

The *DYRK1A* gene, encoded in chromosome 21 Down syndrome critical region, bridges between β -amyloid production and tau phosphorylation in Alzheimer disease

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We scanned throughout chromosome 21 to assess genetic associations with late-onset Alzheimer disease (AD) using 374 Japanese patients and 375 population-based controls, because trisomy 21 is known to be associated with early deposition of β -amyloid (A β) in the brain. Among 417 markers spanning 33 Mb, 22 markers showed associations with either the allele or the genotype frequency ($P < 0.05$). Logistic regression analysis with age, sex and apolipoprotein E (APOE)- $\epsilon 4$ dose supported genetic risk of 17 markers, of which eight markers were linked to the *SAMSN1*, *PRSS7*, *NCAM2*, *RUNX1*, *DYRK1A* and *KCNJ6* genes. In logistic regression, the *DYRK1A* (dual-specificity tyrosine-regulated kinase 1A) gene, located in the Down syndrome critical region, showed the highest significance [OR = 2.99 (95% CI: 1.72–5.19), $P = 0.001$], whereas the *RUNX1* gene showed a high odds ratio [OR = 23.3 (95% CI: 2.76–196.5), $P = 0.038$]. *DYRK1A* mRNA level in the hippocampus was significantly elevated in patients with AD when compared with pathological controls ($P < 0.01$). *DYRK1A* mRNA level was upregulated along with an increase in the A β -level in the brain of transgenic mice, overproducing A β at 9 months of age. In neuroblastoma cells, A β induced an increase in the *DYRK1A* transcript, which also led to tau phosphorylation at Thr²¹² under the overexpression of tau. Therefore, the upregulation of *DYRK1A* transcription results from A β loading, further leading to tau phosphorylation. Our result indicates that *DYRK1A* could be a key molecule bridging between β -amyloid production and tau phosphorylation in AD.

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INTRODUCTION

Alzheimer disease (AD) is the major cause of dementia in the elderly and is pathologically characterized by senile plaques with β -amyloid deposition ($A\beta$) and neurofibrillary tangles harboring hyperphosphorylated tau in the brain. It is well established that familial autosomal-dominant early onset AD is mostly caused by mutations of the amyloid protein precursor (*APP*) and presenilin 1 and 2 (*PS1* and *PS2*) genes (1). In contrast, Down syndrome (DS) is also highlighted as a model condition predisposing to AD, because patients with DS develop early deposition of $A\beta$ in the brain (2). Therefore, it has been speculated that genetic factors related to AD could exist on chromosome 21, independent of the $\epsilon 4$ allele of the apolipoprotein E gene (*APOE- $\epsilon 4$*), a known strong risk for late-onset AD (3,4). Using the candidate approach, it was reported that duplication of the *APP* gene was transmitted in patients with familial autosomal-dominant early onset AD with cerebral angiopathy (5), whereas an association with the *APP* gene, to the best of our knowledge, was not supported in case-control studies (6–8). The *BACE2* gene, encoding β -secretase of *APP*, was not associated with AD; however, recent studies showed weak associations (9–11). In contrast, with the positional approach, genome scans of late-onset AD showed positive linkage on chromosome 21 (12,13). Although this linkage remains controversial (14–16), a locus strongly influencing age at onset was also found on chromosome 21 (17). To search for genetic factors for late-onset AD on chromosome 21, we scanned throughout this chromosome using patients with Japanese late-onset AD and population-based controls, by a stepwise single nucleotide polymorphism (SNP) scan. We report that the *DYRK1A* gene is a genetic factor related to the progression of AD.

RESULTS

Chromosome 21 scan

An exploratory scan of chromosome 21 was performed in 188 AD and 375 controls, using 417 SNPs at an average interval of <100 kb, including at least one SNP in each coding region. Selected SNP markers were distributed between base positions 14 440 543 and 46 915 057 based on NCBI Build 35, whereas no SNP closer to the centromere was included because of the duplicated region in the chromosome 21 sequence (18). Using a threshold of $P < 0.05$ for allele frequency, we detected 14 SNPs, which is less than the predicted 21 markers. Therefore, to reduce type II error, we also tested genotype frequency in both dominant and recessive models (Fig. 1). Finally, the exploratory scan detected 42 SNPs in total (10.0%), among which 14 SNPs were significant in both allele and genotype frequencies, of which one positive region was identified in the Down syndrome critical region (DSCR) (19–21). The confirmatory scan targeting the selected 42 SNPs indicated that 22 SNPs were still significant for either allele or genotype frequency (Table 1). Among those, 17 SNPs were also significant by logistic regression for the risk genotype with age, sex and *APOE- $\epsilon 4$* dose. Genes linked to these SNPs were the

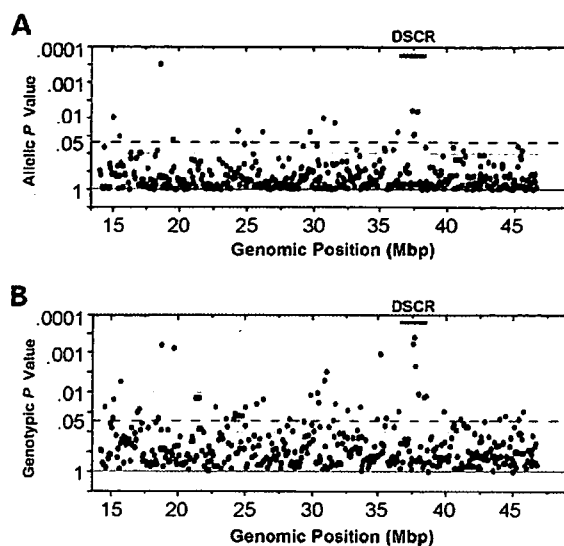


Figure 1. Exploratory scan using 417 markers. (A) P -values for allele frequency in chi-squared test. (B) P -values for genotype frequency in better fitting models. Genomic position is based on NCBI Build 35.

SAMSNI, *PRSS7*, *NCAM2*, *RUNXI*, *DYRK1A* and *KCNJ6* genes and those linked to unknown open reading frames were C21ORF 63, 55 and 5. In logistic regression, the *DYRK1A* gene, located in the middle of the DSCR, showed the highest significance [OR = 2.99 (95% CI: 1.72–5.19), $P = 0.001$], whereas the *RUNXI* gene showed a very high odds ratio [OR = 23.3 (95% CI: 2.76–196.5), $P = 0.038$].

Haplotype analysis of *DYRK1A*

SNPs located in the *DYRK1A* gene region were genotyped to determine the haplotype associated with AD. Linkage disequilibrium was identified in the control group from 30 kb upstream of exon 1 to intron 9, but not in exon 13 genotyped by rs1803439 which was not in Hardy–Weinberg equilibrium, and the AD group showed similar results (Fig. 2). Haplotype analysis indicated that three haplotypes had significantly different frequencies between AD and controls, whereas the permutation test supported significant differences in two haplotypes. Considering the haplotype frequencies, rs18126696 alleles could represent the risk haplotype (Table 2). We also sequenced all coding regions of the *DYRK1A* gene in six patients and three controls homozygous for the risk allele, but no sequence alteration was found.

DYRK1A mRNA in hippocampus of AD

DYRK1A mRNA in the hippocampus was measured by quantitative polymerase chain reaction (PCR) to examine the relation with the occurrence of AD and with the genotype of rs18126696. *DYRK1A* mRNA level in the patients was significantly different ($P < 0.01$), being ~ 7 -fold greater than that in pathological controls (Fig. 3A). In contrast, patients homozygous for the risk rs18126696-c allele showed a tendency for a decrease in *DYRK1A* mRNA level compared with the others,

Table 1. Genes linked to markers associated with AD on chromosome 21

Marker	Association ^a (<i>P</i>)		Logistic regression ^b		Gene
	Allele	Genotype ^c	Odds (95% CI)	<i>P</i> -value	
rs723856	0.019	0.012 (aa)	1.53 (1.08–2.18)	0.0181	<i>SAMSN1</i>
rs2268437	0.008	0.008 (aa)	2.09 (1.24–3.55)	0.0059	<i>PRSS7</i>
rs2212624 ^d	0.058	0.003 (gg)	1.66 (1.17–2.35)	0.0046	<i>NCAM2</i>
rs2833844	0.033	0.030 (cc)	1.74 (1.11–2.73)	0.0166	C21 orf 63
rs28360609 ^d	0.128	0.017 (aa)	3.43 (1.31–8.95)	0.0119	C21 orf 55
rs4816501	0.224	0.004 (tt)	23.3 (2.76–196.5)	0.0038	<i>RUNX1</i>
rs1023367	0.054	0.036 (cc, ct)	1.40 (0.96–2.05)	0.0839	C21 orf 5
rs2835740	0.035	0.001 (cc)	2.99 (1.72–5.19)	0.0001	<i>DYRK1A</i>
rs2835908	0.024	0.056 (cc)	1.55 (0.99–2.43)	0.0546	<i>KCNJ6</i>

^aOne-sided *P*-value in chi-squared test.

^bLogistic regression of risk genotype with age, sex and APOE-ε4 dose under no interaction.

^cRisk genotypes in a better fitting model are shown in parentheses.

^dAD group showed deviation from the Hardy–Weinberg equilibrium.

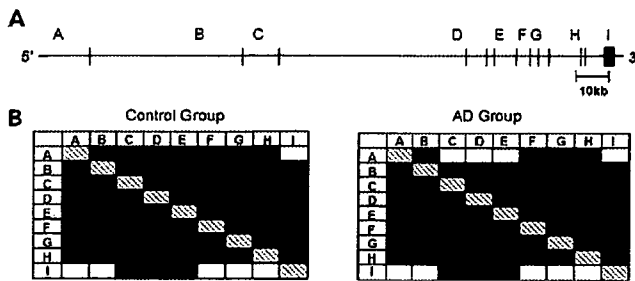


Figure 2. Linkage disequilibrium in *DYRK1A* gene region. (A) Genomic structure of the *DYRK1A* gene is shown. Horizontal bar indicates exons, and letters indicate SNPs, such as rs28360609 (A), rs2251085 (B), rs2835740 (C), rs10470178 (D), rs11701810 (E), rs1024294 (F), rs2835773 (G), rs2835774 (H) and rs1803439 (I). (B) r^2 (upper right) and $|D'|$ values (lower left) were judged significant at less than 0.5 and 0.9, respectively, and significant values are shown by dark boxes.

but this was not significant (Fig. 3B). Thus, the increased expression of *DYRK1A* mRNA is possibly a consequence of AD.

