GluN2 subunit [10,11]. Previous studies have found that Tyr-1325 and Tyr-1472 are the principal tyrosine phosphorylation sites on the GluN2A and the GluN2B subunits, respectively [12,13]. Genetically engineered mice expressing the Y1325F mutation of GluN2A show antidepressant-like behavior, but their other neural functions, such as hippocampal-dependent learning, are normal [12]. Alternatively, mice expressing the Y1472F mutation of GluN2B show a selective impairment in amygdala-dependent fear-learning [13]. Considering the versatile role of the NMDA receptor in various neural functions [6], the phenotypes of these mutant mice are milder than expected: thus these mice provide valuable models in which to dissect the molecular basis of specific behaviors including anxiety-like behavior.

Corticotropin-releasing factor (CRF), which is highly abundant in the amygdala as well as in the paraventricular nucleus of the hypothalamus, plays an important role in regulating anxiety-like behavior [14]. Patients suffering from anxiety disorders often have increased CRF levels in their cerebrospinal fluid [15,16]. In rodents, intracerebro-ventricular delivery of CRF is anxiogenic [17]. Likewise, transgenic mice overexpressing CRF exhibit increased anxiety-like behavior [18]. Conversely, CRF_1 receptor knockout mice have reduced anxiety [17]. Injection of CRF antagonists or CRF_1 receptor antisense oligonucleotide into the amygdala reduces stress-induced anxiety-like behavior [19,20]. These results collectively show that CRF plays a key role in the regulation of anxiety-like behavior particularly in the amygdala. Therefore understanding the molecular mechanism of the regulation of CRF expression in the amygdala is important.

In the present study, using behavioral, pharmacological, and biochemical approaches with knock-in mice in which the Tyr-1472 of GluN2B is mutated to phenylalanine (GluN2B-YF), we have identified Tyr-1472 phosphorylation as a regulator of CRF mRNA expression and anxiety-like behavior.

Results

Enhanced anxiety-like behavior of GluN2B-YF mice

Given that we previously found that GluN2B-YF mice show a selective impairment in amygdala-dependent learning [13], we evaluated amygdala-dependent anxiety-like behavior in GluN2B-YF mice using the elevated plus-maze (EPM) test, one of the most popular behavioral tests for research on anxiety [21]. The measures of anxiety are the percentage of time spent in the open arms and the percentage of open arm entries. In the test, GluN2B-YF mice spent less time in the open arms than wild-type (WT) mice (time in open arms: WT, 41.5 ± 3.9%, n = 28; YF, 29.0 ± 3.5%, n = 31; $F_{(1,57)}$ = 5.516, $p < 0.05$, one-way ANOVA) (Figure 1A). We also

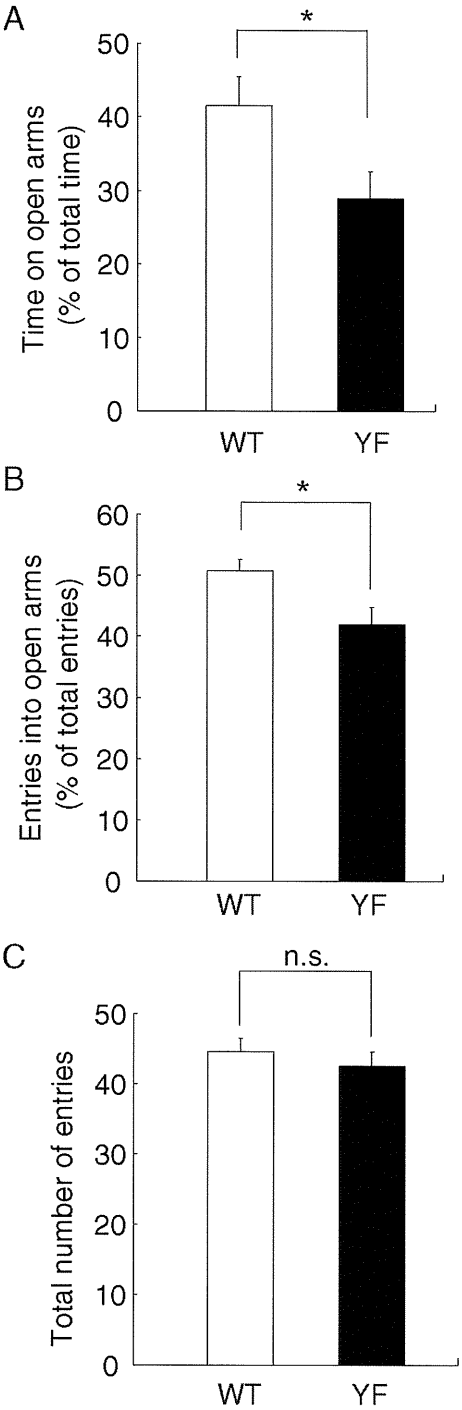


Figure 1 Enhanced anxiety-like behavior of GluN2B-YF mice in the elevated plus-maze test. (A), (B) GluN2B-YF mice spent less time exploring the open arms (WT, n = 28; YF, n = 31; $F_{(1,57)}$ = 5.516, $p < 0.05$, one-way ANOVA) (A) and made fewer entries into the open arms (WT, n = 28; YF, n = 31; $F_{(1,57)}$ = 6.908, $p < 0.05$, one-way ANOVA) (B) during the elevated plus-maze test. **(C)** Total number of entries of GluN2B-YF mice into the open and closed arms was not significantly different from that of WT mice (WT, n = 28; YF, n = 31; $F_{(1,57)}$ = 0.490, $p > 0.4$, one-way ANOVA). The asterisk indicates significant genotype differences. n.s., not significant.

found that GluN2B-YF mice showed a clear preference for closed arms (percentage of entries into open arms: WT, 50.8 ± 1.8%, n = 28; YF, 41.9 ± 2.7%, n = 31; $F_{(1,57)}$ = 6.908, $p < 0.05$, one-way ANOVA) (Figure 1B). There was no significant difference in the number of total arm entries between GluN2B-YF and WT mice (number of total entries: WT, 44.6 ± 1.9, n = 28; YF, 42.6 ± 2.0, n = 31; $F_{(1,57)}$ = 0.490, $p > 0.4$, one-way ANOVA), suggesting that the locomotor activity of the GluN2B-YF mice was unchanged compared to that of WT mice (Figure 1C). Together with the findings that spontaneous activity of GluN2B-YF mice in the open field test was virtually normal compared to that of the WT mice (data not shown), these results suggest that reduced open arm activity in GluN2B-YF mice is due to increased anxiety rather than motor impairment.

Increased CRF expression in the amygdala of GluN2B-YF mice

Several neurochemical systems are implicated in anxiety-related behavior in mice [3,22,23]. Among them, we focused on CRF-mediated signaling because many studies have reported that CRF is involved in the fear-potentiated EPM behavior [21]. Relative to wild-type controls, we found, by real-time PCR, that GluN2B-YF mice had markedly increased levels of CRF mRNA in the amygdala, which regulates the behavioral systems involved in the fear response (WT, 100.0 ± 7.3%, n = 5; YF, 175.3 ± 41.5%, n = 5; $p < 0.05$, Student's t-test) (Figure 2A). Interestingly, slight changes in the levels of CRF mRNA in other brain regions, including the hippocampus, cerebellum, and hypothalamus were not significantly different (hippocampus: WT, 100.0 ± 10.3%, n = 5; YF, 115.7 ± 19.5%, n = 5; $p > 0.2$, Student's t-test; cerebellum: WT, 100.0 ± 25.2%, n = 5; YF, 145.7 ± 70.1%, n = 5; $p > 0.2$, Student's t-test; hypothalamus: WT, 100.0 ± 9.7%, n = 5; YF, 103.8 ± 16.1%, n = 5; $p > 0.5$, Student's t-test) (Figure 2B-D). To confirm the increased CRF levels in the amygdala, we performed an ELISA and found that CRF was indeed increased in the amygdalae of GluN2B-YF mice (WT, 100.0 ± 4.4%, n = 10; YF, 120.4 ± 5.0%, n = 10; $p < 0.01$, Student's t-test) (Figure 2E). As expected, there was no significant difference in the CRF levels determined by ELISA in other brain region such as hippocampus between GluN2B-YF and WT mice (CRF levels in the hippocampus: WT, 100.0 ± 6.9, n = 10; YF, 105.3 ± 7.0, n = 10; $p > 0.5$, Student's t-test). In addition, given that CRF plays a key role in hypothalamus-pituitary-adrenal (HPA) axis activation [14], we examined the HPA axis-regulated plasma ACTH levels by ELISA. We found that the plasma ACTH level of GluN2B-YF mice was similar to that of wild-type mice (WT, 419.5 ± 67 pg/ml (100.0 ± 16.0%), n = 9; YF, 364.7 pg/ml (86.9 ± 13.2%), n = 13; $p > 0.5$, Student's t-test) (Figure 2F). This result suggests that the function of HPA axis is normal in GluN2B-YF mice. Thus, it appears that CRF expression is increased in the amygdala but not in other brain regions of GluN2B-YF mice.

Attenuation of enhanced anxiety-like behavior of GluN2B-YF mice by CRF receptor antagonist

In the amygdala, CRF modulates anxiety-like behavior by binding CRF_1 receptor [16]. If the enhanced anxiety-like behavior of GluN2B-YF mice were due to increased CRF expression, blockade of the CRF_1 receptor might attenuate the enhanced anxiety-like behavior of GluN2B-YF mice. To examine this possibility, we performed the EPM test after injecting mice with the CRF_1 receptor selective antagonist NBI 27914. There was a significant interaction between genotype and NBI 27914-treatment for the time spent on open arms ($F_{(1,37)}$ = 8.30, p = 0.0066, two-way ANOVA). Tukey's post-hoc test revealed that vehicle-injected GluN2B-YF mice showed enhanced anxiety-like behavior relative to vehicle-injected WT mice (time on open arms: WT, 32.7 ± 4.5%, n = 10; YF, 21.3 ± 2.6%, n = 12; $F_{(1,37)}$ = 7.12, $p < 0.05$, two-way ANOVA/Tukey's post-hoc test) (Figure 3). In contrast to the vehicle-injection, we found that the enhanced anxiety-like behavior exhibited by GluN2B-YF mice was attenuated by acute intraperitoneal injection of NBI 27914 (time on open arms: WT, 25.8 ± 2.6%, n = 8; YF, 36.5 ± 7.4%, n = 9; $F_{(1,37)}$ = 2.31, $p > 0.1$, two-way ANOVA/Tukey's post-hoc test) (Figure 3). These results argue that activated CRF_1 receptor-mediated signaling causes the enhanced anxiety-like behavior of GluN2B-YF mice. In addition, in contrast to the case seen in Figure 1, slight decrease in the number of entries into open arms of vehicle-injected GluN2B-YF mice was not significantly different (vehicle-injected WT, 48.5 ± 3.6%, n = 10; vehicle-injected YF, 40.1 ± 2.5%, n = 12; p = 0.08, two-way ANOVA/Tukey's post-hoc test; NBI 27914-injected WT, 40.4 ± 5.0%, n = 8; NBI 27914-injected YF, 42.8 ± 4.9%, n = 9; p = 0.68, two-way ANOVA/Tukey's post-hoc test), probably because of injection stress [19].

