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Deficiency in EP4 Receptor—Associated Protein Ameliorates Abnormal Anxiety-Like Behavior and Brain Inflammation in a Mouse Model of Alzheimer Disease

Risako Fujikawa,^{*†} Sei Higuchi,^{*} Masato Nakatsuji,[‡] Mika Yasui,^{*} Taichi Ikedo,^{*§} Manabu Nagata,^{*§} Kosuke Hayashi,^{*§} Masayuki Yokode,^{*} and Manabu Minami^{*}

From the Departments of Clinical Innovative Medicine,^{*} Neurosurgery,[†] and Gastroenterology and Hepatology,[‡] Kyoto University Graduate School of Medicine, Kyoto; and the Japan Society for the Promotion of Science,[§] Tokyo, Japan

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Address correspondence to
Manabu Minami, M.D., Ph.D.,
Department of Clinical Innovative Medicine, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: mminami@kuhp.kyoto-u.ac.jp.

Microglia are thought to play key roles in the progression of Alzheimer disease (AD). Overactivated microglia produce proinflammatory cytokines, such as tumor necrosis factor- α , which appear to contribute to disease progression. Previously, we reported that prostaglandin E₂ type 4 receptor—associated protein (EPRAP) promotes microglial activation. We crossed human amyloid precursor protein transgenic mice from strain J20^{+/-} onto an EPRAP-deficient background to determine the role of EPRAP in AD. Behavioral tests were performed in 5-month-old male J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mice. EPRAP deficiency reversed the reduced anxiety of J20^{+/-} mice but did not affect hyperactivity. No differences in spatial memory were observed between J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mice. In comparison with J20^{+/-}EPRAP^{+/+}, J20^{+/-}EPRAP^{-/-} mice exhibited less microglial accumulation and reductions in the Cd68 and tumor necrosis factor- α mRNAs in the prefrontal cortex and hippocampus. No significant differences were found between the two types of mice in the amount of amyloid- β 40 or 42 in the cortex and hippocampus. J20^{+/-}EPRAP^{-/-} mice reversed the reduced anxiety-like behavior and had reduced microglial activation compared with J20^{+/-}EPRAP^{+/+} mice. Further research is required to identify the role of EPRAP in AD, but our results indicate that EPRAP may be related to behavioral and psychological symptoms of dementia and inflammation in patients with AD. (*Am J Pathol* 2017; 187: 1848–1854; <http://dx.doi.org/10.1016/j.ajpath.2017.04.010>)

Alzheimer disease (AD), which affects approximately 48 million people around the world, results in memory loss, language difficulties, disorientation, mood swings, poor self-care, and behavioral issues that become increasingly severe as the disease advances.^{1,2} Abnormal accumulation of amyloid- β (A β) released from amyloid precursor protein (APP) is a pathologic hallmark of AD. In addition to direct neuronal injury, accumulation of A β also causes indirect injury to neurons by inducing brain inflammation. Microglia, the resident innate immune cells in the brain, are thought to play important roles in a number of neurodegenerative disorders, including AD, with microglial activation increasing as the disease progresses.¹ Although multiple studies have proposed that microglia are legitimate therapeutic targets,² the molecular mechanisms that govern microglial activation remain poorly understood.

Prostaglandin E₂ type 4 (EP4) receptor—associated protein (EPRAP) is a cytoplasmic signaling partner of EP4.³ In mice, EPRAP is also known as Fem1a, an ortholog of *Caenorhabditis elegans* FEM-1 that participates in sex determination.⁴ Recently, a study that involved EPRAP-deficient (EPRAP^{-/-}) mice⁵ found that EPRAP is present in wild-type microglia and that microglial EPRAP promotes inflammation in the brain. In particular, EPRAP deficiency decreases microglial activation in mice treated with lipopolysaccharide or kainic acid. Application of an EP4 agonist to microglia results in increased uptake of A β ,⁶ although

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little is known regarding the contribution of EPRAP in neurodegenerative disorders. In this study, we examined the role of EPRAP in the pathogenesis of AD using AD model h-APPJ20 (J20^{+/-}) EPRAP^{+/+} mice and J20^{+/-}EPRAP^{-/-} compound mutant mice to determine whether EPRAP suppresses microglial activation and A β accumulation.

