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Regional expression of tyrosinase in central catecholaminergic systems of colored mice

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Abstract: A relationship between coat color and behavioral characteristics has been reported for numerous species. We previously indicated that particular behavioral traits contributing to the genotype at the agouti locus manifest only when possessing a wild-type allele at the albino (i.e., tyrosinase: *Tyr*) locus. The present study was performed to investigate tyrosinase expression with marked activity in central nervous systems. The whole brain of male B10 and B10-c mice, a B10 congenic strain of the albino locus from BALB/c, at 8 to 9 weeks of age was removed, and obtained several regions of brain, especially catecholaminergic. Comparatively large amounts of *Tyr* mRNA and its translation products of approximately 68 kDa were found in the regions obtained, and definitely possessed the enzyme activity for the oxidation of L-tyrosine. The present results indicate the possibility that the amount of catecholamines produced in albino mice is higher than that of colored mice due to the deficit in tyrosinase heritably.

Key words: albino, central nervous system, striatum, tyrosinase

Introduction

As of October 2011, there were 378 loci known to be correlated with the expression of coat color phenotypes in mice, and genes of more than 150 have been cloned [21]. Among them, the *c* or albino (tyrosinase*: Tyr*) locus located on mouse Chromosome 7 [17] as an important determinant of the expression of coat color is known to encode tyrosinase (monophenol monooxygenase: EC 1.14.18.1), a rate-limiting enzyme in the synthetic pathway of melanin pigments [8]. The start point of the melanin synthetic pathway is to oxidize the aromatic amino acid L-tyrosine by tyrosinase, generally expressed in melanocytes of the skin, hair follicles, and the choroid, ciliary body, and iris of the eye, and the retinal pigment epithelium [18]. A loss-of-function mutation in the *Tyr* gene brings about an albino phenotype characterized by a white coat and pinkish-red eyes. Representative albino mouse strains such as inbred BALB/c or outbred ICR originated from Swiss strain mice possess the classic albino mutation, Tyr^{c}, which is considered to be a null mutation, explaining why the mutated protein has no activity *in vivo* and is retained in the endoplasmic reticulum [11, 31]. A point mutation in Tyr^{c} is identified at the residue +387, G-to-C, which results in the substitution of a cysteine for a serine residue at position 103 [17, 29, 35].

Meanwhile, a relationship between coat color and temperament, namely behavioral and emotional characteristics, has been reported for several species. Much attention has focused on the role of the specified subset of the gene locus, e.g., agouti locus, in the docility of

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animals including foxes [16], Norway rats [6], and deermice [12]. Many reports indicated the characteristic behavioral traits in mice with an albino phenotype when compared with a colored mice [4, 15, 19, 25, 30, 32, 33]. We also indicated that novel exploratory activity was suppressed in a manner dependent on the frequency of the dominant wild-type allele at the agouti locus, and the allele-dependent suppression was only observed in colored mice, and not in albino mice [34]. These reports point out that the governance of the *Tyr* locus extends not only the apparent behavioral phenotype, but also central nervous systems controlling behavioral traits markedly. However, the underlying mechanism remains unclear. In particular, the detailed expression profiles have not been confirmed whether tyrosinase in fact has enzyme activity in the central nervous system. The aim of the present study was to investigate tyrosinase expression and its marked activity in central nervous system.

Materials and Methods

Animals and dissection of specific brain regions

C57BL/10JMsHir (B10) mice, and B10.C-*Tyr*^{c}/Hir (B10-c) mice, a C57BL/10 congenic strain of the albino locus from the BALB/c strain [14] only for DOPA reaction test, were obtained from the RIKEN Bioresource Center (Tsukuba, Japan). All experiments were approved by Nihon University Animal Care and Use Committees, and performed in accordance with the Guidelines for Animal Experiments, College of Bioresource Sciences, Nihon University. Both strains were housed in an animal experimental facility with a 12-h light/12-h dark cycle (lights on at 06:00) and a temperature of 23 ± 1°C. Three days after delivery, the litter size was, in principle, adjusted to six pups. Each litter was weaned at 21 days of age, and male mice were housed three/cage with free access to commercial chow (Labo-MR-Stock, Nihon Nosan Kogyo Co., Yokohama, Japan) and tap water. Animals were sacrificed at 8 to 9 weeks of age by carbon dioxide, the whole brain of each mouse was removed, weighed, and frozen at –80°C. The brains frozen were placed onto a Stainless Steel Adult Mouse Brain Slicer Matrix with 2.0-mm coronal section slice intervals (Zivic Instruments, Pittsburgh, PA, USA) and dissected using a surgical blade to obtain the following regions: frontal cortex (FC), striatum (Str), hippocampus (Hip), substantia nigra (SN), hypothalamus (Hypo), and locus ceruleus (LS), according to the Allen Brain Atlas (http://mouse.brain-map.org/static/atlas), and stored separately in 1.5-ml tubes at −80°C.