De-phosphorylation of Tyr-1472 and increased CRF expression in the amygdala of wild-type mice after the EPM test

We next investigated whether the level of Tyr-1472 phosphorylation in the amygdala of wild-type mice was affected by the EPM test. At 10 min after the test, the amygdala was resected, and the total lysates and RNAs were prepared. As shown in Figure 4A, the level of Tyr-1472 phosphorylation was significantly decreased in the amygdala of wild-type mice exposed to the EPM test compared with control wild-type mice (control: 100.0 ± 8.5%, n = 6;

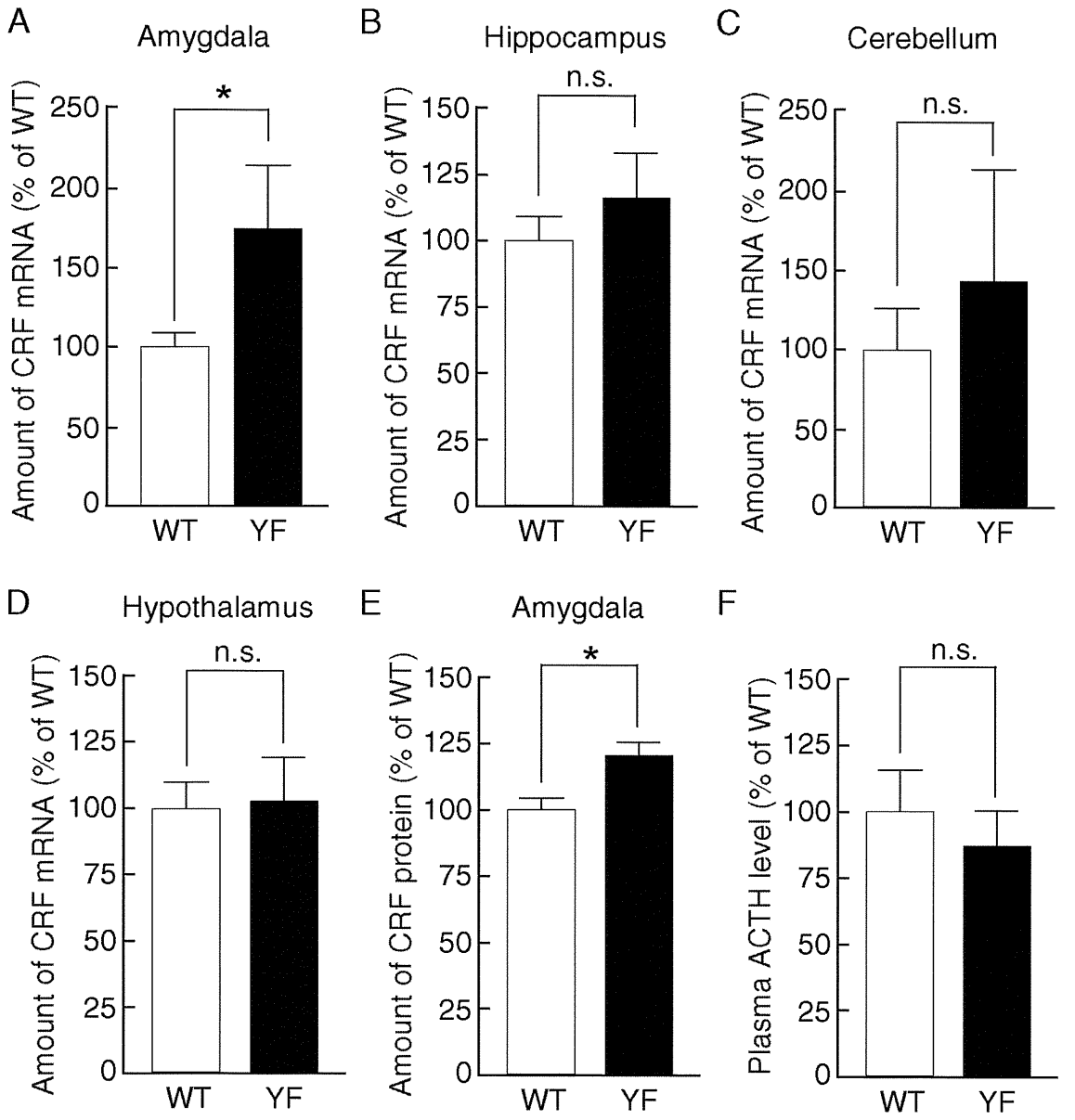


Figure 2 Increased CRF levels in the amygdala but not in other brain regions in GluN2B-YF mice. (A)-(D) Real-time PCR analyses showed that CRF mRNA levels were increased in the amygdala (WT, $n = 5$; YF, $n = 5$; $p < 0.05$, Student's t-test) (A) but not in other brain regions, including the hippocampus (WT, $n = 5$; YF, $n = 5$; $p > 0.2$, Student's t-test) (B), cerebellum (WT, $n = 5$; YF, $n = 5$; $p > 0.2$, Student's t-test) (C), and hypothalamus (WT, $n = 5$; YF, $n = 5$; $p > 0.5$, Student's t-test) (D), of GluN2B-YF mice compared to WT mice. **(E)** Increased expression of CRF in the amygdala was confirmed by ELISA (WT, $n = 10$; YF, $n = 10$; $p < 0.01$, Student's t-test). **(F)** The plasma ACTH level of GluN2B-YF mice was not significantly different from that of WT mice (WT, $n = 9$; YF, $n = 13$; $p > 0.5$, Student's t-test). The asterisk indicates significant genotype differences. n.s., not significant.

EPM test: 85.1 ± 5.3%, $n = 4$; $p < 0.05$, Student's t-test). Interestingly, the level of CRF mRNA in the amygdala was increased by the EPM test (control: 100.0 ± 24.3%, $n = 6$; EPM test: 168.7 ± 17.3%, $n = 4$; $p < 0.05$, Student's t-test) (Figure 4B). These data further support that Tyr-1472 phosphorylation is relevant to the anxiety-like behavior and negative regulation of CRF expression in the amygdala.

Induction of de-phosphorylation of Tyr-1472 and increased CRF expression by NMDA receptor stimulation

We then examined whether NMDA receptor activity is involved in the regulation of CRF expression in the amygdala. Coronal sections including the amygdala were stimulated with 100 μM NMDA for 7 min, followed by a wash-out period of 15 min. Then, the amygdala was rapidly dissected out and RNA was isolated to examine

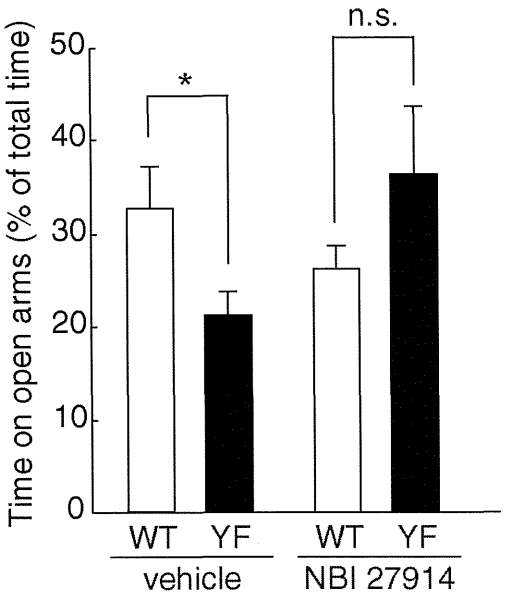


Figure 3 Abrogation of increased anxiety-related behavior of GluN2B-YF mice by intraperitoneal injection of the CRF_1 receptor antagonist NBI 27914. The reduced time spent in the open arms shown by GluN2B-YF mice in the elevated plus-maze test (WT, n = 10; YF, n = 12; $F_{(1,37)}$ = 7.12, $p < 0.05$, two-way ANOVA/Tukey's post-hoc test) was attenuated by intraperitoneal injection of NBI 27914 (WT, n = 8; YF, n = 9; $F_{(1,37)}$ = 2.31, $p > 0.1$, two-way ANOVA/Tukey's post-hoc test). The asterisk indicates significant differences. n.s., not significant.

the level of CRF mRNA expression (Figure 4C). We found that NMDA receptor stimulation increased CRF mRNA expression in wild-type slices (control: 100.0 ± 10.5%, n = 6; NMDA stimulation: 160.1 ± 20.3%, n = 6; $p < 0.05$, Student's t-test). Interestingly, the same stimulation also induced de-phosphorylation of GluN2B Tyr-1472 (the level of Tyr-1472 phosphorylation: control, 100.0 ± 5.8%, n = 5; NMDA stimulation, 42.5 ± 3.1%, n = 5; $p < 0.05$, Student's t-test) (Figure 4D). In contrast to wild-type slices, CRF mRNA expression levels in the slices from GluN2B-YF mice were virtually unchanged by the same NMDA stimulation (control: 153.3 ± 20.5%, n = 6; NMDA stimulation: 143.1 ± 7.5%, n = 6; $p > 0.2$, Student's t-test) (Figure 4C). Thus, NMDA receptor stimulation is a likely trigger for increased CRF mRNA expression through de-phosphorylation of Tyr-1472 in the amygdala.

Discussion

In this study, we showed that Tyr-1472 phosphorylation of GluN2B is a negative regulator of CRF mRNA expression in the amygdala. Behaviorally, deficient Tyr-1472 phosphorylation leads to enhanced anxiety-like behavior, which is consistent with enhanced CRF signaling [14,17,18]. We further demonstrated that acute intraperitoneal injection of NBI 27914, a selective CRF_1 receptor antagonist, attenuated the anxiety-like behavior of GluN2B-YF mice. Given the established role of CRF in anxiety [14,16,17], it is likely that the enhanced anxiety phenotype observed in GluN2B-YF mice is linked to increased CRF expression in the amygdala.

Considering that Tyr-1472 phosphorylation is required for the NMDA receptor-mediated signaling [13], the present finding that GluN2B-YF mice exhibit increased anxiety-like behavior is consistent with previous pharmacological and genetic studies in rodents showing the anxiolytic-like effects of NMDA receptor blockade [24,25]. Strikingly, intra-amygdala injection of an NMDA receptor antagonist, MK-801, prevents stress-induced increases in anxiety-like behavior in the EPM test [25], suggesting that NMDA receptors, especially in the amygdala, play a key role in regulating anxiety-like behavior. Interestingly, we found that the level of CRF was markedly increased in the amygdala of GluN2B-YF mice but not in other brain regions such as the hippocampus, cerebellum, and hypothalamus (Figure 2B-D). Thus, the Tyr-1472 phosphorylation-dependent regulation of NMDA receptors in the amygdala is likely to be responsible for anxiety-like behavior. Tissue specific conditional GluN2B-YF mice would be useful for analyzing the differential roles of Tyr-1472 phosphorylation.

CRF in the amygdala contributes to anxiety because injection of CRF antagonists or CRF_1 receptor antisense oligonucleotides into the amygdala reduces anxiety-like behavior in rats [19,20]. In amygdala-derived neuronal cultures, NMDA receptor stimulation induces CRF release [26], suggesting that a functional NMDA receptor system regulates CRF signaling in the amygdala. In this study, we found that the YF mutation in the Tyr-1472 phosphorylation site leads to increased CRF mRNA expression (Figure 2). Correlating with this, NMDA receptor stimulation induced tyrosine de-phosphorylation of Tyr-1472 and up-regulation of CRF mRNA expression in amygdala slices (Figure 4). Thus, Tyr-1472 phosphorylation links NMDA receptor activity and CRF expression in the amygdala: however, the mechanisms underlying enhanced CRF mRNA expression in GluN2B-YF mice remain to be determined. We previously found that Tyr-1472 phosphorylation regulates NMDA receptor-mediated CaMKII signaling in the amygdala [13]. A simple model would predict that downstream NMDA receptor-mediated CaMKII signaling regulates CRF mRNA expression in the amygdala.

