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	
	SNP2	rs2302475	15553	C/T	Intron5	958	797	0.479	0.520	0.014	1.18 (1.03–1.35)
	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 $PACAP^{-/-}$ mice[8] 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.

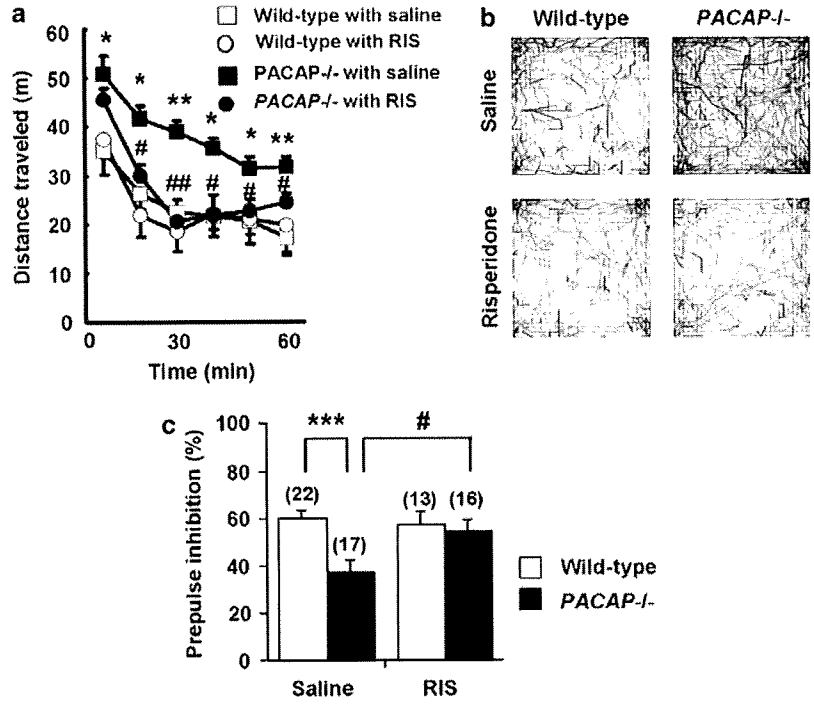

Figure 2 Hyperlocomotion and deficits in the PPI of PACAP$^{-/-}$ mice were normalized by risperidone treatment. (**a**) Locomotor activity in wild-type and PACAP$^{-/-}$ mice that received 0.1 mg/kg risperidone (RIS) or saline. $n=6$ per group. (**b**) Representative locomotor patterns of saline- or 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 ± 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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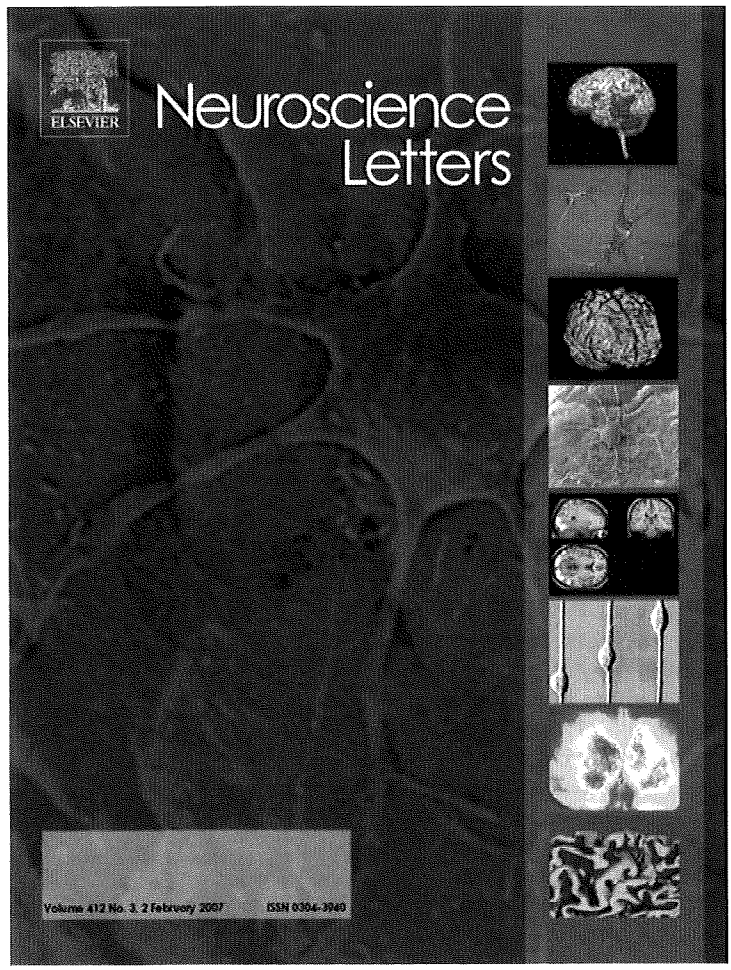

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A new gain-of-function allele in chimpanzee tryptophan hydroxylase 2 and the comparison of its enzyme activity with that in humans and rats