RT-PCR and direct DNA sequencing

Total RNA from the brain sections described above was isolated using TRIzol® Reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's directions and quantified spectrophotometrically after DNase I (Takara Bio, Otsu, Japan) treatment. One μg of total RNA of each sample was reverse-transcribed using the iScript® cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. Three types of primer pair were designed in the regions overlapping exons to confirm the presence of alternative splice variants or exon skip mRNA (Supplementary Table S1, Fig. 1A). For DNA sequencing, 1 μl of cDNA, 10 μl of Platinum® PCR SuperMix (Invitrogen), and 0.5 μM of a pair of primers were mixed in a 200-μl PCR tube. PCR was performed with a thermal cycler, MyCycler (Bio-Rad). PCR conditions consisted of an initial denaturation step at 95°C for 5 min, followed by an additional 45 cycles with a denaturing step at 95°C for 30 s, an annealing step at 60°C for 30 s, and an extension step at 72°C for 60 s. A final extension step was performed at 72°C for 5 min. The PCR products were electrophoresed on a 2.0% agarose gel, and visualized with ethidium bromide. The target DNA fragment was excised in a minimal volume of agarose, and it was extracted by the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). Sample preparation for DNA sequencing was carried out with the BigDye® Terminator v03.1/1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and analyzed with an ABI 3130xl automated DNA sequencer (Applied Biosystems) at the General Research Institute, College of Bioresource Sciences, Nihon University. Data obtained were referred to mouse *Mus musculus* tyrosinase mRNA (NM_011661) published in NCBI Reference Sequence (http://www.ncbi.nlm.nih.gov/).

Real-time quantitative RT-PCR

Quantitative RT-PCR primers for *Tyr* were designed to amplify the 129-bp sequence from exons 4 to 5 (forward: 5'-GCACCTATCGGCCATAACAG-3'; reverse: 5'-GCCTGGATCTGACTCTTGGA-3'). A 127-bp sequence for *Rpl19* was amplified with primers (forward: 5'-AAGACCAAGGAAGCACGAAA-3'; reverse:

