1236-19-01). Fewer than four mice were placed in cages on a reversed light-dark cycle and were permitted food and water *ad libitum*. All behavioral tests were conducted on the F2-F3 generation of both male and female mice produced by intercrossing of the heterozygous mice (*Arhgef4*$^{+/-}$). Mice >11 weeks of age (35 animals, WT: 12, Hetero: 11, Homo: 12) were used in open field, elevated plus-maze, object location memory (OLM), and novel object recognition memory (ORM) tests in a sequence. Behavioral tests were performed in the middle phase of the dark cycle. Between trials, the surface of the arena or maze was cleaned with 70% ethanol and distilled water. Data acquisition and analysis for all tests were performed blinded to genotype.

Open field test

Open field tests were performed as described [17]. Tests were performed in an opaque white plastic arena (33×33 cm, 33 cm high). Mice were placed in the periphery of the arena, and their behavior was recorded for 15 min using a camcorder (HDR-CX100, SONY, Tokyo, Japan). For the measurement of general motor activity, path length (total distance) and speed of movement in the total area were analyzed by Ethovision XT (Noldus, Wageningen, the Netherlands). For anxiety-related behavior, entries to the central area and times spent in the central area (infield, square 20×20 cm) were analyzed.

Elevated plus-maze test

Mice were placed in the center of an elevated plus-maze (4×30 cm arms, 60 cm above floor level, 18 cm high non-transparent side walls), and their paths were recorded by a camcorder (HDR-CX100, SONY, Tokyo, Japan). Time spent in each arm and entries into each arm over 10 min were manually scored and changed to percentage. More details are described in a previous study [18].

Rotarod test

Rotarod tests to measure motor skills of mice were performed as described [19]. Mice were placed on the rotating rod with a start speed of 4 rpm, acceleration rate 20 rpm/min (47600, Ugo Basile, Gemonio VA, Italy) and tested for 14 min. Three times trials each 14 min with 15 min interval were performed. Duration time on the rod before mouse falls off and rod spin speed (rapid per minute, rpm) when mouse falls off were scored and averaged.

Object location memory test

The OLM test was performed as previously described [20, 21] and included training and test sessions. Before training, mice were habituated for 5 min per day for 4 days in an arena (33 cm×33 cm, 33 cm high, less than 45 LUX) and then habituated for 15 min per day over the next 2 days. One side of the experimental box included a spatial cue. In the training session, mice were allowed to freely explore two identical objects placed in the box for 10 min. During the test session 24 h after the training, mice were placed back in the same box, but one of the objects was moved to a new location. Interaction with each object (defined as sniffing and/or head within 1 cm of the object) was manually scored for analysis. Mice that showed more than 10% preference for each object in the training session were excluded from the subsequent memory tests. The discrimination index was calculated as follows: (time exploring the novel object – time exploring the old)/(time exploring novel+old)×100.

Novel object recognition memory test

After the OLM tests, mice were placed in the arena for the novel ORM tests with the same objects situated in the same location, and allowed to explore for 10 min. Twenty-four hours later, the mice were placed back into the experimental box containing an old object and a new object in the same locations and allowed to explore for 5 min. Mice that showed more than a 10% preference for each object in the training session were excluded from the subsequent memory tests.

Statistical analyses

Data normality was assessed with the Kolmogorov-Smirnov test, the D'Agostino & Pearson Omnibus normality test, or the Shapiro-Wilk normality test. One-way analysis of variance (ANOVA) was used to compare more than two groups. Post hoc comparisons were conducted using Dunnett's or Bonferroni's multiple comparison tests. If the data did not follow a Gaussian distribution, a nonparametric Kruskal-Wallis test was used to compare more than two groups. The Student's unpaired *t*-test was used to reveal statistical differences between the two groups. We did not conduct any tests for outliers. The data are presented as mean±SEM. Statistical analyses were performed using GraphPad Prism.

RESULTS

Arhgef4-deficient mice exhibited increase of PSD-95 particle size in neurons

To investigate the role of *Arhgef4* in behaviors, we generated *Arhgef4* KO mice by crossing heterozygous mutant mice (*Arh-*