DYRK1A mRNA and Aβ in transgenic mouse brain

We examined whether Aβ loading is related to *DYRK1A* mRNA level in the brain in PS1^{1213T}KI and Tg-PS1/APP mice. Aβ_{1–40} level in PS1^{1213T}KI mice was low, but Aβ_{1–40} was almost undetectable, whereas both Aβ_{1–40} and Aβ_{1–42} were elevated in Tg-PS1/APP mice (Fig. 4A and B), suggesting that Tg-PS1/APP mice have an Aβ burden in their brain. Quantitative PCR showed that the *DYRK1A* mRNA level was significantly increased in Tg-PS1/APP mice when compared with that in PS1^{1213T}KI mice ($P < 0.05$) by 1.2-fold (Fig. 4C). Thus, the expression of *DYRK1A* mRNA increased along with Aβ loading in the mouse brain.

DYRK1A mRNA, Aβ and tau phosphorylation in cell models

We examined whether Aβ, a major component of senile plaques in the AD brain, induces expression of *DYRK1A*

Table 2. Haplotype case–control study for *DYRK1A* gene

Haplotype ^a	Frequency		<i>P</i> -value		
	Overall	AD	Control	Chi-squared	Permutation
2-1-2-1-1-1-2-1	0.500	0.467	0.532	0.0147	0.013
1-2-1-2-2-2-1-2	0.312	0.337	0.287	0.0395	0.051
1-2-2-1-1-2-1-2	0.065	0.064	0.067	0.8369	0.844
1-1-2-1-1-1-2-1	0.065	0.080	0.050	0.0216	0.017
2-1-2-1-1-1-2-1	0.031	0.025	0.038	0.1582	0.171
1-2-1-2-2-2-1-2	0.016	0.018	0.015	0.6984	0.745
1-2-2-2-2-2-1-2	0.011	0.010	0.013	0.6268	0.632

^aHaplotypes were constructed with markers composed of rs8126696 (allele 1 = c, allele 2 = t)–rs2251085 (c/g)–rs2835740 (c/t)–rs10470178 (a/g)–rs11701810 (a/c)–rs1024294 (c/t)–rs2835773 (a/g)–rs2835774 (a/t). Chi-squared for the overall haplotypes ($df = 6$) was significant by the EM algorithm ($P = 0.040$) as well as by the permutation method ($P = 0.038$).

mRNA in cultured neuroblastoma cells. SH-SY5Y cells were incubated with Aβ, and then total RNA was extracted and quantified (Fig. 5). *DYRK1A* mRNA level was significantly increased by 1.6-fold ($P < 0.05$) with 0.5 μM Aβ_{1–42} and by 1.3-fold ($P < 0.01$) with 25 μM Aβ_{25–35}, compared with the level in non-treated cells, but was not changed with control 25 μM Aβ_{35–25}. Thus, Aβ loading resulted in an increase in the *DYRK1A* transcription. In an *in vitro* experiment, *DYRK1A* protein not only phosphorylates itself, but also has a large repertoire of phosphorylation (22). Therefore, we examined whether *DYRK1A* overexpression induces phosphorylation of tau at the cellular level. An immunoblot of HEK293T cells transiently transfected with the *MAPT* expression vector showed a detectable amount of tau along with those phosphorylated at Thr²¹² (Fig. 6A). Tau phosphorylated at Thr²¹² was increased by co-transfection of the *DYRK1A* expression vector, compared with that of mock vector, whereas tau level was similar (Fig. 6A). Densitometric quantification supported the induction of phosphorylation by 1.5-fold ($P < 0.01$) (Fig. 6B). Thus, the increase in the *DYRK1A* transcription under overexpression of tau induced tau phosphorylation at Thr²¹².

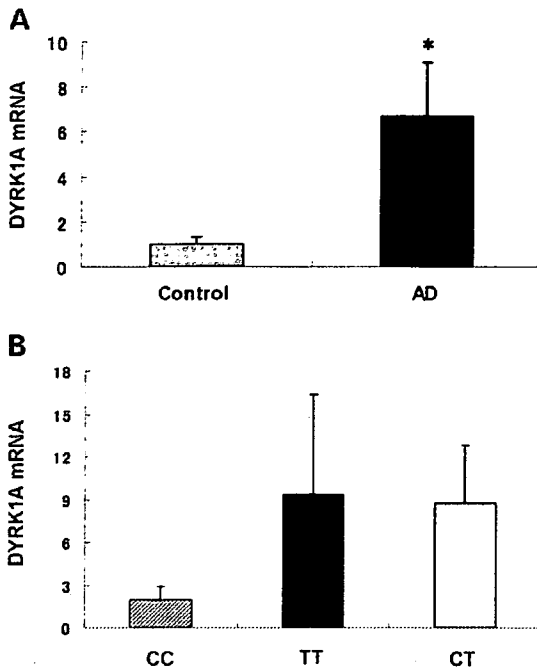


Figure 3. Expression of *DYRK1A* mRNA in human hippocampus. (A) Quantitative real-time PCR of *DYRK1A* mRNA in AD ($n = 22$) and controls ($n = 12$). (B) *DYRK1A* mRNA level in AD brain divided by rs28360609 genotypes, where CC is the risk genotype. *DYRK1A* mRNA level was expressed as the ratio of that of *GAPDH*. Data are shown as mean \pm SEM. * $P < 0.01$ by Mann-Whitney's *U*-test.

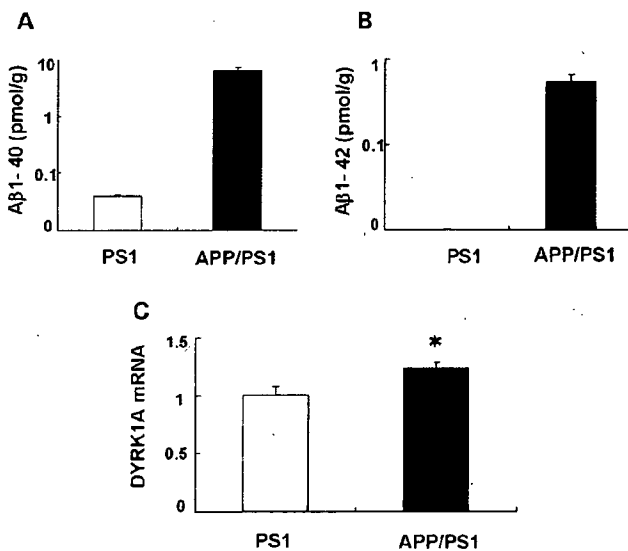


Figure 4. A β -level and expression of *DYRK1A* mRNA in transgenic mouse brain. Heterozygous PS1¹²¹³¹KI (PS1, $n = 6$) and Tg-APP/PS1 (APP/PS1, $n = 6$) mice were sacrificed at 9 months of age. A β -level was measured by ELISA. *DYRK1A* mRNA level was measured by quantitative real-time PCR. (A) A β 1-40 level, (B) A β 1-42 level and (C) amount of *DYRK1A* mRNA. *DYRK1A* mRNA level was expressed as the ratio of that of *GAPDH*. Data are shown as mean \pm SEM. * $P < 0.01$ by Student's *t*-test.

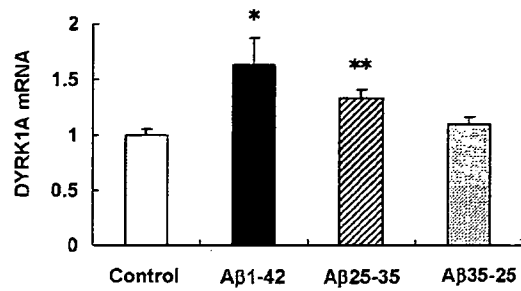


Figure 5. A β -induced expression of *DYRK1A* mRNA in SH-SY5Y cells. SH-SY5Y cells were incubated with A β 1-42, A β 25-35 and A β 35-25. *DYRK1A* mRNA level was measured by quantitative real-time PCR. Values were normalized to those in untreated cells. *DYRK1A* mRNA level was expressed as the ratio of that of *GAPDH*. Data are shown as mean \pm SEM of four independent measurements. ** $P < 0.01$ and * $P < 0.05$ by Student's *t*-test compared with control.

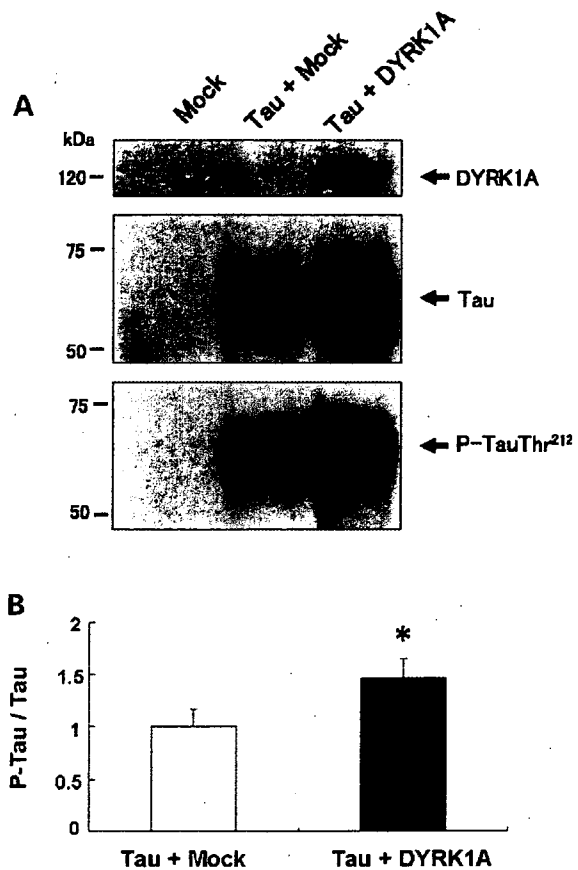


Figure 6. Tau hyperphosphorylation in *DYRK1A*-overexpressing cells. (A) HEK293T cells were transfected with either the *MAPT* expression vector (Tau) or both the *MAPT* and *DYRK1A* expression vectors (Tau + *DYRK1A*). After 24 h incubation, lysates were immunoprecipitated with anti-FLAG M2 agarose and then subjected to immunoblotting with anti-*DYRK1A* (*DYRK1A*), anti-tau (Tau) or anti-phosphotau (P-TauThr²¹²). (B) P-TauThr²¹²/Tau ratio was measured as integrated optical density values. Data are shown as mean \pm SEM of four independent measures. * $P < 0.01$ by Student's *t*-test.