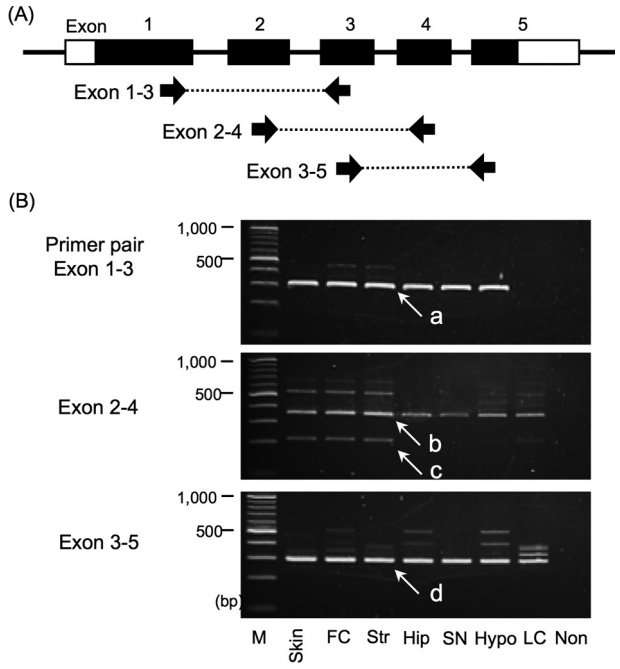

Fig. 1. (A) Diagram showing the tyrosinase gene (*Tyr*) locus, and annealing sites of primers for PCR-amplification of tyrosinase mRNA. Each primer pair was designed in the exons stepped over an exon to check for the presence of splice variants. (B) The expression of *Tyr* mRNA in specific brain regions of B10 mice. PCR products indicated by white arrows (a, b, c, and d) were sequenced to confirm the specific amplification by each primer pair (See DNA sequencing data in Fig. 2). M: DNA size standards with 100-1,000 bp in 100-bp increments. FC: frontal cortex, Str: striatum, Hip: hippocampus, SN: substantia nigra, Hypo: hypothalamus, LC: locus ceruleus, Non: non-template control.

5'-GCCGCTATGTACAGACACGA-3') and used as a control for relative mRNA quantification. To confirm that the primer pair amplifies a single specific PCR product, 10 μl of Platinum® PCR SuperMix (Invitrogen), 1 μl of cDNA, and 0.5 μM of a pair of primers were mixed in a 200-μl PCR tube. PCR was performed with a thermal cycler, MyCycler (Bio-Rad). PCR conditions consisted of an initial denaturation step at 95°C for 5 min, followed by an additional 45 cycles with a denaturing step at 95°C for 30 sec, an annealing step at 60°C for 30 s, and an extension step at 72°C for 60 sec. A final extension step was performed at 72°C for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel, and visualized with ethidium bromide.

SYBR Green I-based real-time quantitative RT-PCR amplification was performed using Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Test samples were assayed in duplicate in 20-μl reaction mixtures containing 10 μl of qPCR reaction mix, 0.5 μM of primers, 2 μl of cDNA, and 6 μl of nuclease-free H_2O. Non-template controls were also included. The thermal profile consisted of 2 min of denaturation at 95°C, followed by 45 cycles of PCR at 95°C for 5 s and 60°C for 20 s. Following amplification, melting curve analysis was performed to verify the authenticity of the amplified product from its specific melting temperature using a skin sample as a positive control, employing melting curve analysis software (ver. 6.0.19) of Rotor-Gene 3000 according to the manufacturer's instructions. The relative amounts of *Tyr* cDNA were normalized with reference to those of *Rpl19* cDNA.

Western blot analysis

The brain sections described above were also homogenized for Western blotting with a Polytron® homogenizer (RT10-35GT: Kinematica; Luzerne, Switzerland) and an ultrasonic homogenizer (UP-5s: TAITEC Corporation; Koshigaya, Saitama, Japan) in 200 μl of ice-cold RIPA buffer containing 1% Triton-X 100, 0.5% sodium deoxycholate, 1% SDS, 150 mM sodium chloride, 50 mM Tris-HCl (pH 8.0), and 2 μl of Protease Inhibitor Cocktail (Sigma-Aldrich Corporate; St. Louis, MO, USA). The protein concentration was quantified using a DC Protein Assay Kit (Bio-Rad) with bovine serum albumin as a standard. Lysates (1.25 μg protein/μl) were mixed with 5× Laemili Sample Buffer containing 10% SDS, 2.5% β-mercaptoethanol, 50% glycerol, 0.05% bromophenol blue, and 0.3 M Tris-HCl (pH 6.8), and boiled at 95°C for 5 min. Twenty μg of protein, except for the skin sample (10 μg of protein), was separated by electrophoresis on SDS-polyacrylamide gels (8%) and transferred to a polyvinylidene difluoride membrane (Immobilon®-P: Millipore Corporation; Billerica, MA, USA). A PiNK Plus Prestained Protein Ladder (Broad range, 10–175 kDa; GeneDirex, Las Vegas, NV, USA) was also eluted onto the gel as a molecular marker. Following transfer, the membrane was divided into 2 parts, at approximately 62 kDa, and blocked with 5% ECL™ Blocking Agent (GE Healthcare, Waukesha, WI, USA). Subsequent immunoblotting of each was carried out with goat polyclonal antibody against mouse tyrosinase (1:50, sc-7834; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal antibody against mouse GAPDH (1:100, sc-25778; Santa Cruz) diluted into 0.5% ECL™ Blocking Agent in PBS. After washing, the mem-

brane was incubated with HRP-conjugated rabbit polyclonal antibody against goat IgG (AP107P; Millipore) and HRP-conjugated donkey polyclonal antibody against rabbit IgG (NA934V; GE Healthcare) as a secondary antibody of each. Detection was carried out with the ECL™ Prime Western Blotting Detection Reagent (GE Healthcare), using ChemiDoc XRS (Bio-Rad). The band densities were measured using ImageJ software (http://rsb.info.nih.gov/ij/).