One major neuroendocrine system underlying an individual's capacity to cope with stress is the HPA axis [14]. Besides the regulation of anxiety-like behavior, CRF is a key coordinator of the HPA axis and an essential component in the mediation of behavioral responses to stress [14]. In contrast to the increased CRF levels in

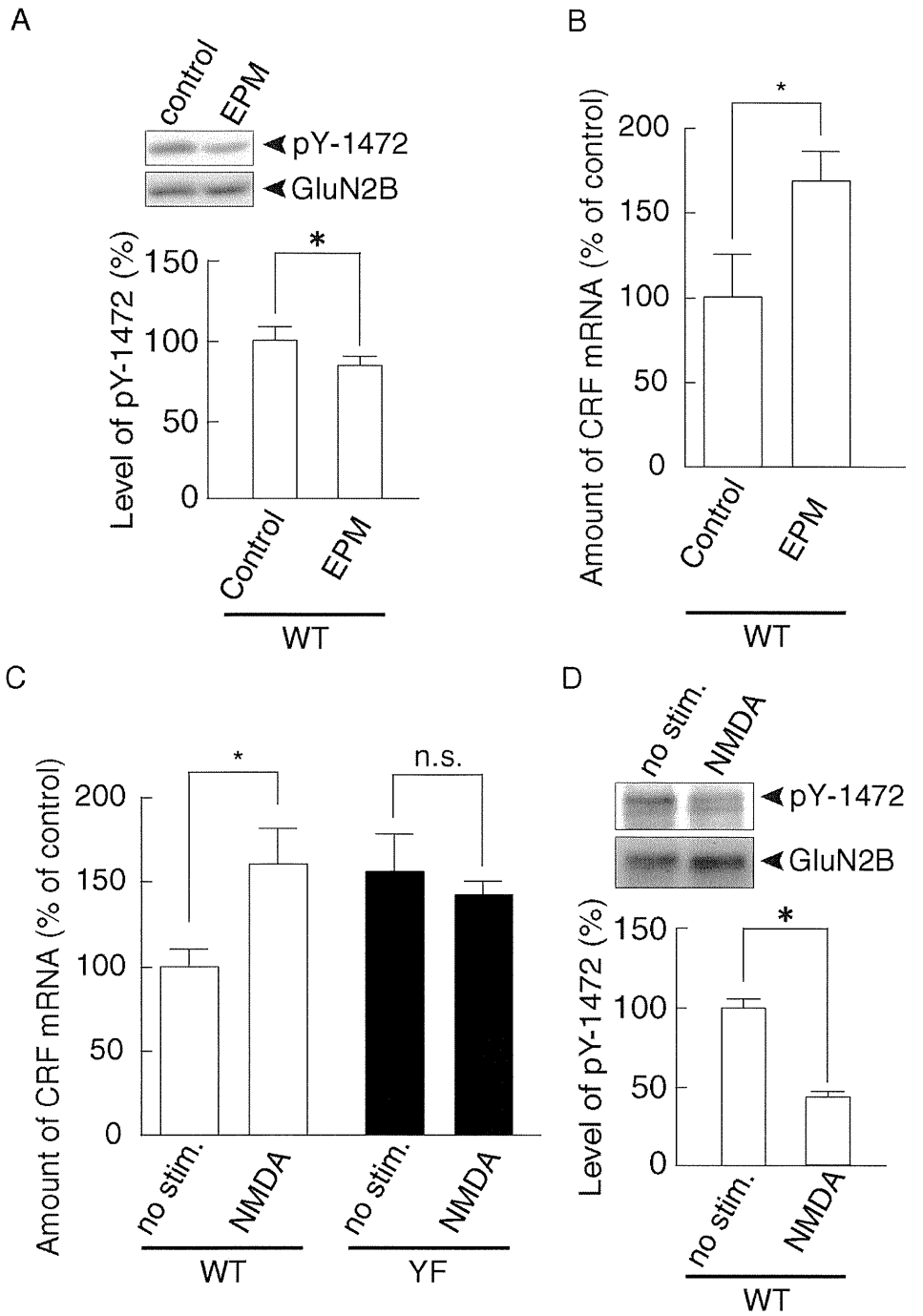


Figure 4 De-phosphorylation of Tyr-1472 and up-regulation of CRF expression after the EPM test and by NMDA-receptor stimulation. **(A)** The level of Tyr-1472 phosphorylation in the amygdala of WT mice was decreased by the EPM test (control, $n = 6$; EPM test, $n = 4$; $p < 0.05$, Student's *t*-test). A representative blot is shown in the upper panel. **(B)** Real-time PCR analyses showed that CRF expression in the amygdala of WT mice was increased after the EPM test (control, $n = 6$; EPM test, $n = 4$; $p < 0.05$, Student's *t*-test). **(C)** Real-time PCR analyses showed that NMDA stimulation (100 μM NMDA for 7 min) increased CRF expression in the amygdalae of WT mice (control, $n = 6$; NMDA stimulation, $n = 6$; $p < 0.05$, Student's *t*-test) but not in GluN2B-YF mice (control, $n = 6$; NMDA stimulation, $n = 6$; $p > 0.2$, Student's *t*-test). **(D)** The same NMDA stimulation also induced de-phosphorylation of Tyr-1472 in the amygdalae of WT mice (control, $n = 5$; NMDA stimulation, $n = 5$; $p < 0.05$, Student's *t*-test). A representative blot is shown in the upper panel. The asterisk indicates significant genotype differences. n.s., not significant.

the amygdala (Figure 2A), we did not find any significant differences in hypothalamic CRF levels between GluN2B-YF mice and WT mice (Figure 2D). Consistent with these findings, the basal levels of plasma ACTH in GluN2B-YF mice were virtually unchanged compared to those of WT mice (Figure 2F). Thus, the function of Tyr-1472 phosphorylation may be different between brain regions. Alternatively, in the hypothalamus, some compensatory event might have occurred to mask the hypothalamic phenotypes in GluN2B-YF mice.

There is widespread interest in CRF_1 receptor antagonists for the treatment of anxiety disorders [21,27]: however, these potential therapies can influence the function of the HPA axis in response to stress [28]. Tyr-1472 phosphorylation in GluN2B regulates anxiety-like behavior through regulation of amygdaloid CRF expression without altering the function of the HPA axis. Therefore, blocking Tyr-1472 phosphorylation in the amygdala may be a clinically effective means of treating anxiety disorders without the potential risks associated with blocking of the function of the HPA axis.

Conclusions

In summary, the present study demonstrates that the cellular interaction between CRF signaling and NMDA receptors, especially Tyr-1472 phosphorylation of GluN2B, is necessary for the regulation of anxiety-like behavior. GluN2B-YF mice should serve as a useful animal model to study the pathogenesis of anxiety disorders and to develop therapeutic drugs for the disease.

Methods

Animals

Heterozygous GluN2B-YF mice [13] were successively backcrossed to C57BL/6J mice to yield subsequent generations with a pure C57BL/6J genetic background. F10 heterozygous mice were crossed to each other to yield homozygous mice and wild-type littermates. Male 8-12-week old mice were used in this study. All experiments and analyses were done in a completely blind manner. Experiments with animals were carried out in accordance with the guidelines for animal use issued by the Committee of Animal Experiments, Institute of Medical Science, University of Tokyo.

Elevated plus-maze (EPM) test

The elevated plus-maze test (EP-3002; O' Hara & Co., Ltd., Tokyo, Japan) consisted of two open arms (25 × 5 cm) and two enclosed arms of the same size extending from a central area (5 × 5 cm) and elevated 50 cm from the ground. The ambient light level was 30 lux. The mice were placed in the central square of the maze facing one of the open arms. Mouse behavior was recorded during a 10 min test period with a Macintosh computer using Image EP 2.13× and Image EPC 2.03s× (O'Hara & Co., Ltd.), a modified software based on the public domain of NIH Image program. The following conventional parameters were recorded: the number of entries into open or closed arms and the time spent in open or closed arms.

Real-time PCR

Mice were anesthetized with halothane and immediately decapitated. Brains were then rapidly removed and frozen by liquid nitrogen. Serial coronal sections (400 μm thickness) were cryosectioned, and the various brain regions were collected with a biopsy puncher. Total RNA was isolated from the brain regions and reverse transcribed with Superscript III (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed with TaqMan primers on an ABI PRISM 7900HT system (Applied Biosystems, Foster City, CA, USA) according to the supplier's protocol. The following intron-spanning primer sets were used: HPRT (internal control), Mm0046968_m1; CRF, Mm01293920_S1 (Applied Biosystems). Relative expression levels were determined according to the 2-ΔΔCt method (Applied Biosystems, User Bulletin).

Measurements of CRF protein level

Serial coronal sections (200 μm) were cryosectioned, and the amygdalae were collected [29]. CRF protein levels were measured by using an enzyme-linked immunoassay kit (Mouse/Rat CRF-HS ELISA kit (YK131), Yanaihara Institute Inc., Shizuoka, Japan) according to the supplier's protocol.

Measurements of plasma adrenocorticotropin-releasing hormone (ACTH) levels

Mice were killed by rapid decapitation, and blood was collected at around 9:00 AM. Serum was isolated and frozen at -80°C until analysis. Basal plasma ACTH levels were measured using an enzyme-linked immunoassay (SRL Inc., Tokyo, Japan).

Intraperitoneal injection of CRF_1 receptor antagonist

A non-peptide selective CRF1 receptor antagonist, NBI 27914 (dissolved at 10 mg/ml in DMSO, 10 mg/kg body weight, Tocris, Bristol, UK) [30] or vehicle (DMSO) was injected intraperitoneally 45 min before the EPM test.

Pharmacological treatment of brain slices

Coronal slices including the amygdala (400 μm thickness) were prepared from 8- to 10-week-old mice and placed in an interface-type holding chamber for at least 3 h. Slices were preincubated in ACSF [13] for 1 h and then stimulated with 100 μM NMDA for 7 min.

Preparation of lysate, immunoprecipitation and immunoblotting

Preparation of lysate from the mouse brain and immunoblotting were performed as described previously [13]. For quantification, the immunoreacted protein bands were analyzed with the NIH image software. The rabbit polyclonal antibody against phospho-Tyr-1472/GluN2B was described previously [31]. The mouse monoclonal antibody against GluN2B was purchased from Millipore (Billerica, MA, USA).

Statistical analysis

All data are expressed as the means ± SEM. Statistical analysis was performed using Student's t test, one-way ANOVA, two-way ANOVA, and Tukey's post hoc test. Differences with $p < 0.05$ were considered as significant.

Abbreviations

ACTH: adrenocorticotropin-releasing hormone; ANOVA: analysis of variance; CRF: corticotropin-releasing factor; EPM: elevated plus-maze; HPA: hypothalamic-pituitary-adrenocortical; NMDA: *N*-methyl-D-aspartate.

Acknowledgements

This research was supported in part by Grants-in-Aid for Scientific Research (T.N., T.Y., and T.M.), by the Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms) (T.Y.), by the Global COE Program (Comprehensive Center of Education and Research for Chemical Biology of the Diseases) (T.M.), and the Strategic Research Program for Brain Sciences (T.M.) from the Japan Society for the Promotion of Science and the Ministry of Education, Science, Sports, Culture and Technology of Japan.

Author details

[1]Division of Oncology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [2]Division of Neuronal Network, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. [3]Division of Systems Medical Science, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan. [4]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), 4-1-8 Hon-cho, Kawaguchi, 332-0012, Japan. [5]Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, Kanazawa University, and Hamamatsu University School of Medicine, Yamada-Oka, Suita, Osaka 565-0871, Japan. [6]Department of Psychiatry, Osaka University Graduate School of Medicine, Yamada-Oka, Suita, Osaka 565-0871, Japan. [7]Molecular & Behavioral Neuroscience Institute and Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109-2200, USA. [8]Division of Genetics, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan.