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Abstract

Tryptophan hydroxylase 2 (TPH2) is a rate-limiting enzyme of neuronal serotonin biosynthesis. Recently, two single nucleotide polymorphisms (SNPs) at the exon 11 coding region that resulted in amino acid substitutions in the C-terminal domain have been reported to affect enzyme activity in humans and mice. We determined 175 base-pair sequences of the exon 11 region in nine primate species from all recognized lineages. All nucleotide sequence substitutions were synonymous, with the exception of one adenine (A) to guanine (G) substitution at the 1404th position in the open reading frame (ORF). This substitution leads to a glutamine (Q) to arginine (R) amino acid substitution at the 468th position within chimpanzee sequences. The frequency of the *G* allele was 0.24 among 66 chimpanzees. Therefore, it is a novel SNP observed in chimpanzees, and we have named these two alleles as *ch468Q* and *ch468R*, respectively. When expressed in HeLa cells, *ch468R* caused an approximate 20% increase in enzyme function during L-5-hydroxytryptophan (5HTP) production ($P < 0.001$). We also surveyed the interspecies difference in enzyme activity among human, chimpanzee, and rat. Although the rat showed an identical amino acid sequence at the C-terminal region as those of human and *ch468Q*, the rat enzyme was more active than those of human or chimpanzee ($P < 0.001$), indicating the importance of substitutions in other regions. Our findings on the chimpanzee SNP will be a useful genetic marker in understanding the individual difference in the serotonin-related behavior.

Keywords: TPH2; SNP; 5HTP; Chimpanzee; Serotonin

Serotonin synthesis is mediated by the rate-limiting enzyme tryptophan hydroxylase (TPH), which catalyzes the pterin-dependent hydroxylation of L-tryptophan to L-5-hydroxytryptophan (5HTP). Additionally, this reaction is the first step in the synthesis of melatonin, which is involved in the regulation of mammalian reproduction and circadian rhythms [27]. The TPH belongs to the family of tetrahydrobiopterin-dependent aromatic amino acid hydroxylases, which includes phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH) [14]. These enzymes share similar protein organization composed of a regulatory N-terminal domain, a catalytic domain, and a short C-terminal oligomerization domain (Fig. 1a) [6–8]. Although the precise manner is often unique for each hydroxylase, they can be activated by similar mechanisms [4,9,10].

Recently, TPH isoform 2 was discovered to be preferentially located in brain dorsal raphe [23,28] and in the peripheral myenteric neurons in the gut [4]. The discovery of TPH2 has renewed great interest in studying the role of this enzyme in the neurochemical function of serotonin. To date, over 500 single nucleotide polymorphisms (SNPs) have been identified in the TPH2 gene in humans and mice, but coding non-synonymous SNPs are only six [3,33]. A number of recent studies have

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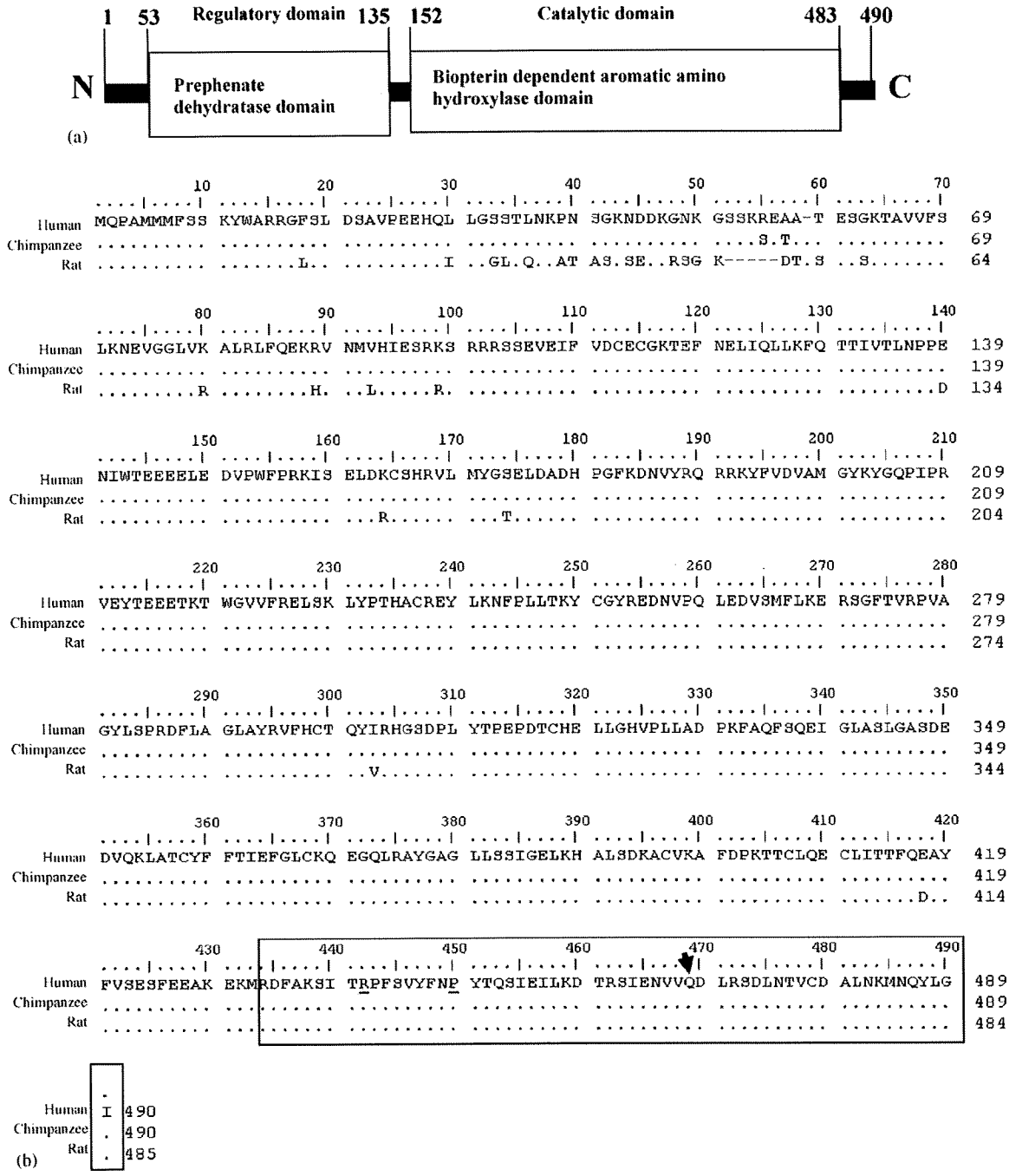