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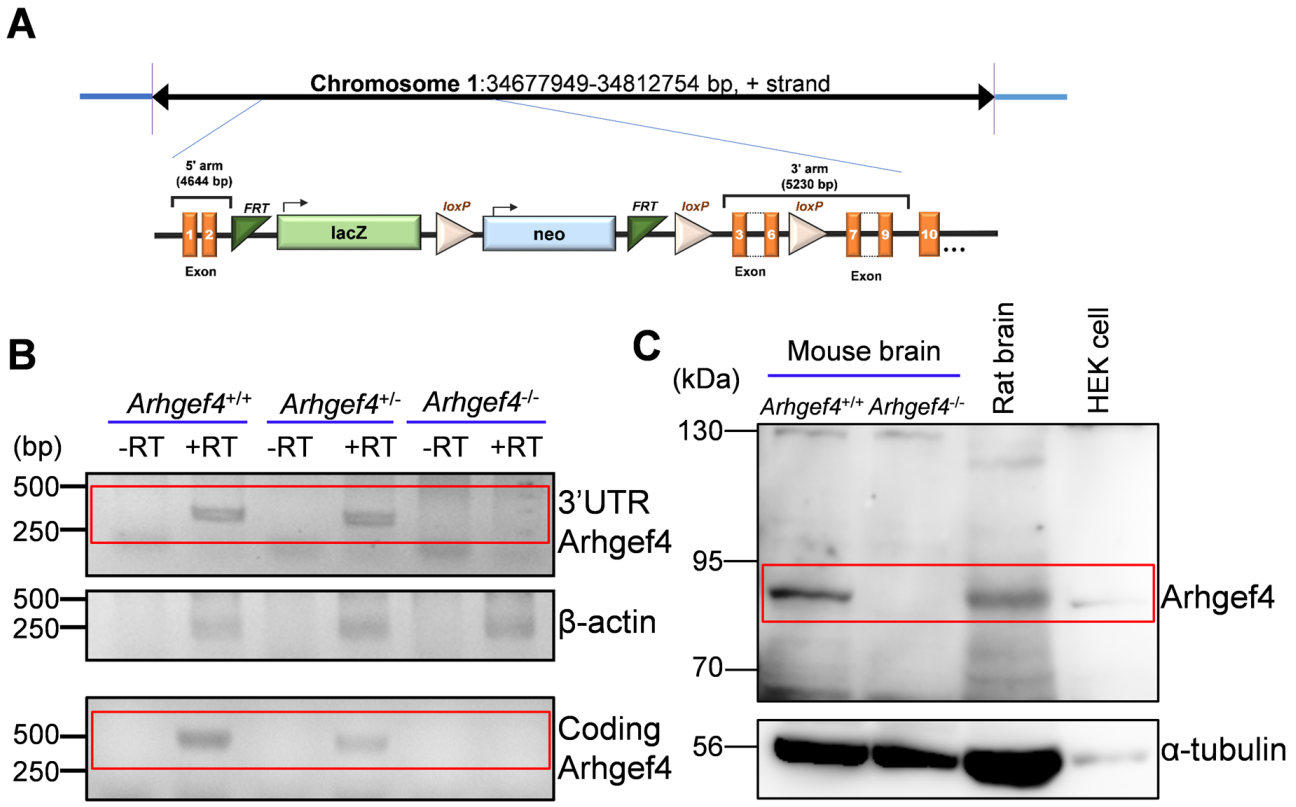

Fig. 1. *Arhgef4* knockout by gene disruption. (A) Chromosomal region of *Arhgef4* gene and diagram of vector for knockout (modified information provided by KOMP). FRT: flippase recognition target, lacZ: β-galactosidase, neo: neomycin, loxP: locus of X-over P1. (B) RT-PCR analysis. Five micrograms of total RNA was transferred to cDNAs of *Arhgef4* and β-actin by reverse transcription. The 3'untranslated regions of their cDNAs near the poly (A) tail or coding regions were amplified by PCR. (C) Forty micrograms of brain lysates from WT mice (*Arhgef4*$^{+/+}$), Homo mice (*Arhgef4*$^{-/-}$), or rats, and 10 μg of human embryonic kidney (HEK) cells were separated by 8% acrylamide gel. The western blotting assay was performed using rabbit polyclonal anti-*Arhgef4* antibodies (specific for N-terminal regions of mouse *Arhgef4*; see Materials and Methods). After the detection of *Arhgef4*, the blots were stripped and subsequently reprobed with anti-α-tubulin antibody.

Table 1. Fold change of *Arhgef4* expression in KO brain

Genotype	Average ΔΔCt (Mean±SD, N=4)	Expression fold change to wild type ($2^{-\Delta\Delta Ct}$)
Arhgef4$^{+/-}$	0.85±0.20	0.555 (0.483~0.637)
Arhgef4$^{-/-}$	4.29±0.46	0.051 (0.037~0.070)

SD, standard deviation.

gef4$^{+/tm1a(KOMP)Wtsi}$, Fig. 1A) [12]. *Arhgef4* deficiency in the brain was examined by performing RT-PCR and western blotting analysis. As shown in Fig. 1B, the *Arhgef4* transcript was not detected in Homo mice brains. We consistently did not detect *Arhgef4* protein in Homo mice brain lysates; whereas *Arhgef4* was detected in the rat brain and HEK cell lysates (Fig. 1C). The quantity of *Arhgef4* transcript in Homo mice was reduced to approximately 0.5% of that in WT mice (Table 1).

Arhgef4 KO homo mice exhibited decreased body weights (data not shown), which is consistent with the data of the International Mouse Phenotyping Consortium[2] (IMPC, www.mousephenotype.org) [22]. PSD-95, a major scaffolding protein in the post-synaptic regions of excitatory synapses, regulates receptors such as NMDA and AMPA and plays a crucial role in experience-dependent plasticity [23, 24]. Our previous study demonstrated that downregulation of *Arhgef4* by RNA interference increases PSD-95 levels in the dendrites, and conversely, overexpression of *Arhgef4* decreases PSD-95 levels [11]. Accordingly, we examined PSD-95 level in hippocampal neurons of Homo mice. The ablation of *Arhgef4* significantly increased the size of PSD-95 particles (Fig. 2C), but not the number of those in the dendrites (data not presented). On the other hand dendritic protrusions significantly decreased in hippocampal neurons of the homozygous mice (Fig. 2D). *Arhgef4* ablation did not change the expression levels of PSD-95 and synaptic proteins, including Homer-1, GluA1, GluN1, or synaptophysin,