Discussion

Genome scanning using case-control studies, based on linkage disequilibrium, is a strategy to identify genetic factors of polygenetic diseases. In general, many susceptibility genes have been reported, but it remains difficult to replicate the results in different studies. This could possibly be caused by selection bias in patients as well as in controls, because hospital-based control subjects often suffer from another disease, leading to an additional background of that disease. Therefore, we used population-based controls to match the phenotypic background. From the exploratory and confirmatory scans, we identified 22 candidate SNPs associated with late-onset AD on chromosome 21. Although we showed their risk effects in logistic regression with age, sex and *APOE-ε4* dose, a known major risk for AD (3), these candidates need to be confirmed, because *P*-values were inconclusive when considering the comparison of multiple loci.

We found associations of AD with markers linked to six known genes, but not with reported candidates, the *APP* and *BACE2* genes. The *SAMSNI* gene encodes a member of putative adaptors and scaffold proteins containing SH3 and sterile alpha motif domains, expressed mainly in immune tissues and hematopoietic cells and also at lower levels in the heart, brain, placenta and lung (23). The *DYRK1A* gene, located in the DSCR, is a candidate gene responsible for learning and memory impairment in patients with DS (24,25). The *PRSS7* gene encodes enteropeptidase (EC 3.4.21.9), an intestinal enzyme initiating activation of pancreatic proteolytic proenzymes such as trypsin, chymotrypsin and carboxypeptidase A, which are highly expressed in the intestines and at a low level in the brain of rat (26), but is downregulated in amniotic fluid cells in patients with DS (27). The neural cell adhesion molecule 2 (*NCAM2*) gene is expressed in fetal and adult brains (28), sharing many features with immunoglobulins and mediating adhesion among neurons and between neurons and muscle (29) and having a potential regulatory role in the formation of selective axonal projections of olfactory sensory neurons in mice (30). The *RUNX1* gene, also called *AML1*, encodes runt-related transcription factor 1, which is required for active repression in CD4-negative/CD8-negative thymocytes, and a defective *RUNX1* gene causes a familial platelet disorder with predisposition to acute myelogenous leukemia (31). The mouse *RUNX1* homolog is expressed in selected populations of post-mitotic neurons of the embryonic central and peripheral nervous systems (32). The *KCNJ6* gene, located in the DSCR, encodes a G protein-coupled inwardly rectifying potassium channel and is expressed in the brain and pancreatic beta cells (33,34). A *kcnj6* mutation was found in the weaver mouse characterized by ataxia with reduced size of the cerebellum because of depletion of granule cell neurons (35).

DYRK1A is a mammalian ortholog of the *Drosophila mini-brain* gene, which is essential for normal post-embryonic neurogenesis (36). In rodents, *DYRK1A* mRNA is expressed ubiquitously in various tissues during development and is also strongly expressed in the adult brain and heart (20, 37–39). In humans, *DYRK1A* mRNA is expressed especially in the brain, and immunoreactive *DYRK1A* is found in the cerebral cortex, hippocampus and cerebellum and is

overexpressed in the DS brain in a dose-dependent manner (40,41). Transgenic mice overexpressing full-length *DYRK1A* mRNA exhibit neurodevelopmental delay, motor abnormalities and cognitive deficit, suggesting a causative role of the *DYRK1A* gene in mental retardation and motor anomalies of DS (24,25). It was noted that all adults with DS over the age of 40 years develop sufficient neuropathology for a diagnosis of AD (42). The identification of the *DYRK1A* gene as a genetic factor strongly supports that the *DYRK1A* gene is involved in the development of AD.

We demonstrated an increase in the *DYRK1A* mRNA level in post-mortem brains, coinciding with the recent report of *DYRK1A* immunoreactivity in the neocortex and hippocampus in AD (41). The risk genotype of the *DYRK1A* gene showed a tendency for a decrease in the *DYRK1A* mRNA level, but our observation needs to be carefully considered because the result might be caused by the reduction of neuronal cells in the AD brain. However, no studies have yet examined the relationship between the *DYRK1A* gene and Aβ. Genetic and pathological evidence strongly supports the amyloid cascade hypothesis that Aβ42, a proteolytic derivative of the APP protein, has an early and pivotal role in all cases of AD. It is thought that Aβ42 forms aggregates that initiate the pathogenic cascade, leading ultimately to neural loss and dementia (43). We demonstrated that Aβ, especially Aβ42, results in an increase of *DYRK1A* transcription in human neuroblastoma cells and is also observed in transgenic mouse models. Therefore, the increase in *DYRK1A* transcription is a common feature of AD and DS and could relate to the cognitive impairment in patients with AD.

The *DYRK1A* enzyme has dual substrate specificity: autophosphorylation for self-activation takes place on the Tyr³²¹ residue in the active loop of the catalytic domain (44) and target protein phosphorylation occurs on serine/threonine residues in several proteins, including STAT3, FKHR, Gli-1, eIF2Be, tau, dynamin, glycogen synthase, 14-3-3, CREB, cyclin L2, Arip4, Hip-1 and PAHX-AP1, indicating that *DYRK1A* may participate in many biological pathways (22). We showed that overexpression of the *DYRK1A* gene phosphorylates tau at Thr²¹² in HEK293T cells overproducing tau, suggesting that tau phosphorylation at Thr²¹² by *DYRK1A* could be a downstream consequence of Aβ overproduction. It was shown in an *in vitro* experiment that *DYRK1A* phosphorylates tau at Thr²¹², which primes tau for phosphorylation by GSK3-β at Ser²⁰⁸, leading to the formation of paired helical filaments composed of highly phosphorylated tau, a component of neurofibrillary tangles (41). However, transgenic mice overexpressing *DYRK1A* did not show this phosphorylation, and this phosphorylation is highly susceptible to dephosphorylation by protein phosphatase-1, which is expressed in the frontal lobes of the brain, indicating that tau phosphorylation at Thr²¹² could be prohibited *in vivo* (45,46). On the contrary, it was noted that peptides of tau phosphorylated at Thr²¹² completely block Aβ binding, and *DYRK1A* mediated phosphorylation of Huntingtin-interacting protein 1 (Hip-1) in response to βFGF, resulting in the blockade of Hip-1-mediated neuronal cell death as well as the enhancement of neurite outgrowth (47,48). Therefore, tau phosphorylation at Thr²¹² could be a protective response against neuronal cell death. Although overexpression of *DYRK1A* could be a common phenomenon

between AD and DS, neuropathological studies might elucidate how the pathway from overexpression of DYRK1A to phosphorylation of tau is related to the severity of Alzheimer pathology.

Our study provides evidence that the *DYRK1A* gene is a genetic factor for AD, whose expression is increased by A β loading in neuroblastoma cells and transgenic mice, resulting in hyperphosphorylation of tau at Thr²¹² under overexpression of tau. The *DYRK1A* gene could be responsible for learning and memory deterioration in DS (24,25), and a *DYRK1A* inhibitor has been proposed as a novel drug to address learning and memory deficit in DS (49). Our findings suggest that *DYRK1A* upregulation is a key phenomenon as a consequence of A β loading in AD, connecting the condition to DS, and we propose a possible relation between the *DYRK1A* gene and memory impairment in AD.

MATERIALS AND METHODS

Sample-set characteristics

Patients with late-onset AD were diagnosed as having definite or probable AD according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (50). Non-demented control subjects, tested by a questionnaire including the date, orientation and past history, were obtained from population-based elderly subjects. Written informed consent to participate in this study was obtained, and then peripheral blood was drawn and subjected to DNA extraction. The number of patients for the scan was 374 (70.6% female), composed of 73 with definite and 301 with probable AD; mean \pm SD age at onset was 73.0 \pm 8.0, range 60–94 years and age at blood drawing was 78.2 \pm 8.3, range 60–98 years. Controls were composed of 375 individuals (54.7% female); age at assessment was 75.5 \pm 4.85, range 66–92 years. Brain hippocampal tissue was also obtained from the post-mortem brains of 22 patients with AD (age: 82.8 \pm 8.5 years, 63.6% female) and 12 pathological controls (age: 89.0 \pm 7.0 years, age at onset: 72.9 \pm 7.2 years and 58.0% female). DNA was extracted from peripheral blood nuclear cells by phenol–chloroform method or using a QIAamp DNA Blood Kit (Qiagen, Tokyo, Japan). The procedure to obtain the specimens was approved by the Genome Ethical Committee of Osaka University Graduate School of Medicine, Ehime University and the Ethical Committee of Fukushima Hospital.