Fig. 1. The schematic TPH2 structure and amino acid sequence alignment. (a) The schematic TPH2 structure including two functional domains. The numbers indicate the amino acid position based on the human coding region. (b) The amino acid alignments of three mammals. The sequenced region (corresponding exon 11 coding region) of primate species is boxed and the chimpanzee-specific Q468R polymorphic site is indicated by an arrowhead. Polymorphic sites in humans (R441H) and mice (P447R) are underlined. Accession numbers of human, chimpanzee, and rat are NP_775489, XP_522470, and NP_776211, respectively.

already reported the association between genetic polymorphisms and affective disorder and suicidal tendency [3,9,24,34]. Additionally, a number of interesting differences in the properties of TPH1 and TPH2 have been revealed, suggesting that these enzymes may have different catalytic control mechanisms or are differentially regulated at the post-translational level [19].

In human and mouse studies, SNPs in TPH2 have been suggested to alter enzyme function [31,32]. The human TPH2 contained an SNP that replaced a wild-type arginine with a histidine (R441H). Expression of the mutant TPH2 in PC12 cells resulted in an approximate 80% decrease in serotonin levels as compared to the wild-type. R441H mutants were identified in unipolar major depression patients. In the mouse TPH2, a wild-type proline has been reported to be replaced by arginine (P447R mutant). Expression of mutant TPH2 revealed an approximate 55% decrease in serotonin levels as compared to wild-type. The mutant P447R homozygous inbred strains (BALB/c and DBA/2J) of mice showed 50–70% decrease in the rate of serotonin synthesis in the brain when compared to the wild-type homozygous strains (C57BL/6 and 129X1/SvJ). Interestingly,

the mice from these strains display significantly different aggressive behavior [16] and responses to antidepressants [2,18]. These results raised the possibility that similar mutations in nonhuman primates may affect their brain serotonin levels.

In general, the nucleotides and amino acids similarities among the TPH2 sequences of human, chimpanzee, and rat that were downloaded from GenBank database were >80% and >90%, respectively. The C-terminal region (highlighted by a box) was identical among the major allele of the species except for a proline to arginine replacement in mouse and for an arginine to histidine replacement in human (Fig. 1a and b).

The close genetic, physiological, and behavioral similarities between humans and nonhuman primates provide the basis for a comparative analysis of the genetic and environmental factors underlying both normative and pathological outcomes in behavioral development [1]. In this study, our primary purpose was to identify a new polymorphic loci in chimpanzees neurotransmitter related genes. Therefore, we sequenced the exon 11 coding region that corresponds to the polymorphic regions in humans and mice for several primate species.

Genomic DNA was extracted from the peripheral blood or buccal mucous membrane obtained from humans (healthy Japanese subjects, $n = 10$; informed consent was obtained in accordance with the guidelines of Gifu University), chimpanzees (*Pan troglodytes*, $n = 66$), gorillas (*Gorilla gorilla*, $n = 10$), orangutans (*Pongo pygmaeus*, $n = 10$), agile gibbons (*Hylobates agilis*, $n = 5$), Japanese macaques (*Macaca fuscata*, $n = 4$), mandrill (*Papio sphinx*, $n = 1$), common marmosets (*Callithrix jacchus*, $n = 3$), tarsier (*Tarsius bancanus*, $n = 1$), and galago (*Galago crassicaudatus*, $n = 1$). Thus, all primate lineages were covered. Most of the nonhuman primate samples were obtained from the Primate Research Institute of Kyoto University and Sanwa Kagaku Kenkyusho Co. Ltd.

We determined the sequences in 10 individuals of chimpanzees and each one individual for the other species. PCR was performed for the amplification of a 295 bp sequence including 175 bp of the exon 11 coding region. Ten microliters of the reaction mixture containing 20 ng DNA, 0.5 μM of each primer, 0.5 U *LA Taq* polymerase, GC buffer I (TaKaRa, Shiga, Japan), and 400 μM of each dNTP was used. The primer sequences employed were 5′-TTCTGTTTATTCTGCAGGGACT-3′ (TPH2F) and 5′- TTAGCCAAGCCATGACACAG-3′ (TPH2R), corresponding to intron 10 and 3′UTR of the human TPH2 genomic sequence. After an initial incubation at 95 °C for 2 min, PCR amplification was performed for 35 cycles consisting of 95 °C for 30 s, 60 °C for 1 min and 74 °C for 2 min; this was followed by a final extension at 74 °C for 10 min. The PCR products were purified by using a PCR purification kit (Roche, Mannheim, Germany), followed by sequencing of both strands at least twice by using an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

The nucleotide sequences of the C-terminal domain (exon 11 coding region of TPH2) in the nine primate species are shown in DDBJ database with accession numbers AB68316–AB68324. A total of 23 nucleotide substitutions were observed. All substitutions were silent, except one substitution within chimpanzees. An adenine (A) at the 1404th position was substituted by guanine (G), and this change caused a replacement of the 468th glutamine (CAG, *ch468Q*) by arginine (CGG, *ch468R*). Both chimpanzee sequences contained one substitution synonymous with that of human sequence at the 1468th position.

