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 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 littersmates. 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
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 (Arhgef4tm1a(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 (Arhgef4tm1a(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 (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’-gggcctgtctgatggatg-3’). Arhgef4 expression was examined on western blots containing 3 μg of brain lysate from each genotyped mouse.

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 (TOPrealTM 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−ΔΔCt 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 B-5-1-2, Sigma-Aldrich, St. Louis, MO, 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)
Ki-Seo Yoo, et al.

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<sup>+/−</sup>). 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-
Arhgef4 Inhibits Memory

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).

![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.](enjournal.org/10.5607/en20049)

Table 1. Fold change of Arhgef4 expression in KO brain

| Genotype | Average ΔΔCt (Mean±SD, N=4) | Expression fold change to wild type (2−ΔΔCt) |
|----------|-----------------------------|--------------------------------------------|
| 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.

Arhgef4 KO homo mice exhibited decreased body weights (data not shown), which is consistent with the data of the International Mouse Phenotyping Consortium2 (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.

2https://www.mousephenotype.org/data/genes/MGI:2442507
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 knockout 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
Arhgef4 Inhibits Memory

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

https://doi.org/10.5607/en20049
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

![Fig. 3. Continued.](https://doi.org/10.5607/en20049)
Arhgef4 Inhibits Memory

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

ACKNOWLEDGEMENTS

This research was supported by grants from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018R1D1A1B07043779) to Hyong Kyu Kim and from the Korea Brain Research Institute (KBRI 20-BR-01-06) to Won-Jong Oh.

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, Retio-unskaia 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 ARH-GEF4, 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 (2000) Isolation of two novel human RhoGEFs, ARHGEF3 and ARH-GEF4, in 3p13-21 and 2q22. Biochem Biophys Res Commun 273:364-369.

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 163:16-24.

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 P, 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 C, 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/datagenes/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, Kramér EA, Azwai S, Chen Y, Magnan CN, Zeller M, Sylvin A, Haettig J, Jia Y, Tran A, Dang R, Post RJ, Chabrier M, Babayan AH, Wu J, 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 M, 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, Beleovsky 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.