[2] https://www.mousephenotype.org/data/genes/MGI:2442507

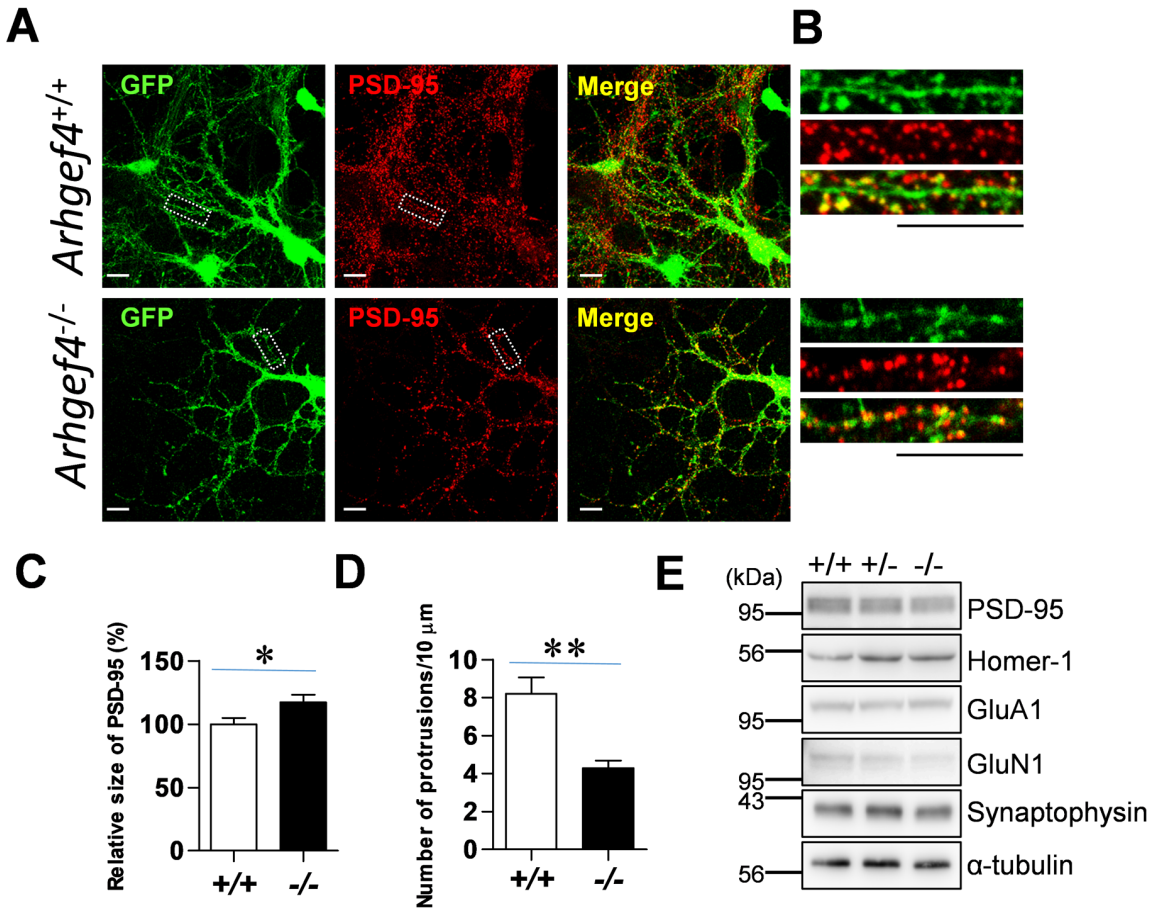

Fig. 2. Lacking *Arhgef4* decreased the number of dendritic protrusions but increased the size of PSD-95 particles. (A) Hippocampal neurons isolated from WT and Homo mice were cultured. Neurons were infected with GFP Sindbis viruses and immunostained with monoclonal PSD-95 antibodies. Scale bar: 10 μm. (B) The selected dendrites were straightened and enlarged. Scale bar: 10 μm. (C) The size of PSD-95 particles was increased in the neurons of *Arhgef4* knockout mice (*Arhgef4*$^{+/+}$: 100.0%±4.93%, N=15 dendrites; *Arhgef4*$^{-/-}$: 117.3%±5.98%, N=14 dendrites, two-tailed unpaired *t*-tests: *p<0.05). (D) The number of dendritic protrusions per 10 μm was decreased in the neurons of *Arhgef4* knockout mice (*Arhgef4*$^{+/+}$: 8.2±0.86, N=15 dendrites; *Arhgef4*$^{-/-}$: 4.3±0.39, N=14 dendrites, two-tailed unpaired *t*-tests: **p<0.01). (E) Ablation of *Arhgef4* gene did not change synaptic protein expression levels, including PSD-95, in the brain. Brain lysates (25 μg of lysate for PSD-95, Homer-1, GluA1, and GluN1, 3 μg of lysate for synaptophysin, α-tubulin) from WT, Hetero, and Homo mice were loaded and subjected to western blotting analysis.

depending on the copy number of the *Arhgef4* gene (Fig. 2E).