Genotyping for the chimpanzee SNP for remaining 56 individuals was performed by using a PCR-RFLP method. After amplification using TPH2F and TPH2R primers, 1 μl of the PCR product was incubated at 37 °C overnight in the reaction mixture having a total volume of 10 μl, containing 1 × NEBuffer 4 with 1.5 U of *HpyCH4*V (New England BioLabs, Beverly, MA). The products were subsequently separated by electrophoresis on a 2.0% agarose gel.

The allelic frequency of *G* allele among 66 chimpanzees was 0.24. The genotypic frequencies of *G/G*, *A/A*, and *G/A* were 0.091, 0.606, and 0.303, respectively. The presence of Hardy–Weinberg equilibrium was examined by using the Chi-square test for goodness of fit. The observed genotypic frequency distribution well accorded with the expectation under the Hardy–Weinberg equilibrium (Chi-square test, $P = 0.364$). To confirm the chimpanzee specificity of the SNP, we also conducted the SNP genotyping in each of the 10 humans, 10 gorillas, 10 orangutans, five agile gibbons, four Japanese macaques, and three common marmosets, but we could not detect the same substitution. If these species have the SNP site in the same frequency as chimpanzee, then it should be detected by the possibility of 99.9% in 10 individuals [15]. For confirmation, we need to survey larger sample size and diverse population.

For elucidating the functional effect of chimpanzee SNP, the human full-length ORF was amplified from the human brain hippocampus cDNA library (TaKaRa, Shiga, Japan) by using the primers 5′-CCCTGCTGCAGAGAAAGAAT-3′ (TPH2Fm) and 5′- AGATCATGCTGGCAACAACA-3′ (TPH2Rm), and was subsequently ligated into a human TPH2/TOPO TA cloning vector (Invitrogen, Carlsbad, CA). The chimpanzee amino acid sequence differed from that of the humans at only three positions. Of these, the two positions – C164A (R54S) and G170A (A56T) – were common in all the chimpanzees surveyed, but the A1404G (Q468R) substitution was polymorphic within chimpanzees. Therefore, two types of chimpanzee TPH2 ORFs were obtained by the mutagenesis of human ORF. The following are the oligonucleotides used to generate the ch468Q and ch468R (lowercase underlined letters represent new codons introduced by site-directed mutagenesis): 5′-AGCAAAagtGAAactGCTACCGAAAG-3′ for R54S and A56T, and 5′-ATGTGGTGcggGACCTTCGCAG-3′ for Q468R.

The rat ORF was amplified by 5′-TCCCCGCGGTTCGAAACCatgcagcccgcaatgatgat-3′ (ratTPH2Fm) and 5′-GGACTAGTCTAGAtcaaatccccaaatattggttcatt-3′ (ratTPH2Rm). The ratTPH2Fm included *Csp45*I restriction sites and a ribosome-binding sequence (ACC) flanking the 5′ region of the TPH2 ORF, and the ratTPH2Rm included the *Xba*I restriction sites flanking the 3′ region of the rat TPH2 ORF.

The human and chimpanzee inserts were obtained by digestion with *Kpn*I and *Not*I, the recognition sites of which were located on the TOPO TA cloning vector, and the rat insert

was obtained by digestion with *Csp*45I and *Xba*I. The inserts were then ligated with the Gateway® pENTR™11 entry vector (Invitrogen, Carlsbad, CA) digested with the same restriction enzymes. The inserts were transferred from the entry vector to the Gateway™ pDEST™ 12.2 destination vector (Invitrogen, Carlsbad, CA) by an LR reaction with Gateway® LR Clonase™ II Enzyme Mix (Invitrogen, Carlsbad, CA).

Transfection of pDEST12.2/TPH2 ORFs to HeLa cells was performed using jet PEI® (PolyPlus-Transfection, Illkirch, France) essentially according to the manufacturer's instructions. HeLa cells (2×10^5 cells/3.5 cm dish) were plated 28 h prior to the treatment. The cells were transfected with 1.7 μg DNA (1.5 μg of TPH2/pDEST12.2 and 0.2 μg of β-GAL/pCMV-SPORT) plus 3.4 μl jet PEI for 48 h.

After transfection of the expression vectors for 48 h, HeLa cells were collected and the TPH activity was determined essentially as described previously [11]. Cells in the monolayer culture were collected in PBS(−) "Ca/Mg-free PBS" and then subjected twice to freezing in liquid nitrogen and thawing on water. The disrupted cells were pre-incubated for 15 min at 30 °C in 0.1 M Tris–HCl (pH 8.0) containing 30 mM DTT, 50 μM $Fe(NH_4)_2(SO_4)_2$, and 4 mg/ml catalase in a total volume of 100 μl. Subsequently, 50 μl of another cocktail was added to yield a final reaction mixture of 250 μM tryptophan, 400 μM 6R-tetrahydrobiopterin, 500 μM NADH, 1 mM NSD-1015, 2 mg/ml catalase, and 50 μg/ml dihydropteridine reductase in 0.1 M K-phosphate buffer (pH 6.9). The enzyme reaction was allowed to proceed for 10 min at 30 °C and was then terminated by adding 1 M perchloric acid.

The 5HTP formed was measured using a high performance liquid chromatography (HPLC) system equipped with a fluorescence monitor (JASCO model, FP920) set at excitation and emission wave lengths of 302 nm and 350 nm, respectively. The solid phase was ODS (4.6 mm × 250 mm, JASCO, Finepak SIL-C18T5), the mobile phase was a 100:5:7 mixture of 40 mM sodium acetate (adjusted to pH 3.5 with formic acid): acetonitrile: methanol and the flow rate was 1 ml/min [10]. To correct the enzyme activity, we employed the β-galactosidase enzyme assay system (Promega, Madison, WI) according to manufacturer's instructions.