Authors' contributions

MD, TT, YK, TM, TY, and TN designed the project. MD, TT, YK, KY, TI, SH, RH, HU, and TN performed experiments and analyzed the data. TM, TY, and TN wrote the manuscript and supervised the project. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 11 November 2010 Accepted: 30 November 2010
Published: 30 November 2010

References

1. Alonso J, Lepine JP: **Overview of key data from the European study of the epidemiology of mental disorders (ESEMeD).** *J Clin Psychiatry* 2007, **68**:3-9.
2. Salzman C, Miyawaki EK, le Bars P, Kerrihand TN: **Neurobiologic basis of anxiety and its treatment.** *Harv Rev Psychiatry* 1993, **1**:197-206.
3. Cortese BM, Phan KL: **The role of glutamate in anxiety and related disorders.** *CNS Spectr* 2005, **10**:820-830.
4. Martinez G, Ropero C, Funes A, Flores E, Blotta C, Landa AI, Gargiulo PA: **Effects of selective NMDA and non-NMDA blockade in the nucleus accumbens on the plus-maze test.** *Physiol Behav* 2002, **76**:219-224.
5. Wiley JL, Cristello AF, Balster RL: **Effects of site-selective NMDA receptor antagonists in an elevated plus-maze model of anxiety in mice.** *Eur J Pharmacol* 1995, **294**:101-107.
6. Cull-Candy S, Brickley S, Farrant M: **NMDA receptor subunits: diversity, development and disease.** *Curr Opin Neurobiol* 2001, **11**:327-335.
7. Choi DW: **Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage.** *Trends Neurosci* 1988, **11**:465-469.
8. McDonald JW, Johnston MV: **Physiological and pathophysiological roles of excitatory amino acids during central nervous system development.** *Brain Res Rev* 1990, **15**:41-70.
9. Collingridge GL, Bliss TVP: **Memories of NMDA receptors and LTP.** *Trends Neurosci* 1995, **18**:54-56.
10. Salter MW, Kalia LV: **Src kinases: a hub for NMDA receptor regulation.** *Nat Rev Neurosci* 2004, **5**:317-328.
11. Chen BS, Roche KW: **Regulation of NMDA receptors by phosphorylation.** *Neuropharmacol* 2007, **53**:362-368.
12. Taniguchi S, Nakazawa T, Tanimura A, Kiyama Y, Tezuka T, Watabe AM, Katayama N, Yokoyama K, Inoue T, Izumi-Nakaseko H, Kakuta S, Sudo K, Iwakura Y, Umemori H, Inoue T, Murphy NP, Hashimoto K, Kano M, Manabe T, Yamomoto T: **Involvement of NMDAR2A tyrosine phosphorylation in depression-related behavior.** *EMBO J* 2009, **28**:3717-3729.
13. Nakazawa T, Komai S, Watabe AM, Kiyama Y, Fukaya M, Arima-Yoshida F, Horai R, Sudo K, Ebine K, Delawary M, Goto J, Umemori H, Tezuka T, Iwakura Y, Watanabe M, Yamamoto T, Manabe T: **NR2B tyrosine phosphorylation modulates fear learning as well as amygdaloid synaptic plasticity.** *EMBO J* 2006, **25**:2867-2877.
14. Keck ME: **Corticotropin-releasing factor, vasopressin and receptor systems in depression and anxiety.** *Amino Acids* 2006, **31**:241-250.
15. Koob GF, Heinrichs SC: **A role for corticotrophin releasing factor and urocortin in behavioral responses to stressors.** *Brain Res* 1999, **848**:141-152.
16. Reul JM, Holsboer F: **Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression.** *Curr Opin Pharmacol* 2002, **2**:23-33.
17. Bale TL, Vale WW: **CRF and CRF receptors: role in stress responsivity and other behaviors.** *Annu Rev Pharmacol Toxicol* 2004, **44**:525-557.
18. Strenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW: **Overproduction of corticotrophin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior.** *J Neurosci* 1994, **14**:2579-2584.
19. Heinrichs SC, Pich EM, Miczek KA, Britton KT, Koob GF: **Corticotropin-releasing factor antagonist reduces emotionality in socially defeated rats via direct neurotropic action.** *Brain Res* 1992, **581**:190-197.
20. Liebsch G, Landgraf R, Gerstberger R, Probst JC, Wotjak CT, Engelmann M, Holsboer F, Montkowski A: **Chronic infusion of a CRH1 receptor antisense oligodeoxynucleotide into the central nucleus of the amygdala reduced anxiety-related behavior in socially defeated rats.** *Regul Pept* 1995, **59**:229-239.
21. Korte SM, De Boer SF: **A robust animal model of state anxiety: fear-potentiated behavior in the elevated plus-maze.** *Eur J Pharmacol* 2003, **463**:163-175.
22. Holmes A: **Targeted gene mutation approaches to the study of anxiety-like behavior in mice.** *Neurosci Biobehav Rev* 2001, **25**:261-273.
23. Mathew SJ, Price RB, Charney DS: **Recent advances in the neurobiology of anxiety disorders.** *Am J Med Genet Part C Semin Med Genet* 2008, **148C**:89-98.

24. Boyce-Rustay JM, Holmes A: **Genetic inactivation of the NMDA receptor NR2A subunit has anxiolytic- and antidepressant-like effects in mice.** *Neuropsychopharmacol* 2006, **31**:2405-2414.
25. Adamec RE, Burton P, Shallow T, Budgell J: **Unilateral block of NMDA receptors in the amygdala prevents predator stress-induced lasting increases in anxiety-like behavior and unconditioned startle-effective hemisphere depends on the behavior.** *Physiol Behav* 1999, **65**:739-751.
26. Cratty MS, Birkle DL: ***N*-methyl-D-aspartate (NMDA)-mediated corticotropin-releasing factor (CRF) release in cultured rat amygdala neurons.** *Peptides* 1999, **20**:93-100.
27. Holmes A, Heilig M, Rupniak NM, Steckler T, Griebel G: **Neuropeptide systems as novel therapeutic targets for depression and anxiety disorders.** *Trends Pharmacol Sci* 2003, **24**:580-588.
28. Habib KE, Weld KP, Rice KC, Pushkas J, Champoux M, Listwak S, Webster EL, Atkinson AJ, Schulkin J, Contoreggi C, Chrousos GP, McCann SM, Suomi SJ, Higley JD, Gold PW: **Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates.** *Proc Natl Acad Sci USA* 2000, **97**:6079-6084.
29. Murphy NP, Maidment NT: **Orphanin FQ/nociceptin modulation of mesolimbic dopamine transmission determined by microdialysis.** *J Neurochem* 1999, **73**:179-186.
30. Baram TZ, Chalmers DT, Chen C, Koutsoukos Y, De Souza EB: **The CRF1 receptor mediates the excitatory actions of corticotropin releasing factor (CRF) in the developing rat brain: in vivo evidence using a novel, selective, non-peptide CRF receptor antagonist.** *Brain Res* 1997, **770**:89-95.
31. Nakazawa T, Komai S, Tezuka T, Hisatsune C, Umemori H, Semba K, Mishina M, Manabe T, Yamamoto T: **Characterization of Fyn-mediated tyrosine phosphorylation sites on GluRε2 (NR2B) subunit of the *N*-methyl-D-aspartate receptor.** *J Biol Chem* 2001, **276**:693-699.

doi:10.1186/1756-6606-3-37
Cite this article as: Delawary *et al.*: NMDAR2B tyrosine phosphorylation regulates anxiety-like behavior and CRF expression in the amygdala. *Molecular Brain* 2010 **3**:37.

Original Research Article

Dementia
and Geriatric
Cognitive Disorders

Dement Geriatr Cogn Disord 2010;30:302–308
DOI: 10.1159/000320482

Accepted: August 16, 2010
Published online: September 25, 2010

KIBRA Genetic Polymorphism Influences Episodic Memory in Alzheimer's Disease, but Does Not Show Association with Disease in a Japanese Cohort

Noriyuki Hayashi[a] Hiroaki Kazui[a] Kouzin Kamino[a, f] Hiromasa Tokunaga[a]
Masahiko Takaya[a] Mikiko Yokokoji[a] Ryo Kimura[c] Yumiko Kito[a] Tamiki Wada[a]
Keiko Nomura[a] Hiromichi Sugiyama[a] Daisuke Yamamoto[a] Tetsuhiko Yoshida[d]
Antonio Currais[g] Salvador Soriano[h] Toshimitsu Hamasaki[b] Mitsuko Yamamoto[a]
Yuka Yasuda[a] Ryota Hashimoto[a, e] Hitoshi Tanimukai[a] Shinji Tagami[a] Masayasu Okochi[a]
Toshihisa Tanaka[a] Takashi Kudo[a] Takashi Morihara[a] Masatoshi Takeda[a]

Departments of [a]Psychiatry and [b]Biomedical Statistics, Osaka University Graduate School of Medicine, [c]Osaka General Medical Center, [d]Osaka National Hospital, and [e]Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, Osaka, and [f]National Hospital Organization, Shoraiso Hospital, Nara, Japan; [g]Department of Neuroscience, MRC Centre for Neurodegeneration Research, Institute of Psychiatry, King's College London, London, UK; [h]Department of Human Anatomy and Pathology, Loma Linda University Medical School, Loma Linda, Calif., USA

Key Words
Alzheimer's disease · Episodic memory · Genetics · Neuropsychological assessment · *KIBRA* gene · Rivermead Behavioral Memory Test

Abstract
Background/Aims: A single-nucleotide polymorphism (SNP) in the *KIBRA* gene, rs17070145, was reported to be significantly associated with episodic memory in cognitively normal cohorts. This observation has expanded genetic studies on *KIBRA* to Alzheimer's disease (AD). Importantly, the association between *KIBRA* and episodic memory in AD has never been addressed. In this study, we investigated whether the *KIBRA* rs17070145 SNP influences AD episodic memory and the disease in a Japanese cohort. ***Methods:*** Blood samples from 346 AD patients and 375 normal cognitive controls were collected and genotyped for rs17070145. Episodic memory was measured in 32 AD patients, diagnosed for the first time, by use of the Rivermead Behavioral Memory Test (RBMT). ***Results:*** We found that *KIBRA* C allele carriers scored significantly lower than *KIBRA* non-C carriers on both RBMT total profile score ($p = 0.042$, effect size = 0.84) and RBMT total screening score ($p < 0.001$, effect size = 1.42). The *KIBRA* gene did not show association with AD in our Japanese cohort. ***Conclusion:*** Our results evidence a strong association between the *KIBRA* gene and episodic memory impairment in AD, but show no influence on AD in our Japanese cohort. We propose that *KIBRA* might have an effect similar to cognitive reserve.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by a progressive deterioration of cognitive abilities and memory loss. For the famil-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1420–8008/10/0304–0302$26.00/0

Accessible online at:
www.karger.com/dem

Takashi Morihara, MD, PhD
Department of Psychiatry, Osaka University Graduate School of Medicine
2-2-D3, Yamadaoka, Suita
Osaka 565-0871 (Japan)
Tel. +81 6 6879 3074, Fax +81 6 6879 3059, E-Mail morihara@psy.med.osaka-u.ac.jp

ial occurrence of the disease (early-onset), the existence of familial AD-responsible genes has been demonstrated, with mutations in 3 genes, *APP, PSEN1* and *PSEN2,* consistently reported. However, the genetic component that underlies sporadic AD (late-onset), which accounts for over 95% of all AD cases, is still poorly understood.

KIBRA (also known as WW and C2 domain-containing protein 1) is a protein mainly expressed in the brain and kidney [1], whose functions are still being characterized, but that, importantly, has been shown to be involved in the control of synaptic plasticity in the brain [2]. Recently, it was reported that KIBRA regulated the Salvador/Hippo/Warts network which restricted tissue size [3]. In 2005, a *KIBRA* gene single-nucleotide polymorphism (SNP), rs17070145, was reported to be significantly associated with episodic memory in 3 independent cognitively normal cohorts from Switzerland and the USA [4]. This result was later confirmed in a German sample of healthy individuals [5], a Japanese sample of healthy individuals [6] and in a cohort in which nearly 50% of individuals had a diagnosis of mild cognitive impairment [7].

These observations led to studies of the *KIBRA* rs17070145 SNP in AD, whose core feature is dysfunction of episodic memory [8]. Recently, Corneveaux et al. [9] reported an association of the *KIBRA* CC genotype (*KIBRA* CC carriers) with increased risk for late-onset AD (n = 702). Conversely, the *KIBRA* T allele (*KIBRA* CT and *KIBRA* TT carriers) was shown to be associated with an increased risk for AD in a Spanish cohort [10]. Despite the available information, *KIBRA* has not yet been established as an AD risk gene, and, importantly, no studies have ever addressed the association between *KIBRA* and episodic memory in AD.

Therefore, in this study, we investigated whether the *KIBRA* SNP rs17070145 influences AD episodic memory and AD in a Japanese cohort.

Methods

Subjects

We collected blood samples from 346 consecutive AD patients who visited Osaka University Hospital between July 27, 2001, and June 10, 2010, and from 375 cognitively normal controls, who were population-based elderly subjects (Suita City, Japan) tested by a questionnaire including the date, orientation and history. Blood samples were collected after written informed consent had been obtained from subjects and/or representatives. This study was approved by the genome ethical committee of the Osaka University Graduate School of Medicine. AD patients met the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria for probable AD [11].