Materials and Methods

Experimental Animals

EPRAP-deficient (EPRAP^{-/-}) mice on a congenic C57BL/6 background were generated as previously described.⁷ J20^{+/-} mice, which express a mutant form of human APP bearing both the Swedish (K670N/M671L) and the Indiana (V717F) mutations (*APPSwInd*),⁸ as well as wild-type (EPRAP^{+/+}) C57BL/6 mice, were from Charles River Laboratories Japan (Yokohama, Japan). We crossed J20^{+/-} mice onto an EPRAP-deficient background. The genetic background of J20^{+/-} mice is C57BL/6, same as that of EPRAP^{-/-} mice. J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mice were bred as littermates. In one cage, there were four mice: an EPRAP^{+/+} mouse, an EPRAP^{-/-} mouse, a J20^{+/-}EPRAP^{+/+} mouse, and a J20^{+/-}EPRAP^{-/-} mouse. Male mice 5 to 8 months old were used for all experiments. For immunohistologic assessment, murine brains were sliced and stained with antibodies against ionized calcium-binding adaptor molecule (Iba)-1 (Wako Pure Chemical Industries, Osaka, Japan). All experiments and animal care were conducted following the guidelines for the Japan's Act on Welfare and Management of Animals (Act no. 105 of October 1, 1973). These studies were approved by the Institutional Animal Care and Use Committees and the ethics committee of Kyoto University (permit number: MedKyo15183).

Behavioral Tests

Behavioral tests were performed between 9:00 AM and 4:00 PM. Neuromuscular strength was tested using a grip strength meter (O'Hara & Co., Tokyo, Japan). Anxiety was evaluated using a light/dark transition box (O'Hara & Co.) and an elevated plus maze (O'Hara & Co.). Spatial memory was tested using a Barnes maze (O'Hara & Co.). All tests were conducted as previously described.⁹

Total RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). mRNA levels of proinflammatory cytokines in the cerebral cortices were measured. The following primers were used: mouse β -actin, forward 5'-CCTGAGCGCAAGTACTCTGTGT-3' and reverse 5'-GCTGATCCACATCTGCTGGAA-3'; Cd68,

forward 5'-CTTCCCACAGGCAGCACAG-3' and reverse 5'-AATGATGAGAGGCAGCAAGAGG-3'; and tumor necrosis factor (Tnf)- α , forward 5'-CATCTTCTCAAAATTCGAGTGACAA-3' and reverse 5'-TGGGAGTAGACAAGGTACAACCC-3'. All experiments were performed in duplicate, and results were normalized using β -actin as a reference gene.

Enzyme-Linked Immunosorbent Assay

A β 40 and 42 in radioimmunoprecipitation assay lysis buffer—soluble fractions of brain homogenates were quantified using the human A β (1 to 40) and human A β (1 to 42) enzyme-linked immunosorbent assay kits (Wako Pure Chemical Industries).

Statistical Analysis

Results are expressed as means $\pm$ SEM. The *t*-test and the Tukey–Kramer test were used to determine statistically significant differences. *P* < 0.05 was considered statistically significant.

Results

J20^{+/-}EPRAP^{-/-} Mice Are Generally Healthy

The J20^{+/-} mouse, which overexpresses human APP with two mutations linked to familial AD, is one of the most widely used models of AD. Brain inflammation and behavioral abnormalities in J20^{+/-} mice arise by 5 to 7 months of age.¹⁰ We generated J20^{+/-}EPRAP^{-/-} compound mutant mice and assessed their general health at 5 months. J20^{+/-}EPRAP^{-/-} mice exhibited lower body weight than wild-type (EPRAP^{+/+}) mice, although body weight did not differ between J20^{+/-}EPRAP^{-/-} mice and J20^{+/-}EPRAP^{+/+} mice (Figure 1A). No significant differences were observed in grip strength (Figure 1B). J20^{+/-}EPRAP^{-/-} mice exhibited no other abnormalities in body temperature, neuronal alignment in the brain, or other features (data not shown).