General motor activity and anxiety-like behavior in Arhgef4 KO mice were not altered

First, we examined the general locomotive activity of *Arhgef4* KO mice by performing open field tests. The KO mice did not show any significant changes in motor activity, as assessed by distance moved and speed, when compared with WT or Hetero mice (Fig. 3A, B). In addition, the time spent in the center of the open field did not differ among genotypes, suggesting that the anxiety level was not affected by *Arhgef4* deletion (Fig. 3C, D). We further examined anxiety-like behavior by performing elevated plus-maze tests and found no significant difference among genotypes (Fig. 3E, F). Finally, we tested motor skills by performing rotarod tests. Latencies to fall from the rotarod were comparable among genotypes, suggesting that *Arhgef4* deficiency does not affect motor function in the rotarod tests (Fig. 3G, H).

Long-term spatial and recognition memories in Arhgef4 KO mice were enhanced

Given that PSD-95 is a key player in synaptic plasticity, which may underlie learning and memory, we examined the long-term memory of *Arhgef4* KO mice in the OLM and novel ORM tests. OLM is dependent on hippocampal function [20, 25-27], whereas ORM engages several brain regions, including the hippocampus and surrounding cortical regions [20]. Notably, we found that both OLM and ORM test results were significantly enhanced in Homo mice compared to WT mice (Fig. 4), suggesting that both spatial

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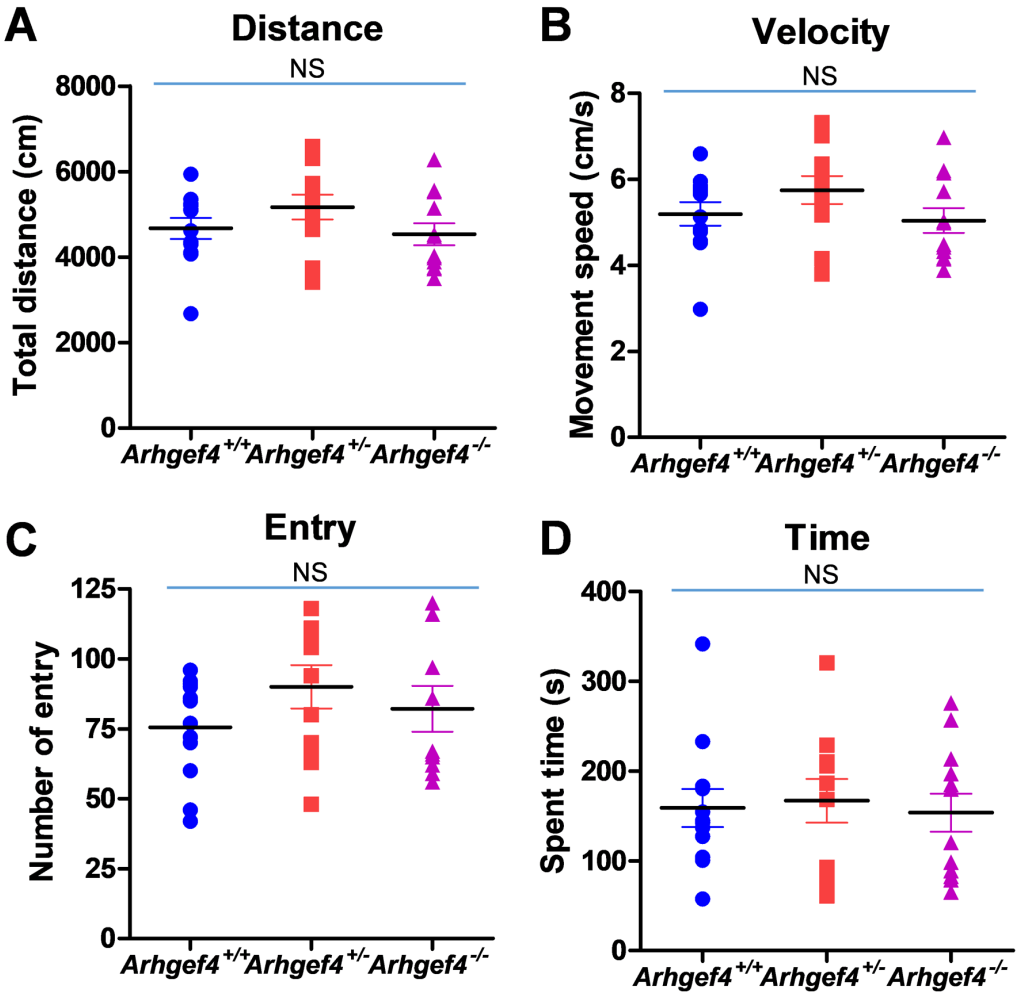