Genotyping and sequencing

An exploratory scan was performed in 188 patients (67.0% female) (age at onset: 75.0 \pm 7.2 and range 60–92 years) and 375 controls matched for age. A confirmatory scan was performed in 374 patients including 175 who underwent exploratory scan, and the data were compared with the genotype data of controls in the exploratory scan. The whole genomic DNA was amplified by degenerate oligonucleotide-primed-PCR and used in the confirmatory scan, because of the small amount of DNA (51). The accuracy of genotyping in the confirmatory scan was monitored by comparison with data obtained in the

Table 3. Primer sequences for *DYRK1A* gene

Exon	Primer sequences (5'–3')		Product size (bp)
	Forward	Reverse	
1	gttttctcacacagtg	ccccactaactgct	207
1	gttttctcacacagtg	ccccactaactgct	207
2	atgtcaaatgatacaaca	tttccaactccataatc	394
3	gcagggtacagaagagggga	agggtaaataggtcacact	258
4	ctcaaatgtcaactgtag	aacaacaagatcactaag	359
5	tgaatagaaatagatggc	tgccaacagaaataaca	445
6	taactgaactctgctttg	atactactactgtcctacc	471
7	gaagttaatacaatggaac	tattcaactgaactcac	413
8	ctgtatgctggatgct	aacacactgattcaagt	372
9	atatgtgaggtttacg	gtaactgctccccac	481
10	taaccagacttcattgt	gtcattctaaggcaccct	433
11	tgaatgtattgggattttgtg	actgtgactgggatgtg	1063
11	tattgggattttgtg		(For sequencing)
11	ctgctcctctgg		(For sequencing)
11	caagattctatggagg		(For sequencing)
11	cgtctactccaatcc		(For sequencing)

exploratory scan. The selected markers were 417 SNPs distributed in chromosome 21, spanning a region of 33 Mb, which was sequenced and reported by the Chromosome 21 Mapping and Sequencing Consortium (18). Mean interval of the markers in NCBI Build 35 was 78.1 kb, and their range was 7.7–240.0 kb, and 15 intervals were over 100 kb where no coding region was predicted on the basis of the SNP information in using SNPbrowser Software Version 3.5 on NCBI Build 35, available from <http://www.appliedbiosystems.com/>. Genotyping was performed by a quantitative genotyping method using the TaqMan SNP Genotyping System (Applied Biosystems, Foster City, CA, USA). DNA obtained from six patients and three controls homozygous for the risk genotype of the *DYRK1A* gene was subjected to direct sequencing of its exons, using the primers listed in Table 3.

Quantitative real-time PCR

Total RNA was isolated from frozen brains using the acid guanidine–phenol–chloroform RNA extraction method provided as ISOGEN (Nippon Gene, Toyama, Japan), and purified using an RNAeasy Mini kit (Qiagen). RNA samples with an A₂₆₀/A₂₈₀ absorption ratio over 1.9 were subjected to cDNA synthesis using a High-Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR was carried out in an ABI PRISM 7900HT (Applied Biosystems), and primers/probe sets for the *DYRK1A* and *GAPDH* genes of human and mouse were purchased from TaqMan Gene Expression Assay Products (Applied Biosystems). All quantitative PCR reactions were duplicated, and the ratio of the amount of *DYRK1A* cDNA to that of *GAPDH* internal control cDNA at a threshold in the mid-log phase of amplification was used to compare the amount of *DYRK1A* mRNA.

Transgenic mice

The PS1^{I213T}KI mouse, with a 'knocked-in' human PS1 I213T mutation in the mouse presenilin 1 gene (52,53), was bred with Tg2576 mice expressing the human *APP* gene harboring

the K670N/M671L Swedish mutation (Taconic) (54). PS1^{1213T}KI and double transgenic (Tg-APP/PS1) mice were maintained on the B6 background. Six heterozygous Tg-APP/PS1 and six PS1^{1213T}KI mice were sacrificed at age 9 months under anesthesia, and their brains were dissected and stored at -80°C until use. All animal procedures were reviewed by the Institutional Animal Care and Use Committee of Shionogi & Co., Ltd. Every effort was made to minimize the number of animals used and their suffering.

Cell culture

Human neuroblastoma (SH-SY5Y) cells were grown in F12 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA), and human embryonic kidney (HEK293T) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% FBS. Amyloid peptides (Sigma-Aldrich, St Louis, MO, USA) were dissolved in phosphate-buffered saline, followed by incubation at 37°C for 72 h. SH-SY5Y cells were incubated for 20 h with A β at $0.5\ \mu\text{M}$ for A β 1–42 and at $25\ \mu\text{M}$ for A β 25–35 and A β 35–25. Total RNA was isolated from harvested cells using an RNeasy Mini kit, and then synthesized cDNA was subjected to quantitative PCR. The human long isoform of *MAPT* cDNA, obtained from Dr Goedert (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK), was cloned in pcDNA3.1 (Invitrogen), and the FLAG epitope-tagged *DYRK1A* expression vector was cloned in pEGFPC2 (55,56). These vectors were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen), followed by their expression for 24 h, and the cells were harvested and subjected to biochemical experiments.

Biochemical experiments

In transgenic mice, the hemisphere of each brain was homogenized in Tris-buffered saline (TBS) composed of 137 mM NaCl and 20 mM Tris, pH 7.6, containing 1% Triton X-100 with Complete™ protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA), followed by ultracentrifugation, and the supernatant was subjected to measurement of A β 1–40 and A β 1–42 levels using a sandwich ELISA kit (Biosource International, Camarillo, CA, USA). In cell experiments, cells were lysed in lysis buffer composed of 150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, protease inhibitor mixture (Sigma-Aldrich) and phosphatase inhibitor cocktail (Pierce, Rockford, IL, USA). After centrifugation at 10 000g for 15 min at 4°C , protein extracts were obtained as the supernatant and quantified using BCA Protein Assay (Pierce). For immunoprecipitation, 300 μg of protein lysate was incubated with 20 μl anti-FLAG M2 agarose (Sigma-Aldrich) with gentle rotation at 4°C overnight, and after centrifugation, the precipitate was dissolved in SDS sample buffer, electrophoresed in 8% SDS-PAGE and blotted onto nitrocellulose membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). After blocking with 5% milk in TBS buffer composed of 0.1% Tween-20, 140 mM NaCl and 10 mM Tris-HCl, pH 7.6, the membranes were incubated overnight at

4°C with primary antibodies, such as polyclonal antibody to phosphotau (P-TauThr²¹²) (Biosource International) diluted to 1:500 or polyclonal antibodies to DYRK1A (Abcam, Cambridge, MA, USA) at 1:200 or to tau (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:500. The membranes were washed and then incubated with peroxidase-conjugated secondary antibodies against mouse, rabbit or sheep immunoglobulin (Promega, Madison, WI, USA), followed by washing and developing with an ECL Plus Western Blotting Detection System (GE Healthcare Bio-Sciences). The immunoreactive bands on films were digitized with an HP 2355 and subjected to densitometric quantification using Image J version 1.36 (National Institute of Health).

Statistical analysis

To reduce type II errors, the exploratory and confirmatory scans were assessed for associations by one-sided chi-squared test for both allele and genotype frequencies in dominant and recessive models, where each α -level was 0.05. For markers showing significant associations in the confirmatory scan, the Hardy-Weinberg equilibrium was tested. The risk genotypes in the better fitting model were given a value of 1 and the other genotypes 0, and then logistic regression was performed along with age, sex and the *APOE*- ϵ 4 dose under no interaction, using StatView software (SAS Institute, Cary, NC, USA). Linkage disequilibrium in the *DYRK1A* gene was also assessed by $|D'|$ and r^2 values; those less than 0.9 and 0.5, respectively, were judged significant (57). Case-control haplotype analysis was performed with the EM algorithm (58) and with the permutation test at 1000 iterations (59), using SNPalyze software (DYNACOM, Japan). Normally distributed variables were compared by Student's *t*-test; otherwise non-parametric Mann-Whitney's *U*-test was applied. A *P*-value less than 0.05 was considered significant.

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ORIGINAL ARTICLE

Pituitary adenylate cyclase-activating polypeptide is associated with schizophrenia

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Pituitary adenylate cyclase-activating polypeptide (PACAP, ADCYAP1: adenylate cyclase-activating polypeptide 1), a neuropeptide with neurotransmission modulating activity, is a promising schizophrenia candidate gene. Here, we provide evidence that genetic variants of the genes encoding PACAP and its receptor, PAC1, are associated with schizophrenia. We studied the effects of the associated polymorphism in the PACAP gene on neurobiological traits related to risk for schizophrenia. This allele of the PACAP gene, which is overrepresented in schizophrenia patients, was associated with reduced hippocampal volume and poorer memory performance. Abnormal behaviors in PACAP knockout mice, including elevated locomotor activity and deficits in prepulse inhibition of the startle response, were reversed by treatment with an atypical antipsychotic, risperidone. These convergent data suggest that alterations in PACAP signaling might contribute to the pathogenesis of schizophrenia.
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Keywords: schizophrenia; PACAP; SNP; hippocampus; memory; PPI

Introduction

Schizophrenia is a common neuropsychiatric disorder affecting 0.5–1% of the general population worldwide. This disease is characterized by psychosis and profound disturbances of cognition, emotion and social functioning. The pathophysiology of schizophrenia is still unclear; however, this disease is highly heritable¹ and several intermediate phenotypes such as neurocognitive dysfunction, abnormal brain morphology and deficits in prepulse inhibition (PPI) of the startle response are known to be useful to identify susceptibility genes for schizophrenia.^{2,3}

The adenylate cyclase-activating polypeptide 1 (ADCYAP1) gene encodes pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide, which is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon family. It exerts multiple activities as a neurotransmitter or neuromodulator via three heptahelical G-protein-linked receptors, one PACAP-specific (PAC1) receptor and two receptors that are shared with VIP (VPAC1 and VPAC2).^{4–6} PACAP induces cyclic AMP accumulation through activation of these receptors.^{4–6} We generated mice lacking the PACAP gene (PACAP^{-/-}); these mice had profound behavioral abnormalities including hyperactivity and explosive jumping in an open field, increased novelty-seeking behavior and deficits in PPI.^{7,8} In addition, the PACAP gene is located on 18p11, which linkage studies have suggested as a locus for schizophrenia and bipolar disorder.⁹ Although previous studies indicated that the PACAP gene could be a good candidate gene for schizophrenia, only one preliminary study has examined a

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possible association with schizophrenia and reported negative results.¹⁰ Here, we present data demonstrating a possible association between PACAP-PAC1 signaling and schizophrenia, using a multidisciplinary approach in both humans and rodents.