EPRAP Deficiency Decreases Abnormal Anxiety-Like Behavior in J20^{+/-} Mice

Several studies reported that J20^{+/-} mice exhibit behavioral and psychological symptoms of dementia (BPSD), including reduced anxiety and increased hyperactivity, similar to other human APP transgenic mice.^{11,12} As previously reported, EPRAP regulates inflammatory activation of microglia, so it could contribute to BPSD associated with brain inflammation in AD.¹³ To determine whether EPRAP is involved in such behavioral abnormalities, we assessed anxiety-like behavior and motor activity in EPRAP^{+/+}, EPRAP^{-/-}, J20^{+/-}EPRAP^{+/+}, and J20^{+/-}EPRAP^{-/-} mice using the light/dark transition test and elevated plus maze,

Figure 1 Prostaglandin E₂ type 4 receptor–associated protein (EPRAP) deficiency does not affect general health in J20^{+/-} mice. **A:** Body weight. **B:** Neuromuscular strength, as evaluated using the grip strength test. Data are expressed as means $\pm$ SEM. $n = 10$ for each group. * $P < 0.05$. EPRAP^{-/-}, EPRAP knockout; EPRAP^{+/+}, wild-type EPRAP; J20^{+/-}EPRAP^{+/+}, J20 transgenic with wild-type EPRAP; J20^{+/-}EPRAP^{-/-}, J20 transgenic with EPRAP knockout.

which are the tests most commonly used for this purpose in mice.

The light/dark transition test is based on the natural aversion of mice to brightly illuminated areas: anxious mice spend less time in the light box. In this study, J20^{+/-}EPRAP^{+/+} mice tended to remain in the light compartment longer than EPRAP^{+/+} and EPRAP^{-/-} mice (Figure 2A), indicating lower levels of anxiety. By contrast, stay time in the light box was significantly reduced in J20^{+/-}EPRAP^{-/-} mice relative to J20^{+/-}EPRAP^{+/+} mice, comparable to the stay times of EPRAP^{+/+} and EPRAP^{-/-} mice (Figure 2A). Next, we measured total distance traveled in the light/dark transition test to assess levels of motor activity in each group. Both the J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mouse groups exhibited hyperactivity (Figure 2B).

The elevated plus maze test is based on the natural aversion of mice for open and elevated areas, with anxious mice spending less time on the open arm than nonanxious mice. J20^{+/-}EPRAP^{+/+} mice stayed on the open arm longer than the EPRAP^{+/+} and EPRAP^{-/-} mice (Figure 2C), indicating lower levels of anxiety. No significant differences were observed between J20^{+/-}EPRAP^{-/-} mice and the remaining groups with regard to performance in this test

(Figure 2C), whereas both J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mice traveled greater distances, indicating hyperactivity (Figure 2D). These results suggest that EPRAP deficiency reversed the reduced anxiety but did not affect hyperactivity in J20^{+/-} mice.

EPRAP Deficiency Does Not Affect Spatial Memory Deficit in J20^{+/-} Mice

J20^{+/-} mice exhibit increasing deficits in spatial memory as they age.^{14,15} In this study, we assessed spatial memory using the Barnes maze. During training, we observed no differences in the learning patterns of J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mice (data not shown). Spatial memory was assessed 1 week (probe test 1) and 1 month (probe test 2) after the last training to investigate the role of EPRAP in short- and long-term memory, respectively. After the platform was removed, mice were allowed to explore the maze board. Time spent in the correct hole during probe tests 1 and 2 tended to decrease for both J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mice relative to EPRAP^{+/+} mice (Figure 2, E and F). No significant differences in time spent around the correct hole during probe tests 1 and 2 were observed between J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mice (Figure 2, E and F). These results suggest that EPRAP deficiency did not prevent spatial memory deficits in J20^{+/-} mice.

Microglial Activation Is Suppressed in J20^{+/-}EPRAP^{-/-} Mice Relative to J20^{+/-}EPRAP^{+/+}

To evaluate the role of EPRAP in the pathophysiology of AD, we examined microglial activation in J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mice. J20^{+/-}EPRAP^{-/-} mice exhibited less accumulation of Iba1-positive microglia in the cortex and hippocampus than J20^{+/-}EPRAP^{+/+} mice (Figure 3, A and B). Quantitative RT-PCR analysis revealed reduced expression of Cd68 and Tnf- α mRNAs in the cortex and the hippocampus of J20^{+/-}EPRAP^{-/-} mice (Figure 3C), suggesting that EPRAP may promote inflammation in AD.

EPRAP Deficiency Does Not Affect the Deposition of A β

Deposition of A β in the brain results in neuronal injury and activation of microglia, important components of the cascade involved in pathogenic progression of AD.¹ J20^{+/-} mice exhibit diffuse A β plaque deposition in the cortex and hippocampus at 5 to 7 months.¹⁶ To clarify the role of EPRAP in the production or clearance of A β , we measured A β using enzyme-linked immunosorbent assay. No significant difference in the amount of A β 40 and A β 42 in the cortex or hippocampus was observed between J20^{+/-}EPRAP^{+/+} and J20^{+/-}EPRAP^{-/-} mice (Figure 4), suggesting that EPRAP exerts little influence on production or clearance of A β .