Fig. 3. General motor activity or anxiety-like behavior was not different among genotypes. (A, B) *Arhgef4* knockout did not change the path distance (*Arhgef4*$^{+/+}$: 4,673 cm±245.6 cm, N=12; *Arhgef4*$^{+/-}$: 5,171 cm±292.1 cm, N=11; *Arhgef4*$^{-/-}$: 4,543 cm±257.4 cm, N=12, one-way ANOVA: $F_{2,32}$=1.558, p=0.2261, NS: not significant) and speed of movement (*Arhgef4*$^{+/+}$: 5.19 cm/sec±0.27 cm/sec, N=12; *Arhgef4*$^{+/-}$: 5.75 cm/sec±0.32 cm/sec, N=11; *Arhgef4*$^{-/-}$: 5.04 cm/sec±0.29 cm/sec, N=12, one-way ANOVA: $F_{2,32}$=1.556, p=0.2264 , NS: not significant) in the total area. (C, D) *Arhgef4* knockout did not change the entry number to the central area (*Arhgef4*$^{+/+}$: 75.58±5.24, N=12; *Arhgef4*$^{+/-}$: 90.00±7.76, N=11; *Arhgef4*$^{-/-}$: 82.17±8.17, N=12, one-way ANOVA: $F_{2,32}$=1.003, p=0.3781 , NS: not significant) and the time spent in the central area (*Arhgef4*$^{+/+}$: 158.9 sec±21.06 sec, N=12; *Arhgef4*$^{+/-}$: 167.0 sec±24.38 sec, N=11; *Arhgef4*$^{-/-}$: 153.7 sec±21.24 sec, N=12, one-way ANOVA: $F_{2,32}$=0.0902, p=0.9140 , NS: not significant). (E) *Arhgef4* knockout did not change the percent of entry into open arms (*Arhgef4*$^{+/+}$: 44.80%±5.11%, N=12; *Arhgef4*$^{+/-}$: 41.81%±2.26%, N=11; *Arhgef4*$^{-/-}$: 37.11%±3.57%, N=12, one-way ANOVA: $F_{2,32}$=1.010, p=0.3754, NS: not significant). (F) *Arhgef4* knockout did not change the percent of time spent in open arms (*Arhgef4*$^{+/+}$: 36.69%±4.36%, N=12; *Arhgef4*$^{+/-}$: 33.01%±3.69%, N=11; *Arhgef4*$^{-/-}$: 27.88%±3.25%, N=12, one-way ANOVA: $F_{2,32}$=0.8914, p=0.420, NS: not significant). (G) *Arhgef4* knockout did not change the duration time on the rod before mice fall off (*Arhgef4*$^{+/+}$: 245.4 sec±70.42 sec, N=12; *Arhgef4*$^{+/-}$: 172.5 sec±33.46 sec, N=11; *Arhgef4*$^{-/-}$: 344.4 sec±62.28 sec, N=12, one-way ANOVA: $F_{2,32}$=2.132, p=0.1352, NS: not significant). (H) *Arhgef4* knockout did not change the mean rpm when mice fall off (*Arhgef4*$^{+/+}$: 32.97 rpm±2.22 rpm, N=12; *Arhgef4*$^{+/-}$: 34.33 rpm±1.42 rpm, N=11; *Arhgef4*$^{-/-}$: 37.31 rpm±1.14 rpm, N=12, Kruskal-Wallis test: p=0.1546, NS: not significant).

memories and recognition memories are affected by *Arhgef4* deletion.

DISCUSSION

In this study, we demonstrated that deficiency of *Arhgef4*, a postsynaptic GEF, does not alter general motor activity and anxiety-like behavior (Fig. 3), but significantly improved long-term spatial and recognition memories (Fig. 4). Our previous report suggested that *Arhgef4* could function as a negative synaptic regulator in cultured neurons [11]. The present study confirms the negative regulation of synaptic PSD-95 by *Arhgef4*.

Consistent with previous reports [6, 7], *Arhgef4* was enriched in the brain in our study. In addition, *Arhgef4* showed marginal