Statistical analysis of enzyme activity was performed with one-way ANOVA followed by Tukey post hoc comparison. All *P*-values reported were two tailed. Statistical significance was defined at $P < 0.05$.

We measured the TPH2 enzyme activity to estimate the production of L-5-hydroxytryptophan (5HTP) in *ch468Q*, *ch468R*, human, and rat. The results of the enzyme activity assay were 49.40 ± 0.45 pmole/10 min/β-gal mU in *ch468Q*, 64.36 ± 1.67 pmole/10 min/β-gal mU in *ch468R*, 50.11 ± 1.72 pmole/10 min/β-gal mU in human, and 65.32 ± 0.99 pmole/10 min/β-gal mU in rat (Fig. 2). The *ch468R* and rat isoform showed significantly higher activity than *ch468Q* and human isoform (ANOVA, $P < 0.05$).

The sequences of the exon 11 coding region were well conserved among human and nonhuman primate species, except one missense substitution at A1404G leading to Q468R in chimpanzees. The enzyme activity assay indicated that the capacity

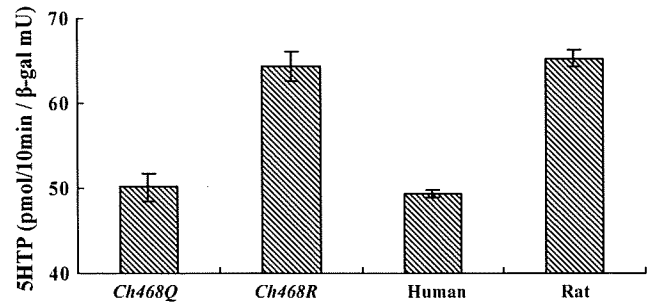

Fig. 2. Inter- and intra-specific comparisons of TPH2 activity. The TPH2 activity assay was conducted *in vitro* with disrupted cells of transfected HeLa cells and expressed as pmoles of 5-hydroxytryptophan (5HTP) per 10 min per β-gal mU. All data are presented as mean ± S.D.

of L-5-hydroxytrytophan biosynthesis was the same in *ch468Q* and human TPH2, whereas it was significantly high in the case of *ch468R*. If there were no significant changes in TPH2 expression levels, it could be a new gain-of-function allele. Although the molecular mechanism by which the *Q468R* mutation enhances catalytic function was not elucidated in the present study, replacement of the non-polar glutamine by the positively charged arginine could suggest the influence of the conformation of the catalytic site of the enzyme in some way [26].

This is the first comparative study on human and rat enzyme activity. In mice, *in vitro* 5HTP synthesis corresponded to their brain serotonin levels [31]. Therefore, although the difference in the activity was produced by an *in vitro* analysis, the high level of rat TPH2 activity probably implies the genetically higher serotonin concentration in serotonergic neurons of rodents than in human and nonhuman primates, if there were no significant differences in the amount of TPH2 protein.

TPH2, similar to all other monoamine-oxygenases, assembles into tetramers [29,30], and the tetramerization domain is present mostly within a 24-residue α-helix in the extreme C-terminal region of the enzymes [4,5]. The region of exon 11 is a part of the biopterin-dependent aromatic amino acid hydroxylase domain and also contains the tetramerization domain. The chimpanzee Q468R substitution is located on the tetramerization domain of the C-terminal. Therefore, the chimpanzee SNP may influence tetramer formation.

This result implies that low enzyme activity in humans and chimpanzees may result in low serotonin concentrations in the brain and may influence differences in the behavioral traits among chimpanzees as observed in mice [31,32]. Neurotransmitters such as dopamine and serotonin have been the prime target for understanding the biological basis of animal behaviors and interactions among animal groups. Primate social colonies are very sophisticated, and social interactions of chimpanzees are an interesting target for anthropologists. However, few genetic markers were reported for understanding primate behavioral traits [1,22].

In this study, we discovered a functional genetic marker for understanding the relationship between serotonin and chimpanzee behaviors including social dominance or aggression. The previous primate studies on the relationship between the serotonergic system and social dominance and aggression were

performed using vervet monkeys and rhesus macaques. Male vervet monkeys with a high rank within a group's social dominance hierarchy demonstrate elevated levels of serotonin in the blood and higher levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid (CSF) [21]. When drugs that increase serotonin transmission were administered to an individual, they acquired a higher dominance status in the colony [25]. Conversely, low CSF 5-HIAA levels were associated with increased ratings of aggression and risk-taking behavior in adolescent male rhesus macaques [19,20]. It is unclear, however, how measurements of whole blood 5-HT or CSF levels of 5-HIAA that relate to the actual functional turnover of 5-HT in discrete regions of the brain are associated with social behavior [17]. Our results might present a potential molecular mechanism for the elevated biosynthesis of 5HTP function in chimpanzee dominance-related behavioral traits.

To elucidate the total neurochemical mechanism for serotonin function, we may also need to conduct a survey on the other serotonin-related genes. Among such genes, tandem repeat polymorphisms in the 5′ promoter regions of serotonin transporter (5HTT) or monoamine oxidase A (MAOA) genes, which are associated with personality traits in humans, have been surveyed in chimpanzees and have shown less or low frequencies polymorphism [12,13]. Therefore, the TPH2 polymorphism reported in this study is particularly important in individual differences in the behaviors among chimpanzees. In future studies, detection of the levels of 5-HIAA in CSF in chimpanzees may facilitate the understanding total serotonin function in the brain. Also, we are searching polymorphic markers relating several serotonin receptor genes in chimpanzees.