We also have a research-oriented clinic for patients with cognitive impairment in the Department of Neuropsychiatry of the Osaka University Medical Hospital. It is also a clinic for early identification of dementia. In this clinic, all patients were examined comprehensively by specialists of geriatric psychiatry, and they underwent standard neuropsychological examinations including the Mini Mental State Examination (MMSE), routine laboratory tests, electroencephalography, cranial magnetic resonance imaging and radionuclear neuroimaging studies. Blood drawing for the genome study was not routine in this clinic. Eighty first-time diagnosed AD patients agreed to it, and 32 out of them agreed to an additional visit for the memory examination by use of the Rivermead Behavioral Memory Test (RBMT) between September 30, 2002, and May 23, 2007 (RBMT-AD specialized clinic subjects). RBMT-AD specialized clinic subjects were excluded from the study if they (1) had the complication of other neurological diseases, (2) had any evidence of focal brain lesions on magnetic resonance images or of cerebral arterial occlusive lesions on magnetic resonance angiography, or (3) did not have a caregiving family member familiar with their everyday life.

Rivermead Behavioral Memory Test

The RBMT, developed by Wilson et al. [12, 13], is a standardized, validated and reliable test for everyday memory, including personal events, name of persons, newspaper articles, places visited, routes followed, schedules and appointments. It is difficult to assess everyday memory with traditional memory tests [14], but the RBMT differs from conventional tests in that each of its 12 items is an analog of an everyday task, rather than a test based on experimental material, such as paired associates or list of words. The Japanese version of the RBMT was developed by Watamori et al. [15], and its reliability and validity have been previously confirmed [16–19]. Concretely, the authors reported that the RBMT can distinguish AD from both mild cognitive impairment and normal control, and strongly correlates with objective memory tests, such as the Everyday Memory Checklist caregiver rating and Clinical Dementia Rating (CDR) memory domain.

Although the RBMT has 4 parallel forms (A, B, C and D) for repeated uses, only the RBMT-A form was administered to subjects in this study. The subtests of the RBMT are (1) remembering a first name and a surname with a facial portrait, (2) remembering to ask for a personal item belonging to the subject, (3) remembering to ask about an appointment, (4) picture recognition, (5) remembering a short story (immediate), (6) remembering a short story (delayed), (7) face recognition, (8) remembering a new route (immediate), (9) remembering a new route (delayed), (10) remembering to deliver a message (immediate and delayed), (11) orientation for time, place and persons, and (12) date. In 8 of the subtests, i.e. points 1–4, 6, 7, 9 and 10 (delayed), the subjects were instructed to remember the information that they were about to be given. The subtests were then conducted 5–30 min after the information had been given. Subtests 2, 3 and 10 are tests of prospective memory. In subtest 2, the subjects were asked to hand in a personal item at the start of the session and instructed to ask for it at the conclusion of the session. The item was then placed out of sight. In subtest 3, subjects were instructed at the beginning of the test session to remember to ask for their next appointment when they heard a buzzer 20 min later. In subtest 10, they had to remember to de-

Table 1. Characterization of the *KIBRA* C carriers and non-C carriers of the RBMT-AD specialized clinic subjects

	CC/CT (n = 12)	TT (n = 20)	p
Mean age ± SD, years	68.5 ± 10.0	72.2 ± 8.1	0.267
Mean age of first abnormal memory loss episode reported by caregivers ± SD, years	63.8 ± 2.6	69.3 ± 2.0[1]	0.104
Male/female, n	5/7	8/12	1.000
APOE ε4+/−, n	10/2	12/8	0.248
CDR score 0.5/1/2, n	1/9/2	3/14/2	1.000
Mean MMSE score ± SD	17.8 ± 2.7	20.4 ± 4.6	0.093
Mean ADAS score ± SD	20.0 ± 5.8	17.6 ± 7.1[1]	0.335
Years of education			
Median	12	10	0.151
IQR	10.25–15	9–14[1]	

p values assessed by t test (continuous variables) and Fisher's exact test (categorical variables). IQR = Interquartile range (Q1–Q3).

[1] One datum was missed.

liver a message in the course of retracing a route around the room. For each subtest of the RBMT, a raw score was given. Then, two kinds of score were produced, a simple pass/fail or screening score ranging in each case from 0 to 1, and a standardized profile score ranging in each case from 0 to 2. A total screening score ranging from 0 to 12 and a total profile score ranging from 0 to 24 were used as indices of overall everyday memory status of the subjects.

Genotyping

Genotyping of *KIBRA* rs17070145 polymorphism was performed by the Taq-Man SNP assay and ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, Calif., USA) as previously described [20–23]. The apolipoprotein E *(APOE)* genotype was determined by the PCR-RFLP method [20–23].

Statistical Analysis

Baseline characteristics are presented as means ± standard deviation, medians or interquartile ranges for continuous variables, and frequencies for categorical variables. Comparisons for continuous variables and categorical variables were performed with the t test and χ^2 test or Fisher's exact test, respectively. The analysis of covariance model was used to investigate the effect of treatment on the RBMT scores with the following covariate: presence of the *KIBRA* SNP C allele (*KIBRA* CT and *KIBRA* CC), *APOE* ε4, age, the age of first abnormal memory loss episode reported by caregivers, gender, CDR stage, MMSE score, Alzheimer's Disease Assessment Scale for Japanese cognitive subscale (ADAS-Jcog) and/or years of education. The best set of covariates was selected by using Akaike's information criterion [24]. All tests were two-sided, and the statistical significance level was set at 5%. Statistical analysis was performed with SAS software version 9.02 (SAS Institute, Cary, N.C., USA), and all p values and confidence intervals (CI) presented are the original and were not corrected for multiple testing. Meta-analysis of *KIBRA* CC AD odds ratio and 95% CI was performed by the Der-Simonian-Laird method.

Results

From the RBMT-AD specialized clinic subjects, we found 1 patient with *KIBRA* CC, 11 patients with *KIBRA* CT and 20 patients with *KIBRA* TT (*KIBRA* non-C carriers). *KIBRA* CC and CT groups (*KIBRA* C carriers) were combined because there was only 1 *KIBRA* CC patient and that patient displayed memory performance similar to that of the *KIBRA* CT group (total profile score was 2, total screening score was 0). A lower frequency of the *KIBRA* C allele was observed, which was in accordance with the National Center for Biotechnology Information database of genetic variation (dbSNP) for the Asian population. Most of the patients were in an early stage of dementia (table 1). No significant differences in age, gender, *APOE* ε4, CDR, MMSE score, ADAS score and years of education were found between *KIBRA* C and *KIBRA* non-C carriers.

When analyzing the RBMT scores of the two groups, we found that C carriers scored significantly lower than non-C carriers on both the profile score ($p = 0.042$, effect size = 0.84) and screening score ($p < 0.001$, effect size = 1.42; table 2), evidencing an association of *KIBRA* rs17070145 polymorphism with episodic memory impairment in our Japanese AD cohort. We then assigned RBMT total scores as dependent variables and *KIBRA* C, age, age of first abnormal memory loss episode reported by caregivers, gender, *APOE* ε4, CDR stage, MMSE score, ADAS-Jcog score and/or years of education as independent variables and performed multiple linear regression analysis. For all the different combinations, we selected the appropriate models to which Akaike's information criteria were the smallest [24]. Model 1 was appropriate for total profile score and model 2 for total screening score. *KIBRA* C was found to be significantly associated with both total profile and screening scores after adjustment with the models shown in table 2.

We also analyzed 346 AD patients and 375 cognitively normal controls. As expected, we found significant differences in gender and *APOE* ε4 allele frequencies (table 3). *KIBRA* rs17070145 genotype and allele distribution in control and AD groups are shown in table 4. The genotype frequencies were in accordance with the Hardy-

Table 2. RBMT scores (total profile score and total screening score) between *KIBRA* C carriers and non-C carriers of the RBMT-AD specialized clinic subjects

	CC/CT	TT	p	Effect size
Total profile score (not adjusted)	2.17, 0.60–3.17	4.26, 3.01–5.51	0.042	0.84
Total profile score (model 1)	1.88, 0.42–3.34	4.22, 3.16–5.29	0.012	1.07
Total profile score (model 2)	1.79, 0.31–3.27	4.28, 3.20–5.35	0.010	1.13
Total screening score (not adjusted)	0.10, 0.00–0.36	0.93, 0.58–1.39	<0.001	1.42
Total screening score (model 1)	0.07, 0.00–0.35	0.91, 0.56–1.37	<0.001	1.54
Total screening score (model 2)	0.05, 0.00–0.31	0.93, 0.58–1.40	<0.001	1.66

Scores are expressed as mean estimates, followed by 95% CI. p values assessed by ANCOVA; model 1: adjusted for *APOE* ε4, years of education and ADAS score (this model is appropriate for total profile score); model 2: adjusted for *APOE* ε4, years of education, ADAS score and age (this model is appropriate for total screening score).

Table 3. Characterization of cognitively normal controls (NC) and AD patients

	NC (n = 375)	AD (n = 346)	p
Mean age ± SD, years	75.5 ± 4.9	75.2 ± 8.6	0.600
Male/female, n	170/205	110/236	<0.001
APOE ε4+/–, n	60/315	172/174	<0.001

p values assessed by t test (continuous variable) and Fisher's exact test (categorical variables).

Table 4. rs17070145 genotype and allele distribution in cognitively normal controls (NC) and AD patients

	CC	CT	TT	p^a	p^b	p^c
NC	13 (3.5)	128 (34.1)	234 (62.4)	0.673	0.414	0.694
AD	16 (4.6)	104 (30.1)	226 (65.3)	0.669		

Results are numbers, with percentages in parentheses.
[a] p for Hardy-Weinberg equilibrium tests (Pearson χ^2 test).
[b] p for genotype distribution (Fisher's exact test).
[c] p for allele distribution (Fisher's exact test).

Weinberg equilibrium. The *KIBRA* SNP did not show any association with AD in our Japanese cohort (table 4), even after adjustment for age, gender and *APOE* ε4 (data not shown).

Figure 1 shows KIBRA CC AD odds ratio and 95% CI in our Japanese cohort and previously reported cohorts. Our cohort's KIBRA CC AD odds ratio was 1.35 (95% CI = 0.64–2.85). Meta-analysis of them was not significant (OR = 1.10, 95% CI = 0.92–1.30).

Discussion

Despite numerous reports evidencing association of *KIBRA* with episodic memory, the relevance of *KIBRA* to AD still remains elusive. In our study, we addressed for the first time whether *KIBRA* genetic variation is associated with episodic memory impairment in AD. Our results evidence a strong association between the *KIBRA* gene and episodic memory impairment in AD and suggest a role for *KIBRA* similar to cognitive reserve, with no impact on diagnosis of AD.