Figure 2 Prostaglandin E₂ type 4 receptor-associated protein (EPRAP) deficiency decreases abnormal anxiety-like behavior but does not affect spatial memory impairment in J20^{+/-} mice. **A and B:** Light–dark transition test. J20^{+/-} EPRAP^{+/+} mice tend to remain in the light compartment longer than EPRAP^{+/+} mice, although no significant difference in light-compartment time is observed between J20^{+/-} EPRAP^{-/-} and EPRAP^{-/-} mice (**A**). Total distance traveled is higher in J20^{+/-} EPRAP^{+/+} and J20^{+/-} EPRAP^{-/-} mice than in other mouse groups (**B**). **C and D:** Elevated plus maze test. J20^{+/-} EPRAP^{+/+} mice remain on the open arm longer than both EPRAP^{+/+} and EPRAP^{-/-} mice. No difference is observed between J20^{+/-} EPRAP^{-/-} mice and the remaining groups (**C**). Total distance traveled is higher in J20^{+/-} EPRAP^{+/+} and J20^{+/-} EPRAP^{-/-} mice than in other mouse groups (**D**). **E and F:** Barnes maze test. Time around the correct hole during probe test 1 (1 week after initial training) (**E**). Time around the correct hole during probe test 2 (1 month after initial training) (**F**). Data are expressed as means ± SEM. *n* = 10 for each group. *P* = 0.07 in EPRAP^{+/+} versus J20^{+/-} EPRAP^{+/+}; *P* = 0.06 in EPRAP^{-/-} and J20^{+/-} EPRAP^{+/+}; and *P* = 0.07 in EPRAP^{+/+} versus J20^{+/-} EPRAP^{+/+} and EPRAP^{+/+} versus J20^{+/-} EPRAP^{-/-}. **P* < 0.05, ***P* < 0.01. EPRAP^{-/-}, EPRAP knockout; EPRAP^{+/+}, wild-type EPRAP; J20^{+/-} EPRAP^{+/+}, J20 transgenic with wild-type EPRAP; J20^{+/-} EPRAP^{-/-}, J20 transgenic with EPRAP knockout.

Discussion

Microglia are thought to play key roles in the pathophysiology and progression of a number of neurodegenerative disorders, including AD, and the number of patients with AD is projected to nearly triple between 2010 and 2050.¹⁷ Therefore, a more detailed understanding of the mechanisms underlying microglial activation is important for the development of new therapies for AD.

Elevated levels of TNF- α have been detected in the plasma and brains of patients with AD.¹⁸ TNF- α stimulation of neuronal cells leads to increased inducible nitric oxide synthase expression and subsequent apoptosis.^{19,20} Previously, we reported that EPRAP promotes induction of inflammation by lipopolysaccharide via regulation of the MKK4–JNK pathways in microglia.⁵ Like lipopolysaccharide, A β binds directly to microglia through cell-surface receptors, such as Toll-like receptor 4.²¹ *In vitro* analyses have revealed that microglia treated with A β increase phosphorylation levels of JNK, as well as production of TNF- α .²²

Patients with AD exhibit not only memory dysfunction but also BPSD, including abnormal levels of anxiety.²³ Patients with AD typically have increased anxiety but sometimes also have the opposite tendency of

disinhibition.²⁴ Antipsychotics and antidepressants have been recommended for the treatment of BPSD,²⁵ although a recent warning by the US Food and Drug Administration regarding the elevated mortality rate of older adults taking atypical antipsychotics necessitates the development of alternative therapies. The underlying mechanism of BPSD and memory deficits may differ. Overactivated microglia may contribute to abnormal anxiety-like behavior. Several studies have observed a correlation between BPSD and cytokine levels in the cerebrospinal fluid of patients with AD.¹³ The serotonin system in the prefrontal cortex plays an important role in the development of anxiety. Previous *in vitro* studies have revealed that TNF- α may increase activity of the serotonin transporter—a protein involved in the termination of serotonergic signaling—via activation of p38 mitogen-activated protein kinase.²⁶ Our data suggest that EPRAP deficiency may alleviate abnormal anxiety in AD by suppressing microglial activation and production of TNF- α .