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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 (Aβ) 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*)-ε4 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 Aβ-level in the brain of transgenic mice, overproducing Aβ at 9 months of age. In neuroblastoma cells, Aβ induced an increase in the *DYRK1A* transcript, which also led to tau phosphorylation at Thr^{212} under the overexpression of tau. Therefore, the upregulation of *DYRK1A* transcription results from Aβ 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 (Aβ) 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 Aβ in the brain (2). Therefore, it has been speculated that genetic factors related to AD could exist on chromosome 21, independent of the ε4 allele of the apolipoprotein E gene (*APOE*-ε4), 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-ε4 dose. Genes linked to these SNPs were the

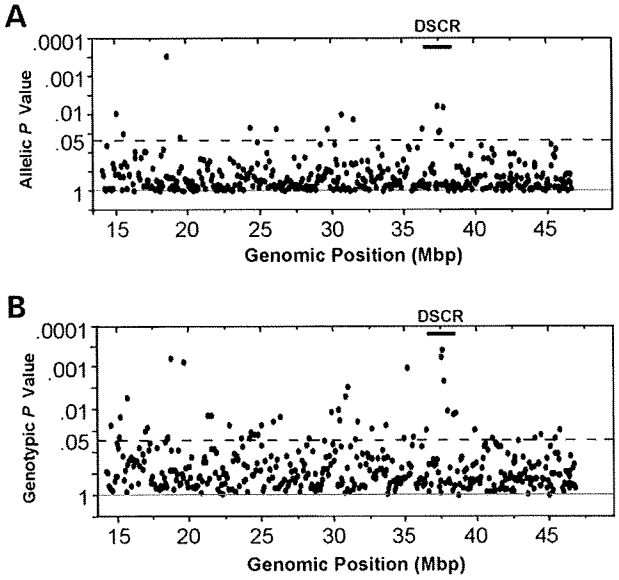

Figure 1. Exploratory scan using 417 markers. (**A**) *P*-values for allele frequency in chi-squared test. (**B**) *P*-values for genotype frequency in better fitting models. Genomic position is based on NCBI Build 35.

SAMSN1, *PRSS7*, *NCAM2*, *RUNX1*, *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 *RUNX1* 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, rs8126696 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 rs8126696. *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 rs8126696-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] (*P*)		Logistic regression[b]		Gene
	Allele	Genotype[c]	Odds (95% CI)	*P*-value	
rs723856	0.019	0.012 (aa)	1.53 (1.08–2.18)	0.0181	*SAMSN1*
rs2268437	0.008	0.008 (aa)	2.09 (1.24–3.55)	0.0059	*PRSS7*
rs2212624[d]	0.058	0.003 (gg)	1.66 (1.17–2.35)	0.0046	*NCAM2*
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	*RUNX1*
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	*DYRK1A*
rs2835908	0.024	0.056 (cc)	1.55 (0.99–2.43)	0.0546	*KCNJ6*

[a]One-sided *P*-value in chi-squared test.
[b]Logistic regression of risk genotype with age, sex and APOE-ε4 dose under no interaction.
[c]Risk genotypes in a better fitting model are shown in parentheses.
[d]AD group showed deviation from the Hardy–Weinberg equilibrium.

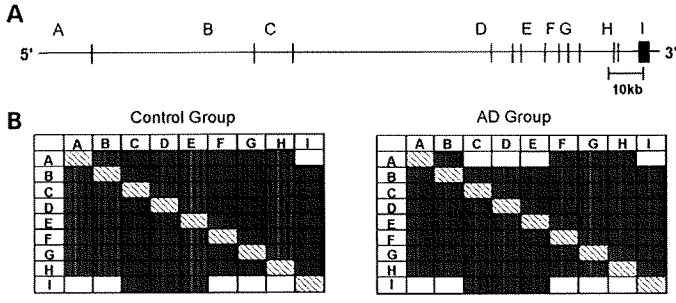

Figure 2. Linkage disequilibrium in *DYRK1A* gene region. (**A**) Genomic structure of the *DYRK1A* gene is shown. Horizontal bar indicates exons, and letters indicate SNPs, such as rs28360609 (A), rs2251085 (B), rs2835740 (C), rs10470178 (D), rs11701810 (E), rs1024294 (F), rs2835773 (G), rs2835774 (H) 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.

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

Haplotype[a]	Frequency			*P*-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

[a]Haplotypes 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 ($P = 0.038$).

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 $PS1^{I213T}KI$ and Tg-PS1/APP mice. Aβ1–40 level in $PS1^{I213T}KI$ mice was low, but Aβ1–40 was almost undetectable, whereas both Aβ1–40 and Aβ1–42 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 $PS1^{I213T}KI$ 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* 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 Aβ1–42 and by 1.3-fold ($P < 0.01$) with 25 μM Aβ25–35, compared with the level in non-treated cells, but was not changed with control 25 μM Aβ35-25. 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 Thr^{212} (Fig. 6A). Tau phosphorylated at Thr^{212} 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 Thr^{212}.