Materials and methods

Subjects

Subjects for the clinical association study were 804 patients with schizophrenia (51.1% males with a mean age of 44.2 years (s.d. 14.5) and a mean age of onset of 24.8 years (s.d. 8.8)) and 967 healthy controls (47.7% males with a mean age of 40.4 years (s.d. 16.1)). All the subjects were biologically unrelated Japanese. Three hundred and fifty-one patients with schizophrenia and 518 controls were from Tokyo Metropolitan (the east part of Japan), and 453 patients with schizophrenia and 449 controls were from Aichi prefecture (the central part of Japan). Patients were recruited at the National Center Hospital of Mental, Nervous, and Muscular Disorders; Nagoya University Hospital; Showa University Hospital and hospitals related to Department of Psychiatry, Nagoya University Graduate School of Medicine or Department of Psychiatry, Showa University School of Medicine. Healthy controls, including hospital and institutional staff, were recruited from local advertisements in Tokyo and Aichi. Magnetic resonance (MR) measurements and neurocognitive tests were performed only on some subjects (MR measurements: 81 patients with schizophrenia and 201 healthy controls; neurocognitive tests: 62 patients with schizophrenia and 139 healthy controls), all of whom were recruited at National Center of Neurology and Psychiatry. Demographic information for the subjects receiving MR measurements and neurocognitive tests is shown in detail in Supplementary Table 1 and Figure 1b. Consensus diagnosis was made for each patient by at least two trained psychiatrists, according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria, based on clinical interview and other available information including medical records and other research assessments. No patient was diagnosed by medical records alone. Controls were healthy volunteers who had no current or past contact to psychiatric services. After a description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees.

Genetic analysis

Venous blood was drawn from subjects and genomic DNA was extracted from whole blood according to standard procedures. Seven single nucleotide polymorphisms (SNPs) in the PACAP gene and three SNPs in the PAC1, VPAC1 and VPAC2 genes were genotyped using the TaqMan 5'-exonuclease allelic discrimination assay, as described previously.^{11,12} Primers and probes for the detection of the SNPs are available on request. Statistical analysis of genetic

association studies was performed using SNPAllyse (DYNACOM, Yokohama, Japan). The presence of Hardy-Weinberg equilibrium was examined by using the χ^2 test for goodness of fit. Allele distributions between patients and controls were analyzed by the χ^2 test for independence. All *P*-values reported are two-tailed. Statistical significance was defined as *P* < 0.05.

Neuroimaging analysis

All MR studies were performed on a 1.5 T Siemens Magnetom Vision plus system (Siemens, Erlangen, Germany). A three-dimensional volumetric acquisition of a T1-weighted gradient echo sequence produced a gapless series of 144 sagittal sections using an MPRage sequence (TE/TR, 4.4/11.4 ms; flip angle, 15°; acquisition matrix, 256 × 256; 1NEX, field of view, 31.5 cm; slice thickness, 1.23 mm).

Data were analyzed with Statistical Parametric Mapping 2 (SPM2) running on MATLAB 6.5. MR images were processed using optimized voxel-based morphometry (VBM) in SPM2 as described in detail previously.^{13,14} Normalized segmented images were modulated by multiplication with Jacobian determinants of the spatial normalization to encode the deformation field for each subject as tissue density changes in normal space. Following modulation, images were smoothed using a 12 mm full-width half-maximum of isotropic Gaussian kernel, because previous studies had proved that this should be a reasonable filter.^{13,15,16} In addition, we confirmed that the results of statistical analyses with three different smoothing filters (6, 8 and 12 mm Gaussian kernels) were essentially the same.

Statistical analyses were performed with SPM2, which implemented a general linear model. A hypothesis-driven regions of interest (ROIs) approach was used to investigate the hippocampus, using an ROI from the Wake Forest University PickAtlas.¹⁷ Our hypothesis is that the PACAP genotype related to the risk of developing schizophrenia is associated with hippocampal volume, because PACAP is associated with hippocampal function in rodents, and hippocampal volume is reported to be reduced in schizophrenia. The genotype and diagnostic effects on hippocampal gray matter volume change were assessed statistically using a single-subject condition and covariate model with a significance level set to 0.05 (corrected for multiple comparisons within the ROI). Age and gender were included in the model to control for confounds. Anatomic localization was according to both MNI coordinates and Talairach coordinates, obtained from M. Brett's transformations (<http://www.mrcctu.cam.ac.uk/Imaging/Common/mnispace.shtml>) and presented as Talairach coordinates.

Neurocognitive tests

Several memory tests, subscales of the Wechsler Memory Scale revised version (logical memory I, logical memory II, visual reproduction I, visual reproduction II, verbal paired associates I (VPAI),

verbal paired associates II, visual paired associates I and visual paired associates II) and the general intelligence IQ (from full scale of the Wechsler Adult Intelligence Scale, revised edition, WAIS-R), were performed by some of the subjects recruited at National Center of Neurology and Psychiatry. In association analysis between SNP3 of the PACAP gene and VPAI, group comparisons of demographic data were performed by using unpaired *t*-tests or χ^2 , as appropriate. There were no differences between genotype groups and demographic variables, for example, age, gender, education years and full-scale IQ, except for gender distribution in patients with schizophrenia ($P=0.026$) (Figure 1b). The effects of the SNP3 genotype of the PACAP gene and diagnosis on scores of memory tests were analyzed by a two-way analysis of covariance (ANCOVA), with age, gender and education years as covariates using SPSS 11.0J for Windows (SPSS Japan Inc., Tokyo, Japan).

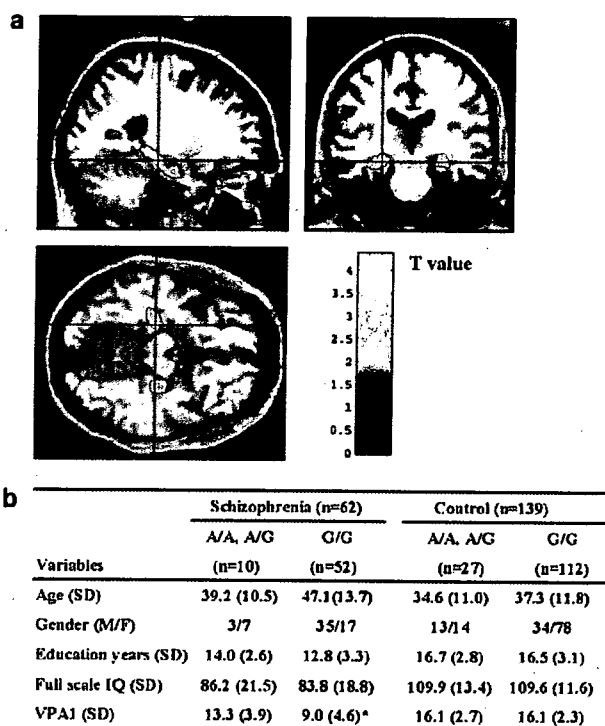


Figure 1 Genetic variation of PACAP is associated with hippocampal morphology and memory in humans. (a) Statistical maps of *t*-transformed hippocampal volume differences derived by optimized VBM of individuals homozygous for the G allele in SNP3 of the PACAP gene, relative to A-carriers, in all subjects, thresholded at $P < 0.05$ (corrected) in coronal, sagittal and axial views. These data show bilateral significant hippocampal volume reduction in individuals homozygous for the G allele. (b) Lower visual associate memory I score in individuals homozygous for the G allele in SNP3 of the PACAP gene, compared to A-carriers, in the schizophrenia group. Means \pm s.d. are shown. VPAI, visual paired associates I. * $P < 0.05$, compared with A-carriers.

When genotype effects on VPAI in controls or patients with schizophrenia were examined separately, a Mann-Whitney *U*-test and ANCOVA with gender as a covariate were used.

Animal study

All animal experiments were carried out in accordance with protocols approved by the Animal Research Committee of Osaka University and by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience. Generation of PACAP^{-/-} mice by a gene targeting technique has been reported previously.⁷ The null mutation was backcrossed onto the genetic background of Crlj:CD1 (Institute of Cancer Research) mice purchased from Charles River (Tokyo, Japan). All wild-type control mice and PACAP^{-/-} mice (homozygous for the mutant PACAP gene) used in locomotor activity and PPI experiments were obtained from the intercross of heterozygous animals. C57BL/6J mice were purchased from Charles River and were allowed to acclimate in our animal facility for at least 5 days before initiation of experiments. Mice were housed in a temperature- (23 \pm 1°C) and light-controlled room with a 12 h light-dark cycle (lights on from 0800 to 2000) and allowed free access to water and food, except during behavioral testing.

Locomotor activity was quantified using an infrared photocell beam detection system, Acti-Track (Panlab, Barcelona, Spain). Following intraperitoneal injection of risperidone (0.1 mg/kg) or an equivalent amount of saline, mice were placed in plastic activity monitoring boxes (30 \times 30 \times 30 cm) and tracked for 60 min, with data being stored permanently; parameters indicative of locomotor activity, such as distance traveled, were assessed. Each mouse was tested individually and had no contact with the other mice. The PACAP mutant cohort used in locomotor activity testing consisted of 12 wild-type mice and 12 PACAP^{-/-} mice ($n=6$ each for saline control and risperidone groups).

Acoustic startle responses for PPI were measured in a startle chamber (SR-LAB; San Diego Instruments, CA, USA) as described.¹⁸ Mice were placed in the startle chamber for 30 min after intraperitoneal injection of risperidone (0.1 mg/kg) or an equal amount of saline. The testing session started with 5 min of acclimatization to the startle chamber in the presence of 65 dB background broadband (white) noise. Testing consisted of forty 120 dB pulses alone and 10 pulses preceded (100 ms) by a prepulse of 66, 68, 71 or 77 dB. Pulses were randomly presented with an average of 15 s between pulses. Twelve no-stimulus trials were included to assess spontaneous activity during testing. PPI was calculated as a percentage score: PPI (%) = (1 - ((startle response for pulse with prepulse) / (startle response for pulse alone))) \times 100. The PACAP mutant cohort used in PPI testing consisted of 35 wild-type mice (saline control group = 22; risperidone group = 13) and 33 PACAP^{-/-} mice (saline control group = 17; risperidone group = 16).