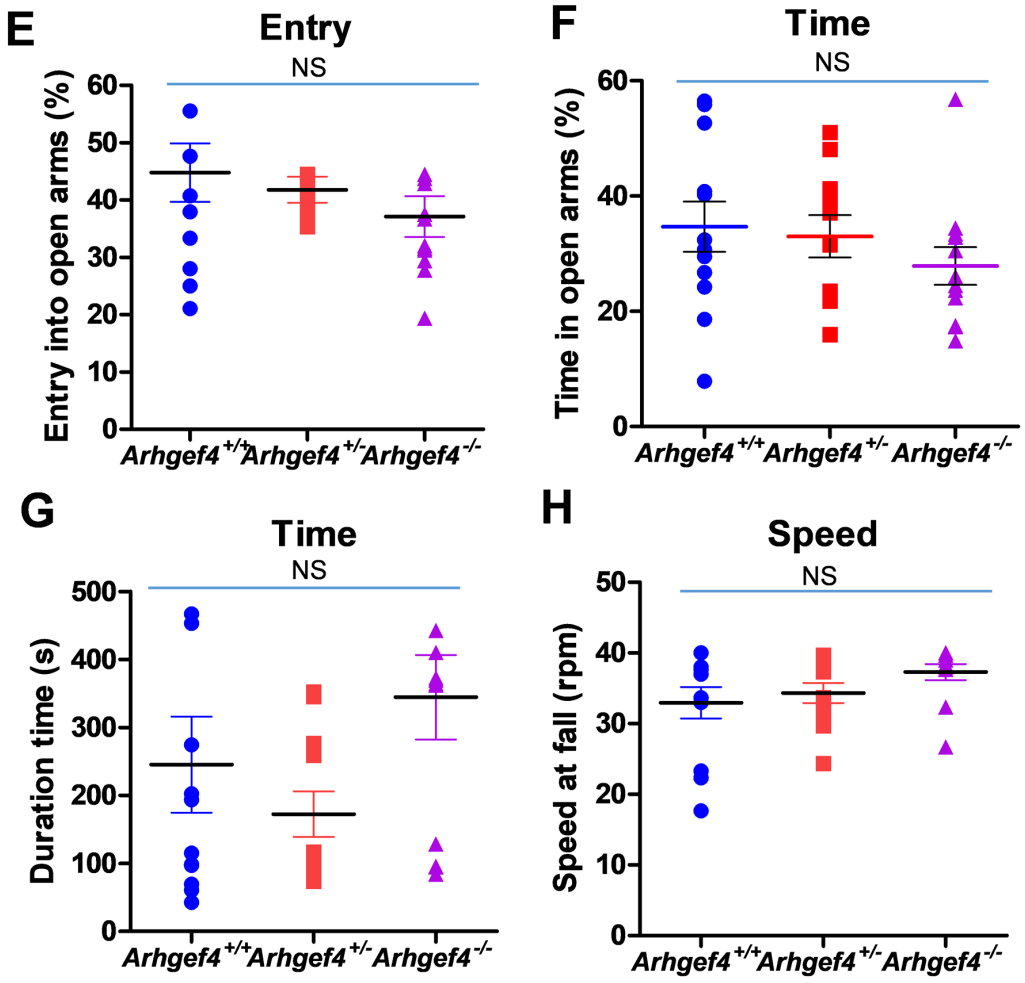

Fig. 3. Continued.

expression in the lung, intestine, and skeletal muscle, but was not detected in the heart and liver by our RT-PCR analysis (data not shown). Our data are also in line with those of the Human Protein Atlas[3] (http://www.proteinatlas.org) [28], which show that *Arhgef4* is highly expressed in the brain and skin. In particular, expression levels of the cerebral cortex and hippocampal formation are higher than in other brain regions. Daily spatial memory such as OLM is dependent on the hippocampus [20, 25-27]. Novel ORM is dependent on some cortex regions such as insular cortex or prefrontal cortex, but only partially on the hippocampus [20]. As indicated by our data, the *Arhgef4* expression pattern is consistent with the specific brain regions associated with memory.

PSD-95, a major scaffolding protein in excitatory synapses interacts with many synaptic proteins including signaling molecules, receptors, and channels, and has a pivotal role in synaptic assembly and function [8, 29]. Moreover, PSD-95 levels at postsynapses in excitatory neurons contribute to a variety of memories in experimental animals [9, 10, 30, 31]. Thus, PSD-95 has been the focus of studies on development and synaptic plasticity. In our data, *Arhgef4* deficiency did not change PSD-95 expression levels (Fig. 2E). This result is consistent with our previous report that *Arhgef4* overexpression can reduce dendritic PSD-95 by sequestering Staufen-containing transporting complexes [11].

Even though there are no effects on viability and mortality, the global *Arhgef4* KOs exhibit hematological defects and smaller body weights (IMPC[4], https://www.mousephenotype.org)[22]. Therefore, we cannot completely exclude any systemic effects on the brain. In order to further consolidate the present results, in-depth analyses of specific brain regions in conditional *Arhgef4* KO mice are required.

In conclusion, *Arhgef4* deficiency enhances long-term memory

[3] https://www.proteinatlas.org/ENSG00000136002-ARHGEF4/tissue
[4] https://www.mousephenotype.org/data/genes/MGI:2442507