There are several memory test batteries available, such as the Auditory-Verbal Learning Test (AVLT) [25], the Revised Wechsler Memory Scale Logical Memory Test [26], the Rey-Osterrieth complex figure [27] and the Takeda Three Colors Combination Test [28]. Association of *KIBRA* rs17070145 with episodic memory was shown for the first time by AVLT in 3 independent cognitively normal cohorts [3], and it has been recently confirmed in a Scottish cohort study (n = 2,091) [29]. In addition, the latter reported no association of the *KIBRA* SNP with the Revised Wechsler Memory Scale Logical Memory Test that rewards relational coding (Lothian Barth cohort, n = 542) [29], suggesting that *KIBRA* is not specific for complex episodic memory such as the Revised Wechsler Memory Scale Logical Memory Test but for simple episodic memory such as the AVLT instead. In our study, we

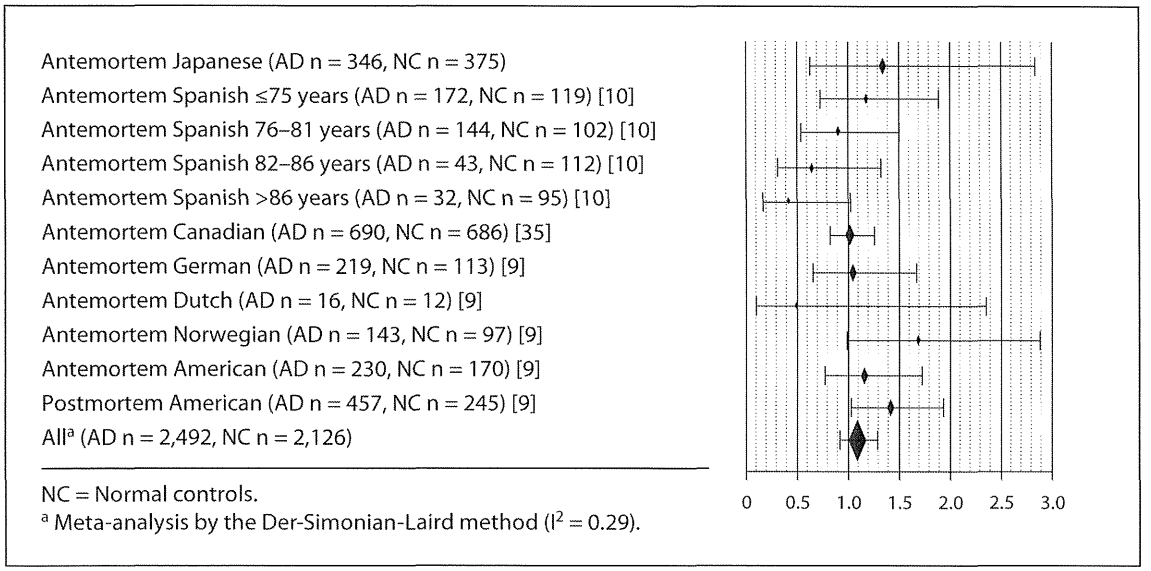


Fig. 1. *KIBRA* CC AD odds ratio and 95% CI in our Japanese cohort and previously reported cohorts.

used the RBMT, which assesses multidimensional aspects of everyday memory such as orientation, prose recall, visual recognition, prospective memory and so on. It also includes both complex episodic memory and simple episodic memory. Our results show that the *KIBRA* rs17070145 polymorphism is strongly associated with episodic memory impairment in our specialized clinic Japanese AD cohort (table 2). MMSE and ADAS-cog showed no differences between *KIBRA* C and non-C carriers (table 1). These results suggest that the *KIBRA* gene specifically seems to affect memory functions but not global cognitive status.

This association remained significant after adjustment for covariant components, which indicates that the *KIBRA* SNP might be an independent risk factor for episodic memory impairment.

Whereas an association of *KIBRA* with episodic memory has been repeatedly reported [4, 5, 7, 29], the impact of *KIBRA* on AD is still controversial (fig. 1). In a Spanish cohort, *KIBRA* CC AD odds ratio decreased continuously with age. *KIBRA* CC AD patients perhaps had earlier onsets and died soon. Hence, we tested the association between *KIBRA* and age of onset and course of the disease in RBMT-AD specialized clinic subjects. We defined the age of first abnormal memory loss episode reported by caregivers as onset age of AD. Although we found no significant differences between *KIBRA* C carriers and non-C carriers, the age of first abnormal memory loss episode reported by caregivers tended to be later in *KIBRA* non-C carriers (table 1). It is possible that the presence of the *KIBRA* T allele delays diagnosis of some AD clinical symptomatology. This effect could be similar to the well-reported effect of cognitive reserve, reflected in years of education. Highly educated individuals have better cognitive performance and, thus, tend to be judged as cognitively normal, albeit AD neuropathology is already present [30–32]. On the other hand, it appears that AD symptomatology progresses faster in people with higher education once AD is diagnosed [33]. Incidentally, in our cohort, duration from first memory loss episode to AD diagnosis was significantly shorter in the *KIBRA* TT group (2.6 ± 1.7 vs. 4.7 ± 1.2 years, $p = 0.001$). We propose that *KIBRA* might have an effect similar to cognitive reserve, particularly in simple word recall.

The impact of *APOE*, an established AD risk gene that accelerates AD brain pathology, on episodic memory was also examined. A recent study reported no differences, suggesting that *APOE* ε4 does not influence episodic memory (AVLT delayed recall) in cognitively normal individuals under 60 years of age [34]. In accordance, our results evidenced no significant differences in both RBMT total profile score (*APOE* ε4–: 3.40 ± 2.12 vs. *APOE* ε4+: 3.64 ± 3.16; $p = 0.831$) and RBMT total screening score (*APOE* ε4–: 0.80 ± 0.63 vs. *APOE* ε4+: 0.86 ± 1.17; $p = 0.873$) between *APOE* ε4 carriers and non-*APOE*-ε4 carriers. This lack of correlation between *APOE* and the episodic memory is intriguing and is in contrast with our findings for *KIBRA*, which seems to

have a less certain effect on AD but a more significant impact on episodic memory in young [4] and elderly subjects [4, 5, 7, 29] and even in mild AD patients, as our study shows (table 2). It is possible that *KIBRA* does not have a direct impact on AD neuropathology but could have an effect on the clinical diagnosis of AD, in a manner similar to cognitive reserve.

Compared to many reports based on Caucasian samples, our cohort evidenced lower frequencies of *KIBRA* CC. Thus, comparison of our results with those based on Caucasian samples must be carried out with caution. As our research-oriented clinic is specialized in the early identification of dementia, we should take selection bias into consideration. Further studies with larger samples, including cognitive functional and pathological data, will be carried out in the future in order to clarify the importance of the *KIBRA* SNP for episodic memory and AD pathology.

Acknowledgements

This work was supported by a Kakenhi (Grant-in-Aid for Scientific Research) on priority areas applied genomics from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Health and Labor Sciences Research Grants for a research on dementia (Dementia-General-003) from the Ministry of Health, Labor and Welfare of Japan.

References

1 Kremerskothen J, Plaas C, Buther K, Finger I, Veltel S, Matanis T, Liedtke T, Barnekow A: Characterization of KIBRA, a novel WW domain-containing protein. Biochem Biophys Res Commun 2003;300:862–867.

2 Buther K, Plaas C, Barnekow A, Kremerskothen J: KIBRA is a novel substrate for protein kinase Czeta. Biochem Biophys Res Commun 2004;317:703–707.

3 Genevet A, Wehr MC, Brain R, Thompson BJ, Tapon N: KIBRA is a regulator of the Salvador/Warts/Hippo signaling network. Dev Cell 2010;18:300–308.

4 Papassotiropoulos A, Stephan DA, Huentelman MJ, Hoerndli FJ, Craig DW, Pearson JV, Huynh KD, Brunner F, Corneveaux J, Osborne D, Wollmer MA, Aerni A, Coluccia D, Hanggi J, Mondadori CR, Buchmann A, Reiman EM, Caselli RJ, Henke K, de Quervain DJ: Common KIBRA alleles are associated with human memory performance. Science 2006;314:475–478.

5 Schaper K, Kolsch H, Popp J, Wagner M, Jessen F: KIBRA gene variants are associated with episodic memory in healthy elderly. Neurobiol Aging 2008;29:1123–1125.

6 Yasuda Y, Hashimoto R, Ohi K, Fukumoto M, Takamura H, Iike N, Yoshida T, Hayashi N, Takahashi H, Yamamori H, Morihara T, Tagami S, Okochi M, Tanaka T, Kudo T, Kamino K, Ishii R, Iwase M, Kazui H, Takeda M: Association study of the KIBRA gene with memory performance in a Japanese population. World J Biol Psychiatry 2010, E-pub ahead of print.

7 Almeida OP, Schwab SG, Lautenschlager NT, Morar B, Greenop KR, Flicker L, Wildenauer D: KIBRA genetic polymorphism influences episodic memory in later life, but does not increase the risk of mild cognitive impairment. J Cell Mol Med 2008;12:1672–1676.

8 Yano M, Umeda S, Mimura M: Preserved priming but insensitivity to perceptual fluency on recognition judgments in Alzheimer's disease. Psychogeriatrics 2008;8:178–187.

9 Corneveaux JJ, Liang WS, Reiman EM, Webster JA, Myers AJ, Zismann VL, Joshipura KD, Pearson JV, Hu-Lince D, Craig DW, Coon KD, Dunckley T, Bandy D, Lee W, Chen K, Beach TG, Mastroeni D, Grover A, Ravid R, Sando SB, Aasly JO, Heun R, Jessen F, Kolsch H, Rogers J, Hutton ML, Melquist S, Petersen RC, Alexander GE, Caselli RJ, Papassotiropoulos A, Stephan DA, Huentelman MJ: Evidence for an association between KIBRA and late-onset Alzheimer's disease. Neurobiol Aging 2010;31:901–909.

10 Rodriguez-Rodriguez E, Infante J, Llorca J, Mateo I, Sanchez-Quintana C, Garcia-Gorostiaga I, Sanchez-Juan P, Berciano J, Combarros O: Age-dependent association of KIBRA genetic variation and Alzheimer's disease risk. Neurobiol Aging 2009;30:322–324.

11 McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM: Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. Neurology 1984;34:939–944.

12 Wilson B, Cockburn J, Baddeley A, Hiorns R: The development and validation of a test battery for detecting and monitoring everyday memory problems. J Clin Exp Neuropsychol 1989;11:855–870.

13 Wilson B, Cockburn J, Baddeley A: The Rivermead Behavioural Memory Test. Elury, Thames Valley Test Company, 1991.

14 Morris P: The Validity of Subjective Reports on Memory: Actions and Absentmindedness. London, Academic Press, 1984, pp 153–172.

15 Watamori T, Hata H, Miyamori T, Eto F: Japanese version of the Rivermead Behavioural Memory Test. Tokyo, Chiba Test Center Co Ltd, 2002.

16 Kazui H, Watamori T, Honda R, Tokimmasa A, Hirono N, Mori E: The validation of Japanese version of the Rivermead Behavioural Memory Test: a test for everyday memory (in Japanese). Shinkei Kenkyu No Shinpo 2002; 46:307–318.

17 Kazui H, Watamori T, Honda R, Mori E: The validation of a Japanese version of the Everyday Memory Checklist (in Japanese). No To Shinkei 2003;55:317–325.

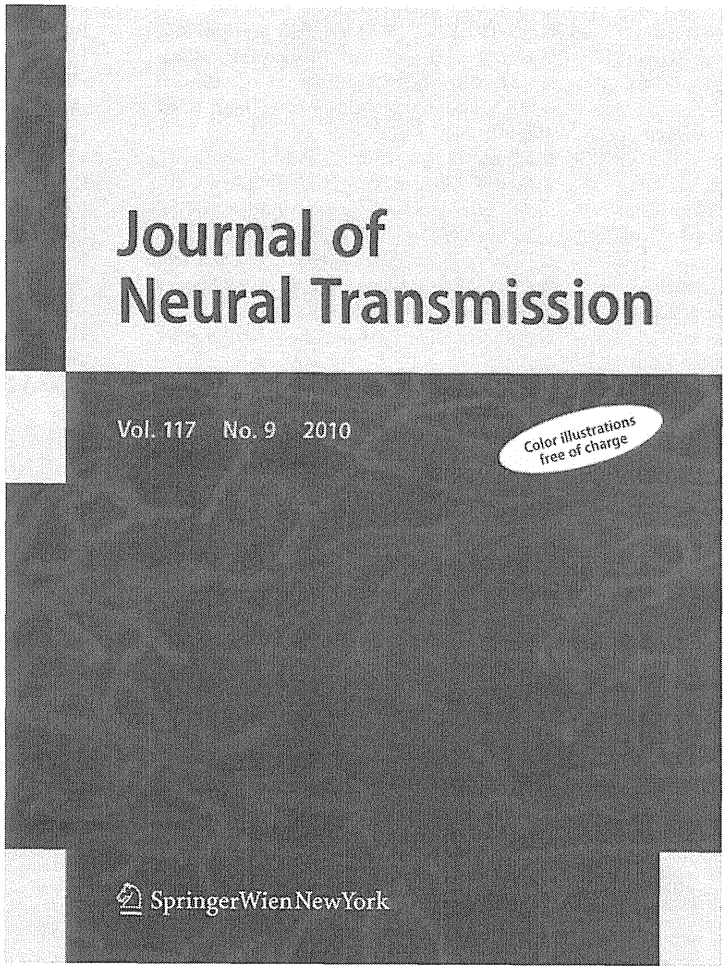
18 Matsuda A, Kazui H, Hirono N, Mori E: Validity of the Japanese version of Rivermead Behavioural Memory Test for evaluation of everyday memory function in patients with mild Alzheimer's disease (in Japanese). No To Shinkei 2002;54:673–678.