Male C57BL/6J mice weighing 20–25 g received once-daily injections intraperitoneally for 14 days with phencyclidine (PCP) (5 mg/kg; $n=13$) or saline for control ($n=12$). PACAP and PAC1 mRNA levels were measured by a real-time quantitative RT-PCR method (TaqMan assay, Applied Biosystems, Tokyo, Japan), using total RNA extracted from the frontal cortex or hippocampus of mice treated with PCP or saline, as described previously.¹⁹ Statistically significant differences were assessed by the Mann-Whitney *U*-test.

Results

Genetic analysis

We examined the possible association between schizophrenia and genetic variations in the PACAP gene. Seven SNPs in the PACAP gene, selected from public databases, were genotyped, and the genotype distributions of all seven SNPs in the PACAP gene were in Hardy-Weinberg equilibrium in both controls and patients with schizophrenia (data not shown). The allele frequencies of the seven SNPs in patients and controls are shown in Table 1. The major allele of SNP3 and the minor allele of SNP5 were in excess in patients with schizophrenia when compared to controls (SNP3: $\chi^2=7.6$, $P=0.0059$, odds ratio=0.74, 95% confidence interval (CI) 0.59–0.92; SNP5: $\chi^2=4.2$, $P=0.041$, odds ratio=1.38, 95% CI 1.01–1.84), whereas no significant association of the other five SNPs with schizophrenia was observed (Table 1). SNP3 was significantly associated with schizophrenia after Bonferroni correction (corrected $P=0.041$). We next examined the possible association between schizophrenia and genes encoding the receptors for PACAP, such as the PAC1, VPAC1 and VPAC2 receptor genes. The genotype distributions of all three SNPs in the PAC1, VPAC1 and VPAC2 genes were in Hardy-Weinberg equilibrium in both controls and patients with schizophrenia, except for that of SNP3 of the VPAC1 gene in controls (data not shown). The

allele frequencies of the three SNPs in each receptor gene in the patients and controls are shown in Table 2. There was significant evidence for an association between a genetic variant of the PAC1 gene and schizophrenia (SNP2: $\chi^2=6.0$, $P=0.014$, odds ratio=1.18, 95% CI 1.03–1.35, corrected $P=0.042$), whereas none of the SNPs in the genes encoding VPAC1 or VPAC2 was associated with schizophrenia (Table 2). The evidence that the genes encoding PACAP and its receptor PAC1 are associated with schizophrenia suggests that signaling through PACAP and PAC1 might be associated with the pathophysiology of schizophrenia.

Intermediate phenotype

As the PACAP gene has been reported to play a role in learning and memory and hippocampal long-term potentiation in rodents,^{20,21} we next examined the possible impact of SNP3 of the PACAP gene, which was associated with schizophrenia, on hippocampal volume in patients with schizophrenia and controls. A genotype effect was found as bilateral reductions of hippocampal volumes (right: $P=0.04$, $t=3.2$; left: $P=0.002$, $t=4.1$) in homozygous G subjects compared with A-carriers (Figure 1a). There was also a diagnostic effect, a significant reduction in left hippocampal volume in patients with schizophrenia compared with controls ($P=0.033$, $t=3.3$). Genotype-diagnosis interaction effects on brain morphology were not found, even at a lenient threshold (uncorrected $P=0.05$). We next estimated the effects of genotypes on hippocampal volume in the control groups and schizophrenic groups, separately. Schizophrenic patients homozygous for the G allele showed a significant reduction in bilateral hippocampal volumes (right: $P=0.013$, $t=3.5$; left: $P=0.005$, $t=3.9$). On the other hand, we found significantly decreased volumes of the bilateral hippocampi in homozygous G subjects compared with the A-carriers, at a lenient threshold (uncorrected $P=0.05$) in controls; however, no voxels could survive after the correction for multiple comparisons. These data

Table 1 Allele frequencies of seven SNPs in the PACAP gene between the patients with schizophrenia and controls

SNP-ID	dbSNP	Distance from SNP1	Major/minor polymorphism	Location	Number of subjects		Minor allele frequency		P-value	Odds ratio (95% CI)
					Controls	Patients	Controls	Patients		
SNP1	rs2846584	—	C/T	5'-region	967	804	0.362	0.373	0.54	
SNP2	rs2231181	712	G/C	5'-UTR	960	795	0.336	0.330	0.69	
SNP3	rs1893154	1071	G/A	Intron1	951	797	<u>0.126</u>	<u>0.097</u>	<u>0.0059</u>	<u>0.74 (0.59–0.92)</u>
SNP4	rs1893153	1149	T/A	Intron1	953	793	0.174	0.163	0.37	
SNP5	rs2856966	3656	A/G	Exon3 (D54G)	953	786	<u>0.047</u>	<u>0.063</u>	<u>0.041</u>	<u>1.38 (1.01–1.84)</u>
SNP6	rs928978	4481	C/A	Intron4	958	798	0.475	0.485	0.58	
SNP7	rs1610037	6581	A/G	3'-region	962	794	0.216	0.211	0.73	

Abbreviations: CI, confidence interval; PACAP, pituitary adenylate cyclase-activating polypeptide; SNPs, single nucleotide polymorphisms.

Minor allele frequencies in controls are shown. Significant results ($P<0.05$) are indicated with underline.

Table 2 Allele frequencies of SNPs in the PAC1, VPAC1 and VPAC2 gene between the patients with schizophrenia and controls

Gene name	SNP-ID	dbSNP	Distance from SNP1	Major/minor polymorphism	Location	Number of subjects		Minor allele frequency		P-value	Odds ratio (95% CI)
						Controls	Patients	Controls	Patients		
PAC1	SNP1	rs1468687	—	T/C	Intron2	950	796	0.287	0.264	0.12	<u>1.18 (1.03–1.35)</u>
	SNP2	rs2302475	15553	C/T	Intron5	958	797	<u>0.479</u>	<u>0.520</u>	<u>0.014</u>	
	SNP3	rs2267742	34598	A/G	Intron12	936	786	0.127	0.133	0.58	
VPAC1	SNP1	rs735773	—	C/G	Intron1	937	784	0.357	0.38	0.16	
	SNP2	rs406360	12972	A/G	Intron4	948	789	0.431	0.433	0.91	
	SNP3	rs3733055	22942	G/T	Exon13 (R445L)	958	801	0.041	0.035	0.33	
VPAC2	SNP1	rs885861	—	C/T	3'-UTR	963	802	0.208	0.232	0.090	1.15 (0.98–1.36)
	SNP2	rs3793224	55026	C/T	Intron4	944	791	0.247	0.232	0.29	
	SNP3	rs3812312	109228	C/T	Intron2	923	781	0.221	0.218	0.85	

Abbreviations: CI, confidence interval; SNPs, single nucleotide polymorphisms.

Minor allele frequencies in controls are shown. Significant results ($P < 0.05$) are indicated with underline.

suggest that SNP3 in the PACAP gene could have an impact on hippocampal morphology.

As the human hippocampus is related to memory function, we also examined the association between SNP3 of the PACAP gene and several subscales of the Wechsler memory scale revised version in patients with schizophrenia and controls (Figure 1b). Two-way ANCOVA on VPAI revealed significant effects of diagnosis ($F = 33.8$, $P < 0.0001$) and genotype of SNP3 ($F = 5.2$, $P = 0.024$), and an interaction between diagnosis and genotype ($F = 6.6$, $P = 0.011$), whereas an effect of genotype was not found in other memory subscales (data not shown). Individuals homozygous for the G allele of SNP3, which was enriched in schizophrenia, had lower scores of VPAI than schizophrenic patients carrying the A allele (Mann-Whitney U -test: $P = 0.015$); however, there was no difference between the two genotypes in the control group ($P > 0.8$). ANCOVA with gender as a covariate did not alter the statistical significance of these results in patients with schizophrenia ($P = 0.029$). These data suggest that the risk SNP of the PACAP gene could be associated with reduced hippocampal volume and poorer memory performance, which are neurobiological traits related to risk for schizophrenia.

Animal study

As our data indicate that PACAP might be associated with schizophrenia, PACAP knockout mice (PACAP^{-/-} mice) could be a possible animal model for schizophrenia. Several schizophrenia-related behaviors in rodents, such as hyperactivity, deficits in PPI, locomotor response to antipsychotics, disturbance in social interaction and cognitive deficits, have been commonly observed in previous pharmacological and genetic animal models for schizophrenia.²² Therefore, we examined the impact of an atypical antipsychotic, risperidone, on hyperactivity and deficits in PPI in PACAP^{-/-} mice. PACAP^{-/-} mice maintained high initial levels of locomotor activity during the open

field test (Figure 2a and b), as reported previously.⁷ When treated with risperidone, hyperlocomotion in PACAP^{-/-} mice was attenuated almost to the normal levels seen in wild-type mice; however, treatment with risperidone had no significant effect on locomotor activity in wild-type mice (Figure 2a and b). Risperidone also reversed the diminished PPI in PACAP^{-/-} mice⁸ to the control level seen in wild-type mice (Figure 2c). Risperidone had no significant effect on PPI levels in wild-type mice (Figure 2c) and startle amplitudes in both PACAP^{-/-} and wild-type mice (data not shown). These results suggest that the abnormal behaviors in PACAP^{-/-} mice, which are believed to be schizophrenia-like phenotypes in rodents, can be rescued by an atypical antipsychotic, risperidone.

The abuse of PCP, an *N*-methyl-D-aspartic acid receptor antagonist, results in positive symptoms, negative symptoms and cognitive impairments, similar to those seen in patients with schizophrenia. Thus, mice chronically treated with PCP have been used as a potential animal model for schizophrenia.²³ To assess a possible change in the expression of PACAP and PAC1 receptor in the pathological state, we performed mRNA expression analysis for PACAP and PAC1 in the frontal cortex and hippocampus of mice chronically treated with PCP. The expression level of PACAP mRNA was significantly reduced in the frontal cortex, but not in the hippocampus (Supplementary Figure 1). On the other hand, increased expression of PAC1 mRNA was observed in both frontal cortex and hippocampus (Supplementary Figure 1). Although the altered expression of PACAP and PAC1 in mouse brains treated with PCP was subtle, these data are considered to be in line with the behavioral abnormalities in PACAP^{-/-} mice, a possible animal model for schizophrenia.