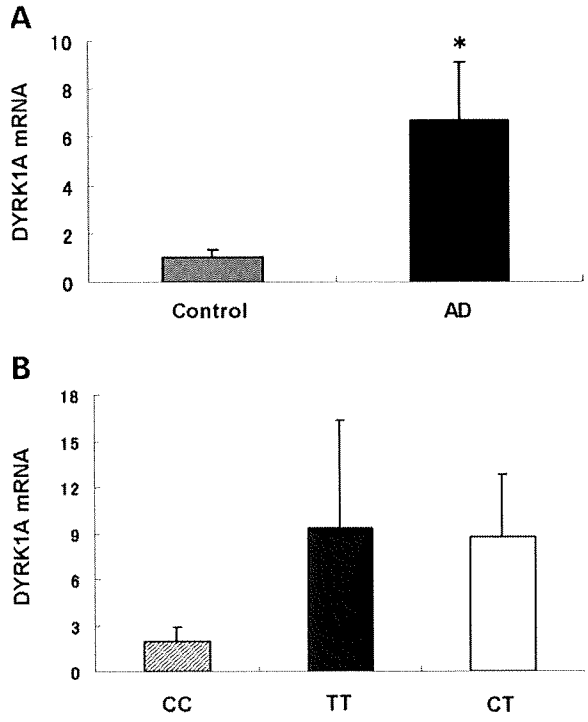

Figure 3. Expression of *DYRK1A* mRNA in human hippocampus. (**A**) Quantitative real-time PCR of *DYRK1A* mRNA in AD ($n = 22$) and controls ($n = 12$). (**B**) *DYRK1A* mRNA level in AD brain divided by rs28360609 genotypes, where CC is the risk genotype. *DYRK1A* mRNA level was expressed as the ratio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

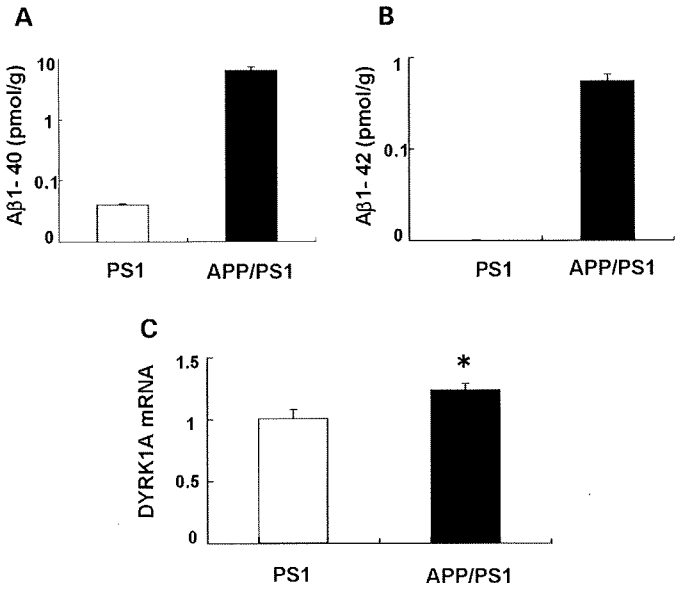

Figure 4. Aβ-level and expression of *DYRK1A* mRNA in transgenic mouse brain. Heterozygous PS1^{I213T}KI (PS1, $n = 6$) and Tg-APP/PS1 (APP/PS1, $n = 6$) mice were sacrificed at 9 months of age. Aβ-level was measured by ELISA. *DYRK1A* mRNA level was measured by quantitative real-time PCR. (**A**) Aβ1–40 level, (**B**) Aβ1–42 level and (**C**) amount of *DYRK1A* mRNA. *DYRK1A* mRNA level was expressed as the ratio of that of *GAPDH*. Data are shown as mean ± SEM. $^{*}P < 0.01$ by Student's *t*-test.

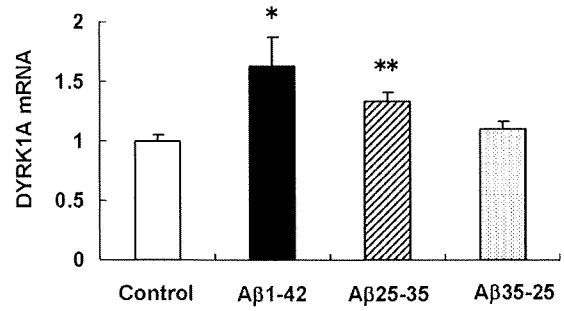

Figure 5. Aβ-induced expression of *DYRK1A* mRNA in SH-SY5Y cells. SH-SY5Y cells were incubated with Aβ1–42, Aβ25–35 and Aβ35–25. *DYRK1A* mRNA level was measured by quantitative real-time PCR. Values were normalized to those in untreated cells. *DYRK1A* mRNA level was expressed as the ratio of that of *GAPDH*. Data are shown as mean ± SEM of four independent measurements. $^{**}P < 0.01$ and $^{*}P < 0.05$ by Student's *t*-test compared with control.