19 Kazui H, Matsuda A, Hirono N, Mori E, Miyoshi N, Ogino A, Tokunaga H, Ikejiri Y, Takeda M: Everyday memory impairment of patients with mild cognitive impairment. Dement Geriatr Cogn Disord 2005;19:331–337.

20 Kimura R, Kamino K, Yamamoto M, Nuripa A, Kida T, Kazui H, Hashimoto R, Tanaka T, Kudo T, Yamagata H, Tabara Y, Miki T, Akatsu H, Kosaka K, Funakoshi E, Nishitomi K, Sakaguchi G, Kato A, Hattori H, Uema T, Takeda M: The DYRK1A gene, encoded in chromosome 21 Down syndrome critical region, bridges between beta-amyloid production and tau phosphorylation in Alzheimer disease. Hum Mol Genet 2007;16:15–23.

21 Kimura R, Yamamoto M, Morihara T, Akatsu H, Kudo T, Kamino K, Takeda M: SORL1 is genetically associated with Alzheimer disease in a Japanese population. Neurosci Lett 2009;461:177–180.
22 Aidaralieva NJ, Kamino K, Kimura R, Yamamoto M, Morihara T, Kazui H, Hashimoto R, Tanaka T, Kudo T, Kida T, Okuda J, Uema T, Yamagata H, Miki T, Akatsu H, Kosaka K, Takeda M: Dynamin 2 gene is a novel susceptibility gene for late-onset Alzheimer disease in non-APOE-epsilon4 carriers. J Hum Genet 2008;53:296–302.
23 Taguchi K, Yamagata HD, Zhong W, Kamino K, Akatsu H, Hata R, Yamamoto T, Kosaka K, Takeda M, Kondo I, Miki T: Identification of hippocampus-related candidate genes for Alzheimer's disease. Ann Neurol 2005;57:585–588.
24 Akaike H: A new look at the statistical model identification. IEEE Trans Automat Contr 1974;AC-19:716–723.
25 Rosenberg SJ, Ryan JJ, Prifitera A: Rey Auditory-Verbal Learning Test performance of patients with and without memory impairment. J Clin Psychol 1984;40:785–787.
26 Wechsler D: Wechsler Memory Scale: Revised. Toronto, Psychological Corporation, Harcourt Brace, and Jovanovich, 1997.
27 Osterrieth PA: Le test de copie d'une figure complexe. Arch Psychol 1944;30:206–356.
28 Takeda S, Nakagome K, Tajime K, Saito M: Effectiveness of the Takeda Three Colors Combination Test as a screening test for dementia. Psychogeriatrics 2009;9:4–10.
29 Bates TC, Price JF, Harris SE, Marioni RE, Fowkes FG, Stewart MC, Murray GD, Whalley LJ, Starr JM, Deary IJ: Association of KIBRA and memory. Neurosci Lett 2009; 458:140–143.
30 Katzman R, Aronson M, Fuld P, Kawas C, Brown T, Morgenstern H, Frishman W, Gidez L, Eder H, Ooi WL: Development of dementing illnesses in an 80-year-old volunteer cohort. Ann Neurol 1989;25:317–324.
31 Stern Y: What is cognitive reserve? Theory and research application of the reserve concept. J Int Neuropsychol Soc 2002;8:448–460.
32 Roe CM, Xiong C, Miller JP, Morris JC: Education and Alzheimer disease without dementia: support for the cognitive reserve hypothesis. Neurology 2007;68:223–228.
33 Bruandet A, Richard F, Bombois S, Maurage CA, Masse I, Amouyel P, Pasquier F: Cognitive decline and survival in Alzheimer's disease according to education level. Dement Geriatr Cogn Disord 2008;25:74–80.
34 Caselli RJ, Dueck AC, Osborne D, Sabbagh MN, Connor DJ, Ahern GL, Baxter LC, Rapcsak SZ, Shi J, Woodruff BK, Locke DE, Snyder CH, Alexander GE, Rademakers R, Reiman EM: Longitudinal modeling of age-related memory decline and the APOE epsilon4 effect. N Engl J Med 2009;361:255–263.
35 Li H, Wetten S, Li L, St Jean PL, Upmanyu R, Surh L, Hosford D, Barnes MR, Briley JD, Borrie M, Coletta N, Delisle R, Dhalla D, Ehm MG, Feldman HH, Fornazzari L, Gauthier S, Goodgame N, Guzman D, Hammond S, Hollingworth P, Hsiung GY, Johnson J, Kelly DD, Keren R, Kertesz A, King KS, Lovestone S, Loy-English I, Matthews PM, Owen MJ, Plumpton M, Pryse-Phillips W, Prinjha RK, Richardson JC, Saunders A, Slater AJ, St George-Hyslop PH, Stinnett SW, Swartz JE, Taylor RL, Wherrett J, Williams J, Yarnall DP, Gibson RA, Irizarry MC, Middleton LT, Roses AD: Candidate single-nucleotide polymorphisms from a genomewide association study of Alzheimer disease. Arch Neurol 2008;65:45–53.

ISSN 0300-9564, Volume 117, Number 9



J Neural Transm (2010) 117:1119–1122
DOI 10.1007/s00702-010-0452-1

BIOLOGICAL PSYCHIATRY - SHORT COMMUNICATION

Plasma levels of vascular endothelial growth factor and fibroblast growth factor 2 in patients with major depressive disorders

M. Takebayashi · R. Hashimoto · K. Hisaoka · M. Tsuchioka · H. Kunugi

Received: 16 April 2010 / Accepted: 12 July 2010 / Published online: 6 August 2010

Abstract We investigated the plasma levels of VEGF and FGF-2, important factors for regulation of neuroplasticity such as neurogenesis, in patients in remission from major depressive disorders (MDD). The plasma VEGF levels were significantly higher in the MDD patients than in the matched control subjects, while no significant difference in plasma FGF-2 levels was found. In particular, the MDD patients with family history of psychiatric disorders, but not patients without such a family history, showed significantly higher values of plasma VEGF than the controls. Although this is a preliminary study, altered VEGF levels might be involved in the pathophysiology of MDD.

Keywords VEGF · FGF-2 · Plasma · Family history · Mood disorders

M. Takebayashi (✉)
Department of Psychiatry, National Hospital Organization (NHO) Kure Medical Center, 3-1 Aoyama, Kure 737-0023, Japan
e-mail: mtakebayashi@kure-nh.go.jp

M. Takebayashi · K. Hisaoka · M. Tsuchioka
Division of Psychiatry and Neuroscience, Institute for Clinical Research, NHO Kure Medical Center, Kure, Japan

R. Hashimoto · H. Kunugi
Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan

R. Hashimoto
Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, Osaka, Japan

R. Hashimoto
Kanazawa University School of Medicine, Kanazawa, Japan

R. Hashimoto
Hamamatsu University School of Medicine, Hamamatsu, Japan

R. Hashimoto
Department of Psychiatry, Osaka University Graduate School of Medicine, Osaka, Japan

R. Hashimoto · H. Kunugi
JST, CREST, Tokyo, Japan

Introduction

Recent morphological and histological studies have suggested that there is an organic impairment of the neuron–glia–vascular network in the brain, so called neuroplasticity, and that this impairment is associated with mood disorders (Pittenger and Duman 2008). Neuroplasticity is a fundamental mechanism of neuronal adaptation, structurally linked to adult neurogenesis, gliogenesis, and synaptogenesis. Increasing evidence demonstrates that multiple neurotrophic/growth factor systems, such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF-2) play an important role in neuroplasticity, and that the dysregulation of these neurotrophic/growth factor systems might be involved in mood disorders, including major depressive disorders (MDD) (Evans et al. 2004; Takebayashi et al. 2006; Warner-Schmidt and Duman 2007; Castren and Rantamaki 2010).

This aim of this study was to examine whether the plasma levels of VEGF and FGF-2 are associated with MDD, because these two major growth factors are important for regulating neuroplasticity, particularly neurogenesis. In addition, we also measured plasma levels of

transforming growth factor β-1 (TGF-β1), another growth factor involved in neuroplasticity and immunity.

Materials and methods

Sixteen Japanese patients with MDD (8 males and 8 females; mean age ± SD = 53.2 ± 13.0 years; age range = 25–75 years) were recruited from Musashi Hospital at the National Center of Neurology and Psychiatry (NCNP). All but one of the patients were outpatients. The diagnosis of MDD and evaluation of symptoms were determined according to the DSM-IV (American Psychiatric Association 1994) after a clinical interview and a review of clinical records. All patients were under partial or full remission when their blood was drawn. All but one of the patients were being treated with psychotropic agents, such as antidepressants and/or mood stabilizers. The following antidepressant drugs were being administered to patients: paroxetine ($N = 4$), fluvoxamine ($N = 4$), amoxapine ($N = 4$), sulpiride ($N = 2$), trazodone ($N = 2$), milnacipran ($N = 2$), imipramine ($N = 2$), clomipramine ($N = 1$), amitriptyline ($N = 1$). Three patients received lithium (133 mg/day) as a mood stabilizer, eight patients received combined treatment with more than two types of antidepressants or an antidepressant plus lithium. We estimated that each of the patients was receiving a dose equivalent to 150 mg of imipramine for each antidepressant, based on a previous paper (Takebayashi et al. 2006). Eight patients had a family history (FH) of a first-degree relatives with a psychiatric disorder (MDD = 4, schizophrenia = 3, bipolar disorder = 1), while the other eight patients did not.

Sixteen race, gender, and age-matched healthy control subjects (8 males and 8 females; mean age ± SD = 53.8 ± 12.5 years; age range = 25–71 years) were recruited from local advertisements. Subjects with any other diagnosed mental or physical illness were excluded from the study. Probands who had first-degree relatives with psychiatric disorders were also excluded from the control group.

All subjects, both patients and controls, were non-smokers. Subjects with hypertension, obesity, or blood chemistry values outside normal ranges were excluded. The Ethics Committee of the NCNP approved this study. Written informed consent was obtained from all subjects after a full written and verbal explanation of the study.

The blood samples from the patients and the controls were drawn into tubes with ethylenediaminetetraacetic acid around noon and stored without blood cells in different storage tubes at −80°C until they were assayed. The free levels of VEGF, FGF-2 and TGF-β 1 were measured using the enzyme linked immunosorbent assay (ELISA) (VEGF, Quantikine; FGF-2, Quantikine HS; R&D systems, Minneapolis, MN; TGF-β1, Emax Immunoassay System, Promega, Madison, WI) according to the manufacturer's instructions.

Regarding statistical analysis, the data were described in terms of the means ± standard deviations (SD). A Student's t test was used to analyze the differences between the two groups. In the case of three groups, one-way analysis of variance (ANOVA) was used to determine statistical tendencies. When a significant tendency was suggested, differences between groups were analyzed using a Fisher's protected least significant differences post hoc test. The relationship between two variables was examined using Pearson's correlation coefficient. p values <0.05 were considered to be statistically significant.