These results using animal models support the notion that PACAP is associated with the pathophysiology of schizophrenia.

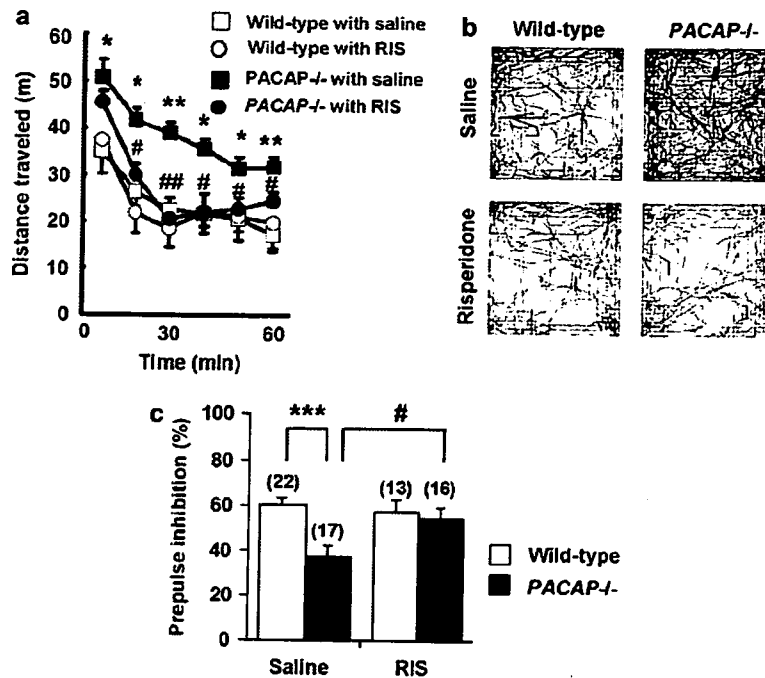


Figure 2 Hyperlocomotion and deficits in the PPI of PACAP^{-/-} mice were normalized by risperidone treatment. (a) Locomotor activity in wild-type and PACAP^{-/-} mice that received 0.1 mg/kg risperidone (RIS) or saline. $n=6$ per group. (b) Representative locomotor patterns of saline- or 0.1 mg/kg risperidone-treated wild-type and PACAP^{-/-} mice during 25–30 min of a 60 min recording in an open field test. (c) PPI levels induced by a 77 dB prepulse in wild-type and PACAP^{-/-} mice after pretreatment with risperidone (0.1 mg/kg) or saline. Numbers of animals for experiments are shown in parentheses. Data are given as means \pm s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to wild-type. * $P < 0.05$, ** $P < 0.01$, compared with saline in PACAP^{-/-} mice.

Discussion

Our findings support the possibility that PACAP is a potential schizophrenia susceptibility gene. Clinical association between schizophrenia and the genes encoding PACAP and PAC1 and an association between intermediate phenotypes, hippocampal volume and visual associate memory performance and a risk SNP in the PACAP gene have been demonstrated in our study. There are several limitations in our results. We screened control subjects with no past or current visits to psychiatric services; however, we could not exclude the possibility that they have an undiagnosed or untreated psychiatric disorder. The obtained evidence for association was not very strong, especially in the association between the genotype and visual associate memory performance ($P < 0.05$ level). When we applied corrections for multiple testing for several memory tests, this positive association became negative. This association is not conclusive, although the association between the risk allele for schizophrenia and poorer memory performance might be attractive. Thus, replication studies should be conducted to confirm our findings. We do not know whether SNP3 alters the expression/function of the PACAP gene. Accordingly, there remains the possibility that other polymorphisms, which are in linkage disequilibrium to this polymorphism, are truly responsible for giving susceptibility.

Studies aiming to identify susceptibility genes for schizophrenia are faced with the confounds of subjective clinical criteria and the likelihood of allelic and locus heterogeneity. Although schizophrenia is substantially heritable, the mode of inheritance is complex, involving numerous genes of small effect and a nontrivial environmental component. The concept of intermediate phenotype (endophenotype) assumes that neurobiological deficits occur across the schizophrenia spectrum in schizophrenia patients, schizotypal patients and clinically unaffected relatives of schizophrenia patients. The intermediate phenotype approach is an alternative method for measuring phenotypic variation that may facilitate the identification of susceptibility genes in the context of complexly inherited traits. Using this approach, we showed an association between the PACAP gene and two intermediate phenotypes, hippocampal volume and visual associate memory, in addition to the genetic association with schizophrenia. Our study could be a successful example of using this strategy to find susceptibility genes for complex diseases.

The hyperactivity and deficits in PPI observed in PACAP^{-/-} mice^{7,8} are believed to be schizophrenia-like behaviors in rodents. PAC1 knockout mice also show abnormal behaviors, including elevated locomotor activity and abnormal social behavior.^{24,25} Our genetic findings, which demonstrate an association

between schizophrenia and two genes, PACAP and PAC1, are supported by the abnormal behaviors in knockout mice of PACAP and PAC1. Risperidone, an atypical antipsychotic, has the advantage of better extrapyramidal tolerability than conventional antipsychotics, but also has advantages in cognitive disturbances and the treatment of negative and depressive symptoms.²⁶ Our previous study showed that haloperidol, a representative conventional antipsychotic, rescued hyperactivity,⁷ but did not rescue deficits in PPI.⁸ As risperidone treatment rescued both of these abnormalities in PACAP^{-/-} mice, and as risperidone is a combined D2 and 5-HT_{2A} receptor antagonist, either dopamine or serotonin signaling, or both, could be relevant to the abnormal behaviors in PACAP^{-/-} mice.

Our convergent evidence suggests that investigation of PACAP-PAC1 signaling in the brain could provide a clue to elucidating the possible mechanisms of pathophysiology in schizophrenia.

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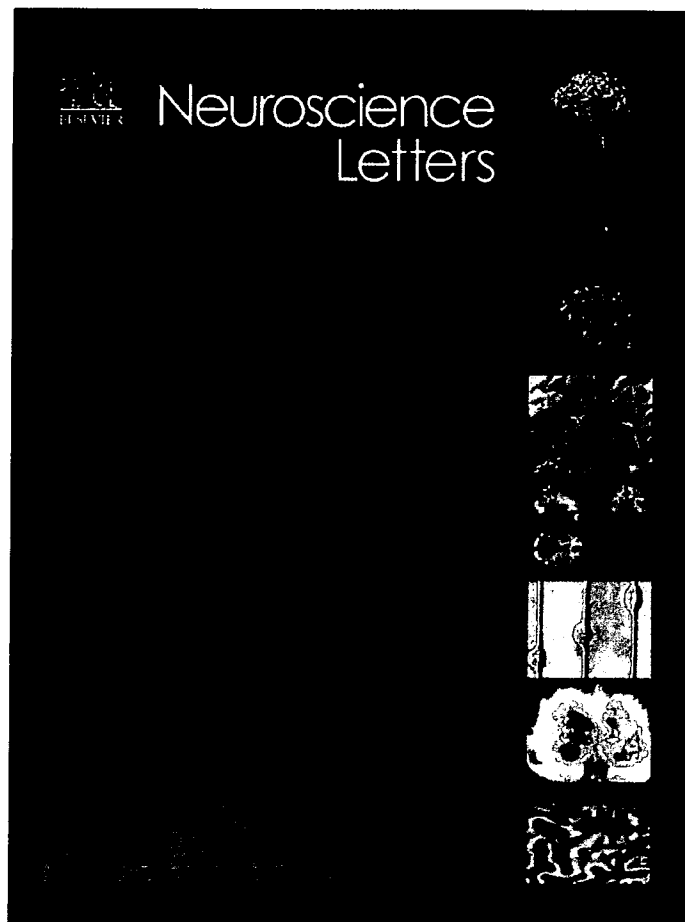
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A possible association between the Val158Met polymorphism of the catechol-*O*-methyl transferase gene and the personality trait of harm avoidance in Japanese healthy subjects

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Abstract

Catechol-*O*-methyltransferase (COMT) is an enzyme that degrades various biogenic amines, which have been hypothesized to be associated with personality traits. We investigated a possible relationship between the COMT Val158Met polymorphism and personality traits assessed by the Temperament and Character Inventory (TCI) in 139 healthy subjects in a Japanese population. The number of Met alleles of the COMT Val/Met genotype tended to relate to harm avoidance (HA) scores parametrically, while no significant difference was observed between genotype groups in either novelty seeking, reward dependence, persistence, self-directedness, cooperativeness or self-transcendence. These results suggest that the Val/Met polymorphism of the COMT gene may play a role in HA in Japanese population.

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Keywords: Catechol-*O*-methyltransferase (COMT); Temperament and Character Inventory (TCI); Harm avoidance; Polymorphism

Genetic factors significantly contribute to the determination of human personality traits, although environmental influence is also important. Personality traits assessed by self-report questionnaires show moderate heritability [6]. Such inheritance is ultimately attributable to functional variants of the genes programming brain development and function [5]. Catechol-*O*-methyl transferase (COMT) is an enzyme involved in monoamine metabolism, and a common single nucleotide polymorphism (SNP) in the COMT gene, producing an amino acid substitution of methionine (Met) to valine (Val) at position 108/158 (Val158Met), affects dopamine regulation in the prefrontal cortex [19]. This polymorphism impacts on the stability of the enzyme, such that the Val allele is associated with signif-

icantly higher enzyme activity than the Met allele [4]. Several studies have revealed that the Val allele is associated with poorer performances, compared with the Met allele, in cognitive tasks of frontal function such as the Wisconsin Card Sorting Test (WCST) and N-back task [7,8]. The underlying mechanism of such behavioral differences may be related to lower prefrontal dopamine levels arising from the higher dopamine catabolism mediated by the Val allele [4,23]. Thus, it is likely that the Val/Met polymorphism of the COMT gene could be associated with a personality trait; however, the relationship between the Val/Met polymorphism of the COMT gene and the personality traits using the Temperament and Character Inventory (TCI) has not been studied in Japanese population [1,2,9,14–16,21,22]. In this study, we examined the relationship between the Val158Met of the COMT gene and the personality traits measured by TCI in Japanese healthy subjects.