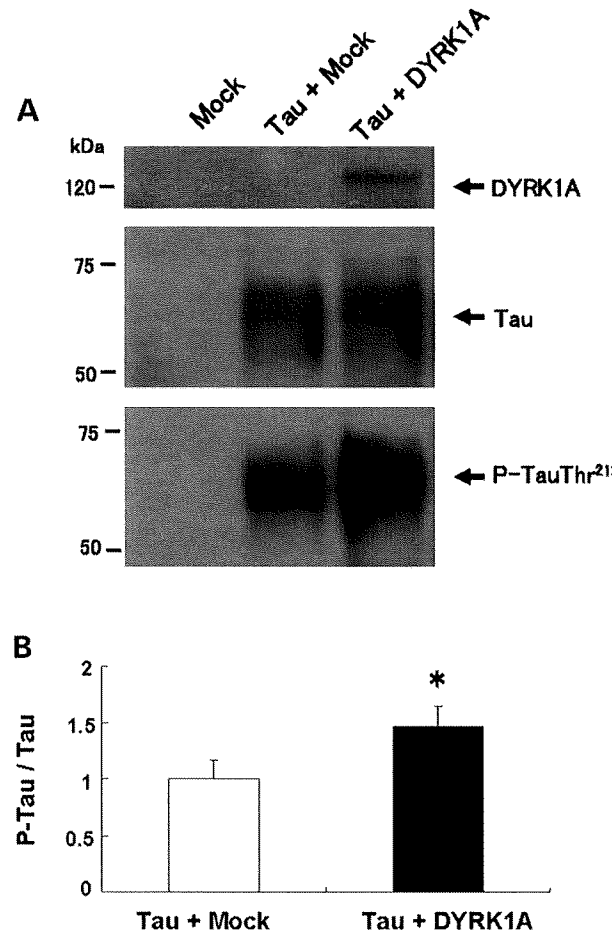

Figure 6. Tau hyperphosphorylation in DYRK1A-overexpressing cells. (**A**) HEK293T cells were transfected with either the *MAPT* expression vector (Tau) or both the *MAPT* and *DYRK1A* expression vectors (Tau + DYRK1A). After 24 h incubation, lysates were immunoprecipitated with anti-FLAG M2 agarose and then subjected to immunoblotting with anti-DYRK1A (DYRK1A), anti-tau (Tau) or anti-phosphotau (P-TauThr^{212}). (**B**) P-TauThr^{212}/Tau ratio was measured as integrated optical density values. Data are shown as mean ± 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 *SAMSN1* 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 minibrain* 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 Tyr^{321} residue in the active loop of the catalytic domain (44) and target protein phosphorylation occurs on serine/threonine residues in several proteins, including STAT3, FHKR, Gli-1, eIF2Bε, 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 Thr^{212} in HEK293T cells overproducing tau, suggesting that tau phosphorylation at Thr^{212} by DYRK1A could be a downstream consequence of Aβ overproduction. It was shown in an *in vitro* experiment that DYRK1A phosphorylates tau at Thr^{212}, which primes tau for phosphorylation by GSK3-β at Ser^{208}, 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 Thr^{212} could be prohibited *in vivo* (45,46). On the contrary, it was noted that peptides of tau phosphorylated at Thr^{212} 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 Thr^{212} 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 Aβ loading in neuroblastoma cells and transgenic mice, resulting in hyperphosphorylation of tau at Thr^{212} 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 Aβ 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 ± SD age at onset was 73.0 ± 8.0, range 60–94 years and age at blood drawing was 78.2 ± 8.3, range 60–98 years. Controls were composed of 375 individuals (54.7% female); age at assessment was 75.5 + 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 ± 8.5 years, 63.6% female) and 12 pathological controls (age: 89.0 ± 7.0 years, age at onset: 72.9 ± 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 Fukushimura Hospital.

Genotyping and sequencing

An exploratory scan was performed in 188 patients (67.0% female) (age at onset: 75.0 ± 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 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.

Table 3. Primer sequences for *DYRK1A* gene

	Primer sequences (5′–3′)		
Exon	Forward	Reverse	Product size (bp)
1	gtttttcttcacacagtg	cccccactaactgct	207
1	gtttttcttcacacagtg	cccccactaactgct	207
2	atgtcaaatgatacaaaca	ttttcccaatccataatc	394
3	gcaggttacagaagaggga	agggtaaatagtcacact	258
4	ctcaaatgtcaactgtag	aacaacaagattcactaag	359
5	ttgaatagaaatagatggc	tgtccaacagaaataaaca	445
6	taactgaactctgcgtttg	atacctacactgtcctacc	471
7	gaagttaatcaatggaac	tattcaaactgacctcac	413
8	ctgtatgctggatgtct	aacacactgatttcaagt	372
9	attatgtgagtgtttacg	gtaactgctccccac	481
10	ttaaccagacttcattgt	gtcattctaaaggcacct	433
11	tgaatgtatttgggattttgtgt	actgtgactgggatgtgg	1063
11	tatttgggattttgtg		(For sequencing)
11	ctgctcctcttgg		(For sequencing)
11	caagattctatggagg		(For sequencing)
11	cgtctactccaatcc		(For sequencing)

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 A_{260}/A_{280} 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^{I213T}KI and double transgenic (Tg-APP/PS1) mice were maintained on the B6 background. Six heterozygous Tg-APP/PS1 and six PS1^{I213T}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 Aβ at 0.5 μM for Aβ1–42 and at 25 μM for Aβ25–35 and Aβ35–25. Total RNA was isolated from harvested cells using an RNAeasy 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 Aβ1–40 and Aβ1–42 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 10 000*g* 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-TauThr^{212}) (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*-ε4 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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Conflict of Interest statement. None of the authors has any conflict of interest.

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Progressive changes of white matter integrity in schizophrenia revealed by diffusion tensor imaging

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Abstract

Recent magnetic resonance imaging (MRI) studies using diffusion tensor imaging (DTI) have suggested reduced fractional anisotropy (FA) in the white matter (WM) of the brain in patients with schizophrenia. We tried to examine whether such reduction in FA exists and whether such changes in FA progress in an age-dependent manner in a Japanese sample of chronic schizophrenia. FA values were compared between 42 patients with chronic schizophrenia and 42 controls matched for age and gender, by using DTI with voxel-by-voxel and region-of-interest analyses. Correlations of FA values with age and duration of illness were examined. Patients with schizophrenia showed lower FA values, compared to controls, in the widespread WM areas including the uncinate fasciculi and cingulum bundles. A significant group-by-age interaction was found for FA in the WM, i.e., age-related reduction of FA was more pronounced in schizophrenics than in controls. A significant negative correlation between FA and duration of illness was also found in the WM. Our data confirmed decreased FA in schizophrenics, compared to controls in the widespread WM areas. Such decreased FA values in schizophrenia might be attributable, at least in part, to progressive changes after the onset of the illness.

Keywords: Magnetic resonance imaging (MRI); DTI; Fractional anisotropy (FA); Aging

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