Results

As shown in Table 1, plasma VEGF levels were significantly higher in the MDD patients than in control subjects ($p = 0.019$), while no significant difference in plasma FGF-2 levels was found between these two groups ($p = 0.823$). In contrast, the TGF-β 1 level in the MDD patients (526 ± 158 pg/ml) did not differ from the control subjects (455 ± 90.9 pg/ml; $p = 0.126$) (data not shown). One-way ANOVA indicated that there was a significant difference among the MDD patients with a FH, and the MDD patients without a FH, and the control subjects ($F = 3.4, p = 0.047$) with regard to the plasma levels of VEGF. In fact, the plasma VEGF levels in the MDD patients with a FH were higher than those of the control subjects (85.8 ± 73.2 pg/ml; $p = 0.021$), while the plasma VEGF levels in the MDD patients without a FH did not differ from the control subjects (71.0 ± 33.8 pg/ml; $p = 0.11$) (Fig 1). No significant differences in plasma FGF-2 and TGF-β1 levels were found among the three groups (FGF-2, $F = 0.33, p = 0.721$; TGF-β1, $F = 1.95$, $p = 0.160$). No significant correlation was detected between the levels of VEGF, FGF-2 or TGF-β1 and age ($N = 32$; VEGF, $r = 0.17$, $p = 0.34$; FGF-2, $r = 0.078, p = 0.67$; TGF-β1, $r = 0.17, p = 0.34$) in any of the subjects. There were also no gender-related differences in the levels of VEGF, FGF-2 and TGF-β1 ($N = 32$, VEGF, $p = 0.569$: FGF-2, $p = 0.396$: TGF-β1, $p = 0.155$). The age of onset ($r = 0.425$, $p = 0.10$), period of morbidity

Table 1 Plasma VEGF and FGF-2 levels in remitted patients with MDD and normal subjects

Group	VEGF (pg/ml)	FGF-2 (pg/ml)
Control ($N = 16$)	39.0 ± 26.9*	3.19 ± 3.28
MDD ($N = 16$)	78.4 ± 55.6*	3.51 ± 4.62

* $p < 0.05$, Comparison between two groups of control and MDD, values are means ± SD

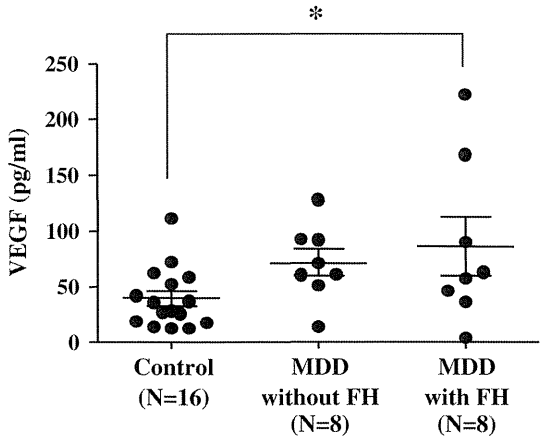


Fig. 1 Higher plasma VEGF concentrations were seen in MDD patients in remission who had a family history (*FH*) of psychiatric disorders compared with control subjects (*control*). The *horizontal lines* indicate the mean ± SD. * Significance level = $p < 0.05$

($r = -0.251$, $p = 0.349$), and antidepressant dose (imipramine equivalents/day) ($r = -0.146$, $p = 0.59$) did not correlate with the plasma levels of VEGF ($N = 16$). Nor was there any correlation between the age of onset (FGF-2, $r = -0.197$, $p = 0.464$; TGF-β1, $r = 0.122$, $p = 0.652$), period of morbidity (FGF-2, $r = -0.211$, $p = 0.432$; TGF-β1, $r = 0.122$, $p = 0.679$) and antidepressant dose (FGF-2, $r = 0.327$, $p = 0.217$; TGF-β1, $r = 0.203$, $p = 0.452$) and the plasma levels of FGF-2 or TGF-β1. There were also no significant differences between the MDD patients with a FH and those without a FH in terms of age (55.0 ± 11.7 vs. 52.5 ± 13.9 years old; $p = 0.921$), age of onset (45.4 ± 15.7 vs. 45.1 ± 14.5 years old; $p = 0.97$), period of morbidity (9.7 ± 9.1 vs. 7.4 ± 8.0 years; $p = 0.60$), and antidepressant dose (157 ± 167 vs. 76.6 ± 49.3 mg/day; $p = 0.22$).

Discussion

We found that plasma VEGF levels were significantly higher in remitted patients with MDD than in matched healthy control subjects. It was interesting to note that the VEGF levels were especially high in the MDD patients with a FH, but not in those without a FH, in comparison to the controls. The increased VEGF plasma protein concentration found in this study is comparable to other studies that found an increase in VEGF mRNA in peripheral leukocytes of MDD patients during SSRI treatment (Iga et al. 2007) and increased VEGF protein in the serum of unmedicated depressed patients with comorbid borderline personality disorder (BPD) (Kahl et al. 2009). In contrast, other studies have shown the serum VEGF levels of MDD patients during SSRI treatment (Ventriglia et al. 2009) and plasma VEGF levels of depressed patients receiving pharmacotherapy to not be altered (Dome et al. 2009). Adding to this controversy, studies in animal models have demonstrated that antidepressants induced an increase in the production of VEGF in the brain, likely resulting in elevated plasma levels of VEGF (Warner-Schmidt and Duman 2007). Although it is difficult to explain the discrepancies, differences in patient age (the mean age of patients in our present study is much higher than previous studies), race, and the duration of pharmacotherapy might have been responsible. Therefore, additional studies are required to investigate the relationship between plasma VEGF levels and drug responses in drug-naïve MDD patients without BPD to clarify whether blood levels of VEGF are associated with state-dependent condition (i.e., the drug medication, psychiatric symptoms or other factors). In addition, none of the previous reports took the patients' FH or genetic factors into consideration. Our study was therefore the first to identify a possible association between plasma VEGF levels and a FH of MDD.

Currently, the mechanism underlying the increase in VEGF levels in the MDD patients with a FH is unknown. Some speculate that a genetic factor could be associated with the increased VEGF production in patients with MDD. Previous studies have shown that there was no significant effect of age, gender and family history (psoriasis) on plasma VEGF levels in healthy subjects and psoriasis patients (Larsson et al. 2002; Barile et al. 2006). So far, there have not been any reports showing a significant association between VEGF gene polymorphisms and MDD, although several studies have looked for such an association (Iga et al. 2007) (Tsai et al. 2009). As previously mentioned, antidepressants induce an increase in the production of VEGF through a neurogenic action (Warner-Schmidt and Duman 2007). Although the mechanism is unknown, our findings suggest that a genetic factor might be associated with the pathway linking VEGF production to antidepressant in pathophysiology of the MDD patients with a FH. Further studies are needed to clarify this hypothesis.

Regarding FGF-2 and TGF-β1 levels, there were no significant differences among the MDD patients with a FH, those without a FH and the control subjects. A previous study demonstrated that the whole blood levels of TGF-β1 in the MDD patients did not differ from the control subjects (Takebayashi et al. 2006). In contrast, another study demonstrated an increase in serum FGF-2 levels in depressed patients with BPD who were receiving pharmacotherapy (Kahl et al. 2009). This previous study did not examine the differences between patients with BPD and those without BPD, so further investigations are needed to determine the impact of BPD on the FGF-2 levels. Further, postmortem and animal studies have suggested that

antidepressant treatment could induce FGF-2 production in the brain (Evans et al. 2004; Bachis et al. 2008). Similarly, additional studies in drug-naïve MDD patients will be needed.

A variety of cells, not only glia and neurons, but also macrophages, T cells and smooth muscle cells, produce VEGF, FGF-2 and TGF-β1 (Kahl et al. 2009). However, the source of increased VEGF was not the focus of our study and remains unclear.

This study is a preliminary report that has a number of limitations, including the small number of patients and the variety of medications that were being used. Recent biochemical studies on mood disorders have revealed alterations in neurotrophic factor systems such as BDNF and GDNF in the brain, as well as a blood marker (Shimizu et al. 2003; Takebayashi et al.2009), although a direct link between either of these proteins and MDD is still unclear. Taken together with our results, dysregulation of multiple neurotrophic/growth factor systems, including VEGF, may be involved in the etiology of complex mood disorders. Further in-depth studies will be necessary.

Acknowledgments This study was supported by the Research Grants for Nervous and Mental Disorders from the NCNP, Health and Labor Sciences Research Grants (Research on Psychiatric and Neurological Diseases and Mental Health), a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), and JST, CREST.

References

American Psychiatric Association (1994) Diagnostic and statistical manual of mental disorders, 4th edn. American Psychiatric Press, Washington, DC

Bachis A, Mallei A, Cruz MI, Wellstein A, Mocchetti I (2008) Chronic antidepressant treatments increase basic fibroblast growth factor and fibroblast growth factor-binding protein in neurons. Neuropharmacology 55(7):1114–1120

Barile S, Medda E, Nistico L, Bordignon V, Cordiali-Fei P, Carducci M, Rainaldi A, Marinelli R, Bonifati C (2006) Vascular endothelial growth factor gene polymorphisms increase the risk to develop psoriasis. Exp Dermatol 15(5):368–376

Castren E, Rantamaki T (2010) The role of BDNF and its receptors in depression and antidepressant drug action: reactivation of developmental plasticity. Dev Neurobiol 70(5):289–297

Dome P, Teleki Z, Rihmer Z, Peter L, Dobos J, Kenessey I, Tovari J, Timar J, Paku S, Kovacs G, Dome B (2009) Circulating endothelial progenitor cells and depression: a possible novel link between heart and soul. Mol Psychiatry 14(5):523–531

Evans SJ, Choudary PV, Neal CR, Li JZ, Vawter MP, Tomita H, Lopez JF, Thompson RC, Meng F, Stead JD, Walsh DM, Myers RM, Bunney WE, Watson SJ, Jones EG, Akil H (2004) Dysregulation of the fibroblast growth factor system in major depression. Proc Natl Acad Sci USA 101(43):15506–15511

Iga J, Ueno S, Yamauchi K, Numata S, Tayoshi-Shibuya S, Kinouchi S, Nakataki M, Song H, Hokoishi K, Tanabe H, Sano A, Ohmori T (2007) Gene expression and association analysis of vascular endothelial growth factor in major depressive disorder. Prog Neuropsychopharmacol Biol Psychiatry 31(3):658–663

Kahl KG, Bens S, Ziegler K, Rudolf S, Kordon A, Dibbelt L, Schweiger U (2009) Angiogenic factors in patients with current major depressive disorder comorbid with borderline personality disorder. Psychoneuroendocrinology 34(3):353–357

Larsson A, Skoldenberg E, Ericson H (2002) Serum and plasma levels of FGF-2 and VEGF in healthy blood donors. Angiogenesis 5(1–2):107–110

Pittenger C, Duman RS (2008) Stress, depression, and neuroplasticity: a convergence of mechanisms. Neuropsychopharmacology 33(1):88–109

Shimizu E, Hashimoto K, Okamura N, Koike K, Komatsu N, Kumakiri C, Nakazato M, Watanabe H, Shinoda N, Okada S, Iyo M (2003) Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. Biol Psychiatry 54(1):70–75

Takebayashi M, Hisaoka K, Nishida A, Tsuchioka M, Miyoshi I, Kozuru T, Hikasa S, Okamoto Y, Shinno H, Morinobu S, Yamawaki S (2006) Decreased levels of whole blood glial cell line-derived neurotrophic factor (GDNF) in remitted patients with mood disorders. Int J Neuropsychopharmacol 9(5):607–612

Takebayashi M, Hisaoka K, Tsuchioka M (2009) Glial dysfunction in mood disorders: the role of GDNF. In: Shirayama Y, Chaki S (eds) Recent developments on depression research. Research Signpost, Kerala, pp 125–143

Tsai SJ, Hong CJ, Liou YJ, Chen TJ, Chen ML, Hou SJ, Yen FC, Yu YW (2009) Haplotype analysis of single nucleotide polymorphisms in the vascular endothelial growth factor (VEGFA) gene and antidepressant treatment response in major depressive disorder. Psychiatry Res 169(2):113–117

Ventriglia M, Zanardini R, Pedrini L, Placentino A, Nielsen MG, Gennarelli M, Bocchio-Chiavetto L (2009) VEGF serum levels in depressed patients during SSRI antidepressant treatment. Prog Neuropsychopharmacol Biol Psychiatry 33(1):146–149

Warner-Schmidt JL, Duman RS (2007) VEGF is an essential mediator of the neurogenic and behavioral actions of antidepressants. Proc Natl Acad Sci USA 104(11):4647–4652