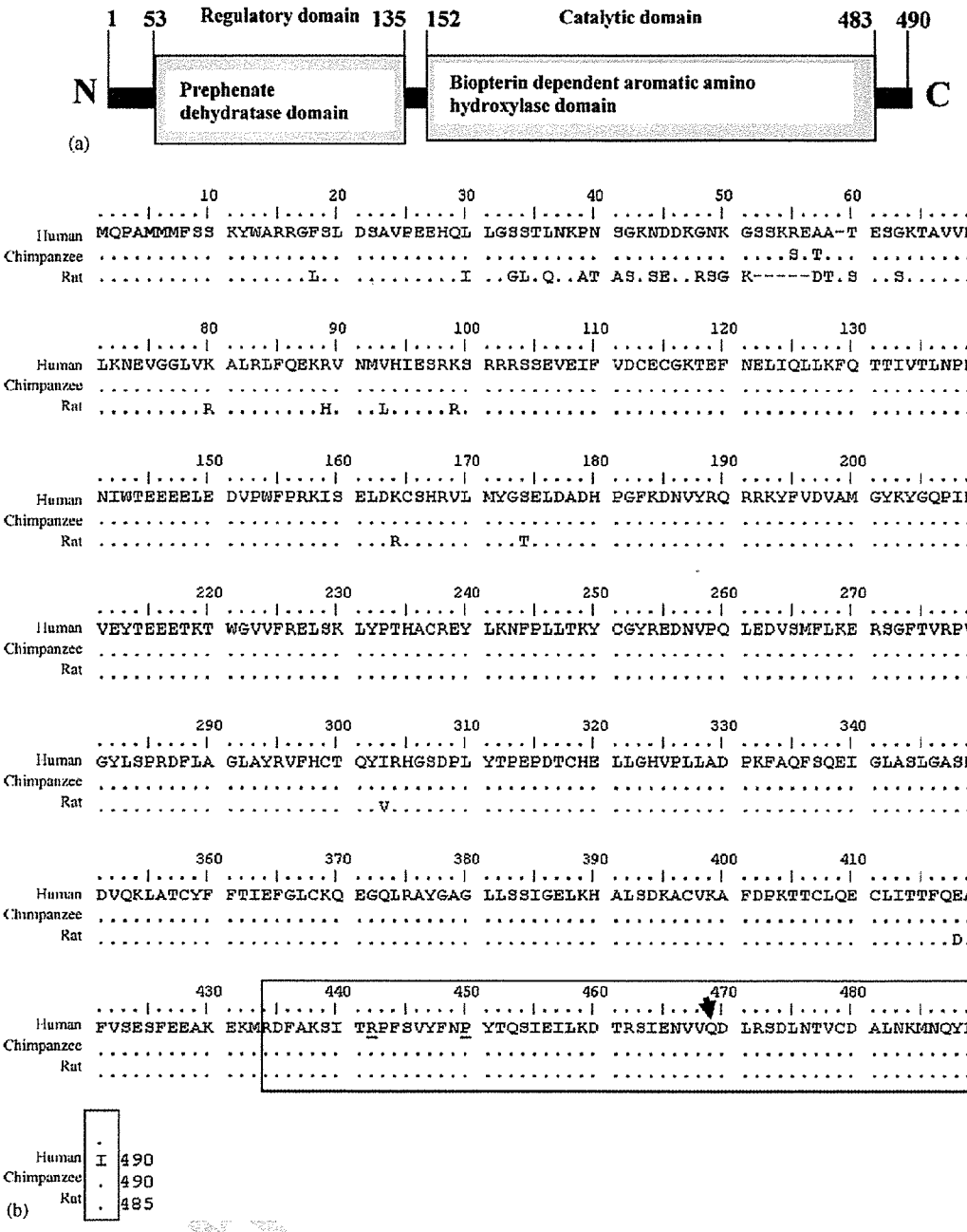

Fig. 1. The schematic TPH2 structure and amino acid sequence alignment. (a) The schematic TPH2 structure including two functional domains. The numbers indicate the amino acid position based on the human coding region. (b) The amino acid alignments of three mammals. The sequenced region (corresponding 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

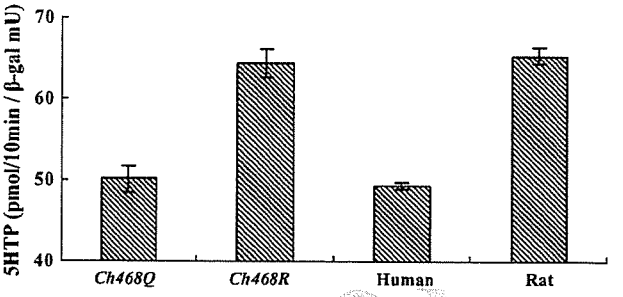

Fig. 2. Inter- and intra-specific comparisons of TPH2 activity. The TPH2 activity assay was conducted *in vitro* with disrupted cells of transfected HeLa cells and expressed as pmoles of 5-hydroxytryptophan (5HTP) per 10 min per β-gal mU. All data are presented as mean ± S.D.

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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