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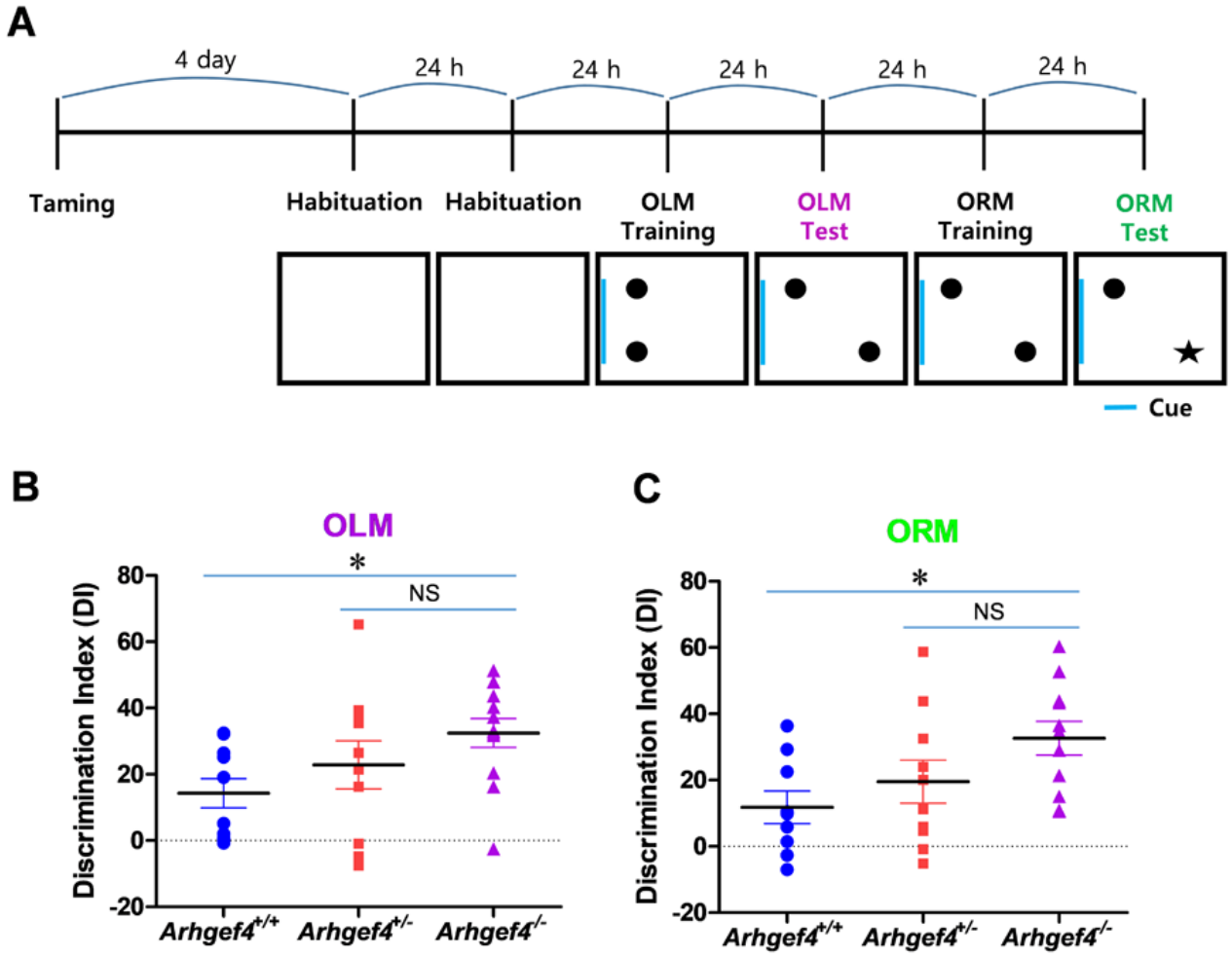

Fig. 4. Object location memory (OLM) and novel object recognition memory (ORM) were increased in *Arhgef4* knockout mice. (A) Experimental time lines for the OLM and ORM tests. (B) OLM increased in *Arhgef4* knockout mice (*Arhgef4*$^{+/+}$: 14.29±4.45, N=10; *Arhgef4*$^{+/-}$: 22.90±7.26, N=10; *Arhgef4*$^{-/-}$: 35.65±3.2, N=11, one-way ANOVA: $F_{2,28}$=4.454, p=0.0209; Dunnett's multiple comparison tests: *p<0.05, NS: not significant). (C) ORM increased in *Arhgef4* knockout mice (*Arhgef4*$^{+/+}$: 11.80±4.91, N=9; *Arhgef4*$^{+/-}$: 19.52±6.51, N=10; *Arhgef4*$^{-/-}$: 32.63±5.08, N=11, one-way ANOVA: $F_{2,27}$=3.608, p=0.0409; Dunnett's multiple comparison tests: *p<0.05, NS: not significant).

through increasing synaptic PSD-95 levels, indicating its role as a negative regulator of synaptic plasticity.

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REFERENCES

1. Kiraly DD, Eipper-Mains JE, Mains RE, Eipper BA (2010) Synaptic plasticity, a symphony in GEF. ACS Chem Neurosci 1:348-365.
2. Miller MB, Yan Y, Eipper BA, Mains RE (2013) Neuronal Rho GEFs in synaptic physiology and behavior. Neuroscientist 19:255-273.
3. Ma XM, Kiraly DD, Gaier ED, Wang Y, Kim EJ, Levine ES, Eipper BA, Mains RE (2008) Kalirin-7 is required for synaptic structure and function. J Neurosci 28:12368-12382.
4. Papadopoulos T, Korte M, Eulenburg V, Kubota H, Retiounskaia M, Harvey RJ, Harvey K, O'Sullivan GA, Laube B, Hülsmann S, Geiger JR, Betz H (2007) Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. EMBO J 26:3888-3899.
5. Kawasaki Y, Senda T, Ishidate T, Koyama R, Morishita T,