Activation of microglia by A β promotes disease progression; however, A β itself also damages neurons.²⁷ Elevated levels of A β lead to a reduction in long-term potentiation in the hippocampus, the most critical area for spatial memory.²⁸ Moreover, research using neuronal cell

Figure 3 Prostaglandin E₂ type 4 receptor-associated protein (EPRAP) deficiency decreases microglial activation and proinflammatory cytokine expression in the brains of J20^{+/+} mice. **A** and **B**: The number of ionized calcium-binding adaptor molecule (Iba)-1 positive microglial cells is lower in the brains of J20^{+/+}-EPRAP^{-/-} mice than in the brains of J20^{+/+}-EPRAP^{+/+} mice. Prefrontal cortex (**A**). Hippocampus (**B**). **C**: Levels of Cd68 and tumor necrosis factor (Tnf)-α mRNAs in the prefrontal cortex of J20^{+/+}-EPRAP^{+/+} and J20^{+/+}-EPRAP^{-/-} mice. The difference in CD68 mRNA levels in the cortex between J20^{+/+}-EPRAP^{+/+} and J20^{+/+}-EPRAP^{-/-} mice is not significant. Data are expressed as means ± SEM. *n* = 6 for each group (**C**). *P* = 0.07. **P* < 0.05, ***P* < 0.01. Scale bars = 50 μm (**A** and **B**). EPRAP^{-/-}, EPRAP knockout; EPRAP^{+/+}, wild-type EPRAP; J20^{+/+}-EPRAP^{+/+}, J20 transgenic with wild-type EPRAP; J20^{+/+}-EPRAP^{-/-}, J20 transgenic with EPRAP knockout.

cultures has revealed that Aβ induces apoptosis of neuronal cells via oxidative stress.²⁹ Further reductions in Aβ would therefore be required to protect the hippocampal neurons that store spatial memory.

Previous studies have revealed that administration of an EP4 agonist increased microglial uptake of Aβ.⁶ In this study, however, EPRAP deficiency did not affect the amount of Aβ, suggesting that this factor does not contribute to the EP4 signal associated with Aβ clearance in microglia. EPRAP

was identified as a novel protein associated with EP4 in macrophages,³ although our recent research suggests that the proinflammatory effects of EPRAP in microglia occur independent of PGE₂–EP4 signaling.⁵ Furthermore, EPRAP is present throughout the whole cytoplasm in microglia,⁵ although EP4 localizes in a punctate perinuclear area in microglia.³⁰ Indeed, the results of this study further support the notion that microglial EPRAP function is not associated with EP4 signaling in AD.

Drugs such as acetylcholinesterase inhibitors can improve cognitive function in patients with AD, although such treatments cannot cure the disease.³¹ On the basis of the Aβ cascade hypothesis, treatments have been developed that use γ-secretase inhibitors and antibodies against Aβ peptide; however, clinical trials of these drugs have been wholly unsuccessful.^{32,33} Therefore, further research regarding the pathogenesis of AD, not limited to suppressing Aβ, is urgently required. The results of this study suggest that EPRAP deficiency ameliorates brain inflammation and BPSD in patients with AD. The molecular mechanisms of BPSD are less well understood than those of the memory deficit in AD; however, treatment of BPSD is essential for improving quality of life for patients, decreasing the burden on caregivers, and minimizing medical expense. Further study is required to clarify the mechanisms underlying microglial activation via the EPRAP-related signaling pathway and to develop advanced therapies for the treatment of BPSD in patients with AD.

Figure 4 Prostaglandin E₂ type 4 receptor-associated protein (EPRAP) deficiency does not affect the amount of amyloid β (Aβ). No significant differences in Aβ levels were observed in the cortex (**A**) or hippocampus (**B**). Data are expressed as means ± SEM. *n* = 6 for each group. EPRAP^{-/-}, EPRAP knockout; EPRAP^{+/+}, wild-type EPRAP; J20^{+/+}-EPRAP^{+/+}, J20 transgenic with wild-type EPRAP; J20^{+/+}-EPRAP^{-/-}, J20 transgenic with EPRAP knockout.

In summary, EPRAP deficiency suppresses microglial activation and reverses the reduced anxiety in J20^{+/-} mice. The reduced anxiety seen in J20 mice is not clearly compatible with human AD, which is typically associated with increased anxiety; however, our results suggest that EPRAP may be related to the pathogenesis of AD. Further investigation may elucidate the role of EPRAP in the development and progression of human neurodegenerative diseases, as well as other health conditions.

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