One hundred and thirty-nine healthy subjects participated in the study. A Japanese version of TCI, a full version of the Wechsler Adult Intelligence Scale-Revised (WAIS-R) [20,24]

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Table 1
Demographic information and COMT Val/Met genotype

Variables	Val/Val (n=77)	Val/Met (n=45)	Met/Met (n=17)
Age	37.2 (12.9)	35.9 (12.3)	33.2 (5.9)
Gender (M/F)	29/48	13/32	5/12
Education years	16.6 (2.9)	16.2 (2.2)	17.4 (4.1)
IQ	110.8 (11.5)	110.3 (11.8)	107.0 (15.2)
WCST PE	4.3 (5.1)	3.7 (4.1)	3.5 (3.7)

Mean \pm S.D. There was no significant difference among genotypes for any variable. WCST PE: Wisconsin Card Sorting Test Preservative Errors.

and the Wisconsin Card Sorting Test [10,13] were administered to all subjects. Subjects with significant medical problems, history of head of trauma, neurosurgery and alcohol or substance abuse were excluded. They had no current or past contact with psychiatric services. All subjects were biologically unrelated Japanese. After a description of the study, written informed consent was obtained from every subject. The study protocol was approved by an institutional ethical committee. Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. The subjects were genotyped using the TaqMan 5'-exonuclease allelic discrimination assay, as described previously [18]. Primers and probes for detection of the SNP were: forward primer 5'-GACTGTGCCGCCATCAC-3'; reverse primer 5'-CAGGCATGCACACCTTGTC-3'; probe 1 5'-VIC-TTTCGCTGGCGTGAAG-MGB-3'; and probe 2 5'-FAM-CGCTGGCATGAAGMGB-3'. PCR cycling conditions were: at 95 °C for 10 min, 50 cycles of 92 °C for 15 s and 60 °C for 1 min. Statistical tests were carried out using SPSS for Windows version 11.0 (SPSS Japan, Tokyo) and a power analysis program (R version 2.5.1: <http://www.r-project.org/index.html>). Group comparisons of demographic data were performed by using analysis of variance (ANOVA) or χ^2 , as appropriate. The effects of the COMT genotype on scales of TCI, IQ or WCST were assessed by ANOVA or multiple regression. A Spearman rank order correlation test was used for comparisons between HA score and age, gender and education. Statistical significance was defined as $p < 0.05$. As the statistical considerations are essential to carry out the association study between genetic polymorphisms and personality [12,17], we further applied correction of multiple comparisons and power analyses to avoid type 1 and 2 errors.

We examined the effects of the COMT genotype on the following measures of the TCI in a cohort of 139 normal subjects: novelty seeking (NS), harm avoidance (HA), reward dependence (RD), persistence (P), self-directedness (SD), cooperativeness (C) and self-transcendence (ST). Table 1 gives the means and standard deviations of age, education years, full scale of IQ, and preservative errors of the WCST and gender distribution for groups defined by COMT Val/Val ($n=77$), Val/Met ($n=45$) and Met/Met ($n=17$) genotypes. There were no differences among genotype groups and demographic variables or tasks of general academic ability, for example, IQ or preservative errors in the WCST (Table 1). An ANOVA detected a significant effect of genotype on HA ($F=4.08$, $df=2,136$, $P=0.019$) (Fig. 1), but no significant difference was observed among genotype

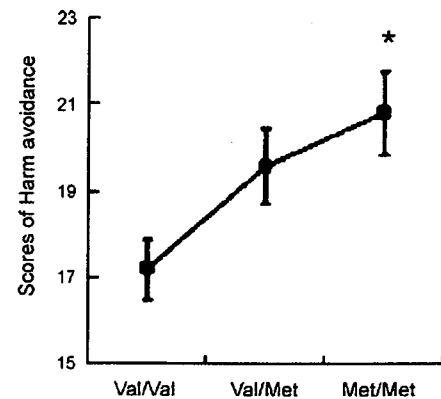


Fig. 1. Harm avoidance and COMT Val/Met genotype. Scores of harm avoidance in healthy individuals with Val/Val ($n=77$), Val/Met ($n=45$) and Met/Met ($n=17$) genotypes are shown (mean \pm S.D.). * $P < 0.05$, significant difference compared with Val/Val.

groups in NS, RD, P, SD, C or ST (Table 2). Post hoc comparisons showed that Val/Val homozygote subjects had lower scores than Met/Met ($p=0.013$) and Val/Met ($p=0.09$) subjects. Prior reports have shown the effect of age on the HA score [3]. When we examined the effect of age, gender or education years on the HA score, we found that age was negatively correlated with HA ($Rho = -0.378$, $p < 0.00001$), whereas gender and education years were not (gender: $Rho = 0.073$, $p = 0.39$, education years: $Rho = -0.048$, $p = 0.57$). Multiple regression with the number of Met alleles and age as continuous factors revealed both factors to be parametrically related HA scores ($r^2 = 0.22$, $F = 19.9$, $p < 0.00001$, Met allele: $t = 2.5$, $P = 0.013$; age: $t = -5.5$, $P < 0.00001$). Thus, the number of Met alleles was positively correlated with a higher HA score in our sample.

There is a risk of type I errors occurring: some comparisons that are statistically significant at a 5% level may be because of chance. When multiple comparison was applied in the effect of genotype on HA score, 0.71% level is appropriate. Thus, the COMT genotype effect on HA in the Japanese population (ANOVA: $p = 0.019$, Multiple regression: $p = 0.013$) should be considered to be a trend level. Replication studies in Japanese populations are necessary to confirm the present results.

Kim et al. [14] reported a significant association between the Val/Met polymorphism and harm avoidance only in female

Table 2
COMT Val/Met genotype and Temperament and Character Inventory (TCI)

Scales	Val/Val (n=77)	Val/Met (n=45)	Met/Met (n=17)
NS (novelty seeking)	21.5 (3.9)	22.1 (5.0)	20.5 (4.3)
HA (harm avoidance)	17.2 (6.2)	19.6 (5.7)	20.8 (4.0)*
RD (reward dependence)	15.1 (3.9)	15.7 (3.8)	14.8 (2.9)
P (persistence)	4.4 (2.0)	4.7 (1.7)	4.6 (2.0)
SD (self-directedness)	30.1 (6.4)	28.7 (5.5)	29.8 (5.0)
C (cooperativeness)	29.0 (4.4)	28.3 (5.6)	29.8 (4.1)
ST (self-transcendence)	10.3 (4.7)	11.2 (4.6)	9.8 (4.6)

Mean \pm S.D. Significantly different compared with Val/Val (* $P < 0.05$).

subjects in a Korean population, which is a close ethnicity to Japanese. Their results indicated a higher HA score in subjects with a Val/Val genotype. These data are opposite to our results, showing a higher HA score in subjects with a Met/Met genotype. Thus, we examined the association between the Val/Met SNP of the COMT gene and HA in male and female subjects separately. Individuals of both sexes with a Met/Met genotype showed higher HA scores than Val/Val or Val/Met individuals (male: Val/Val 16.4, Val/Met 19.6, Met/Met 20.6; female: Val/Val 17.7, Val/Met 19.6, Met/Met 20.9); however, this difference did not reach statistical significance (male: $p=0.06$, female: $p=0.13$). As our sample size is small, a power analysis was performed to evaluate the statistical power. The power of our sample to detect average differences of HA scores between genotypes in female subjects was calculated using a one-tailed alpha value of 0.05. Our sample size had a power (0.8) to detect average differences between Val/Val and Val/Met: 3.4, Val/Val and Met/Met: 4.6, and Val/Met and Met/Met: 4.2. As the average differences of HA score between genotypes in the Korean study were 3.3 (Val/Val and Val/Met), 5.8 (Val/Val and Met/Met), and 2.5 (Val/Met and Met/Met), our sample size had 0.8 of power at least in the comparison between Val/Val and Met/Met.

There were several studies to investigate the relationship between the COMT Val/Met polymorphism and personality traits in other ethnic populations. Benjamin et al. [1,2] showed that the COMT Val/Met polymorphism and interaction of the COMT genotype and 5-HTTLPR were associated with persistence (RD2) and NS. Subsequently, Tsai et al. [22] reported that the COMT Val/Met genotype was associated with NS and RD but not with HA and Lichtenberg et al. [15] failed to find an association between the COMT Val/Met genotype and personality traits. We could not replicate the association between the COMT genotype and other personality traits such as NS, P in other ethnicity. These inconsistencies may relate to sample differences, false positive results, false negative results and possible genetic and allelic heterogeneity. In addition, other genetic factors such as the polymorphisms of the 5HTT, DRD2, DRD3, and DRD4 genes, environmental factors, and gene and environment interactions might influence personality traits [17]. Replication studies using a larger sample size and/or Japanese, Korean or Caucasian cohorts would be required to draw any conclusion.

In this study, we reported a possible association between the Val/Met polymorphism of the COMT gene and HA assessed by the TCI in a Japanese population. The number of Met alleles was positively correlated with a higher HA score. The Met-type COMT protein has lower catecholamine metabolism activity than the Val-type protein, which might lead to a hyper-dopaminergic state and higher activity in the prefrontal cortex. Exposure of rodents to stressful stimuli increased cortical dopamine, while diazepam, an anxiolytic benzodiazepine, could reverse the effect of dopamine increase by stressful stimuli [11], indicating that hyper-dopaminergic transmission in frontal cortex might induce anxious state. Thus, higher activity in the prefrontal cortex could explain the higher HA score and higher anxiety in normal subjects.

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