Iwayama Y, Higuchi O, Akiyama T (2000) Asef, a link between the tumor suppressor APC and G-protein signaling. Science 289:1194-1197.
6. Hamann MJ, Lubking CM, Luchini DN, Billadeau DD (2007) Asef2 functions as a Cdc42 exchange factor and is stimulated by the release of an autoinhibitory module from a concealed C-terminal activation element. Mol Cell Biol 27:1380-1393.
7. Thiesen S, Kübart S, Ropers HH, Nothwang HG (2000) Isolation of two novel human RhoGEFs, ARHGEF3 and ARHGEF4, in 3p13-21 and 2q22. Biochem Biophys Res Commun 273:364-369.
8. Sheng M, Kim E (2011) The postsynaptic organization of synapses. Cold Spring Harb Perspect Biol 3:a005678.
9. Fitzgerald PJ, Pinard CR, Camp MC, Feyder M, Sah A, Bergstrom HC, Graybeal C, Liu Y, Schlüter OM, Grant SG, Singewald N, Xu W, Holmes A (2015) Durable fear memories require PSD-95. Mol Psychiatry 20:913.
10. Nithianantharajah J, Komiyama NH, McKechanie A, Johnstone M, Blackwood DH, St Clair D, Emes RD, van de Lagemaat LN, Saksida LM, Bussey TJ, Grant SG (2013) Synaptic scaffold evolution generated components of vertebrate cognitive complexity. Nat Neurosci 16:16-24.
11. Oh JY, Lim CS, Yoo KS, Park H, Park YS, Kim EG, Lee YS, Kaang BK, Kim HK (2018) Adenomatous polyposis coli-stimulated GEF 1 (Asef1) is a negative regulator of excitatory synaptic function. J Neurochem 147:595-608.
12. Skarnes WC, Rosen B, West AP, Koutsourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T, Jackson D, Severin J, Biggs P, Fu J, Nefedov M, de Jong PJ, Stewart AF, Bradley A (2011) A conditional knockout resource for the genome-wide study of mouse gene function. Nature 474:337-342.
13. Mouse Genome Informatics (2020) Diagrams of general structure of IKMC primary alleles and derivative alleles [Internet]. The Jackson Laboratory, Bar Harbor. Available from: http://www.informatics.jax.org/mgihome/nomen/IKMC_schematics.shtml.
14. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.
15. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.
16. Yoo KS, Lee K, Oh JY, Lee H, Park H, Park YS, Kim HK (2019) Postsynaptic density protein 95 (PSD-95) is transported by KIF5 to dendritic regions. Mol Brain 12:97.
17. Bailey KR, Crawley JN (2009) Anxiety-related behaviors in mice. In: Methods of behavioral analysis in neuroscience (Buccafusco JJ, ed). 2nd ed. CRC Press, Boca Raton, FL.
18. Komada M, Takao K, Miyakawa T (2008) Elevated plus maze for mice. J Vis Exp (22):1088.
19. Deacon RM (2013) Measuring motor coordination in mice. J Vis Exp (75):e2609.
20. Vogel-Ciernia A, Wood MA (2014) Examining object location and object recognition memory in mice. Curr Protoc Neurosci 69:8.31.1-8.31.17.
21. Lee YS, Ehninger D, Zhou M, Oh JY, Kang M, Kwak C, Ryu HH, Butz D, Araki T, Cai Y, Balaji J, Sano Y, Nam CI, Kim HK, Kaang BK, Burger C, Neel BG, Silva AJ (2014) Mechanism and treatment for learning and memory deficits in mouse models of Noonan syndrome. Nat Neurosci 17:1736-1743.
22. International Mouse Phenotyping Consortium (2020) Gene: Arhgef4 [Internet]. International Mouse Phenotyping Consortium. Available from: https://www.mousephenotype.org/data/genes/MGI:2442507.
23. Xu W (2011) PSD-95-like membrane associated guanylate kinases (PSD-MAGUKs) and synaptic plasticity. Curr Opin Neurobiol 21:306-312.
24. El-Husseini AE, Schnell E, Chetkovich DM, Nicoll RA, Bredt DS (2000) PSD-95 involvement in maturation of excitatory synapses. Science 290:1364-1368.
25. Vogel-Ciernia A, Matheos DP, Barrett RM, Kramár EA, Azzawi S, Chen Y, Magnan CN, Zeller M, Sylvain A, Haettig J, Jia Y, Tran A, Dang R, Post RJ, Chabrier M, Babayan AH, Wu JI, Crabtree GR, Baldi P, Baram TZ, Lynch G, Wood MA (2013) The neuron-specific chromatin regulatory subunit BAF53b is necessary for synaptic plasticity and memory. Nat Neurosci 16:552-561.
26. Barrett RM, Malvaez M, Kramar E, Matheos DP, Arrizon A, Cabrera SM, Lynch G, Greene RW, Wood MA (2011) Hippocampal focal knockout of CBP affects specific histone modifications, long-term potentiation, and long-term memory. Neuropsychopharmacology 36:1545-1556.
27. Haettig J, Stefanko DP, Multani ML, Figueroa DX, McQuown SC, Wood MA (2011) HDAC inhibition modulates hippocampus-dependent long-term memory for object location in a CBP-dependent manner. Learn Mem 18:71-79.
28. The Human Protein Atlas (2020) ARHGEF4 [Internet]. The Human Protein Atlas. Available from: https://www.proteinatlas.org/ENSG00000136002-ARHGEF4/tissue.
29. Won S, Levy JM, Nicoll RA, Roche KW (2017) MAGUKs: multifaceted synaptic organizers. Curr Opin Neurobiol 43:94-101.
30. Migaud M, Charlesworth P, Dempster M, Webster LC,

Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RG, Morrison JH, O'Dell TJ, Grant SG (1998) Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature 396:433-439.

31. Elkobi A, Ehrlich I, Belelovsky K, Barki-Harrington L, Rosenblum K (2008) ERK-dependent PSD-95 induction in the gustatory cortex is necessary for taste learning, but not retrieval. Nat Neurosci 11:1149-1151.