DOPA reaction test and immunohistochemistry

The DOPA reaction test was referred to the method of Gershoni-Baruch *et al.* [10]. The whole brain was divided sagittally and notches were coronally made at 1-mm intervals, and fixed immediately in a solution which consisted of 10% formalin and 15% sucrose in 10 mM phosphate-buffered saline (pH 7.4) at 4°C for 3 h. After fixation, each brain was embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek USA ; Torrance, CA, USA). The cryostat sections with a 20-μm thickness adhered to a slide glass were rinsed in PBS and incubated in 0.1% L-3,4-dihydroxyphenylalanine (DOPA) in 10 mM phosphate-buffered saline (pH 7.4) at 37°C for 24 h, and then they were rinsed in water. After the DOPA reaction, specimens were examined by light microscopy. Subsequently, immunohistochemistry was carried out with anti-tyrosinase antibody (Santa Cruz) as a primary antibody and RITC-conjugated donkey polyclonal antibody against goat IgG (1:100, sc-2094: Santa Cruz) as a secondary antibody. Then, specimens were examined by fluorescence microscopy.

Statistical analysis

Statistical analysis between brain regions was performed using ANOVA with Duncan's new multiple range test, using the software package Super ANOVA (Abacus Concepts, Berkeley, CA, USA).

Results

Previous observations indicate that mis-splicing of the *Tyr* gene expressed in melanocytes occurs at a rate of approximately 10% during the process of mRNA processing [27]. First, PCR products amplified by 3 types of primer pair to confirm the presence of alternative splice variants or exon skip mRNA were electrophoresed, and 3 bands that had the expected length of each and 1 band that had a shorter length than expected (Fig. 1B) were sequenced. As a result, the sequence of *Tyr* mRNA in all regions investigated, except for *Tyr* exon 1–3 in LC, was arguably in accordance with the sequence already known (NCBI Reference Sequence; NM_011661). Also, the *Tyr* exon 3 skipped-type mRNA variant was confirmed in FC and Str such as skin (Fig. 2).

Tyr mRNA expression in the central catecholamine-related regions tended to be markedly higher than that in the adrenal gland and thyroid gland, which produce biologically active substances from L-tyrosine as a major source. In brain regions investigated in the present study, *Tyr* mRNA in Str, approximately 39% of the skin, was significantly higher than those in Hip, SN, Hypo, and LC. *Tyr* mRNA in FC was approximately 53% of Str, but no significant difference was found between regions (Fig. 3). Tyrosinase protein in the specific brain regions was detected as a single band (approximately 68 kDa), which was consistent with the skin, despite the fact that a lower molecular weight protein (approximately 62 kDa) was also detected in the Hip. The level in the FC was significantly higher than that in SN and LC, and the levels in Str, Hip, and Hypo were relatively higher but no significant difference was detected among regions in the present study (Fig. 4).

As a result of the DOPA reaction test, a number of pigmented spots were found in specimens of Str from B10 mice, but not in those from B10-c mice. Furthermore, immunohistochemical analysis indicated that pigmented spots caused by exposure to a DOPA solution were co-localized with signals staining with anti-tyrosinase antibody (Fig. 5).

Discussion

The present study revealed that *Tyr* mRNA in comparatively large quantities expresses in the brain regions observed. Particularly, the expression in Str was approximately 39% of the skin, while no brain organs such as the adrenal and thyroid glands, which produce biologically active substances from L-tyrosine, had extremely low levels. Tyrosinase protein in accordance with the molecular weight observed in the skin was also identified in the brain, and clearly possessed the enzyme activity for the oxidation of L-tyrosine. In melanocytes, it is clear that the mouse tyrosinase gene consists of 5 exons which contain protein-coding sequences in all exons, bracketing 4 introns, and processes approximately 1.8 Kb mRNA, but over 10% of *Tyr* transcripts are

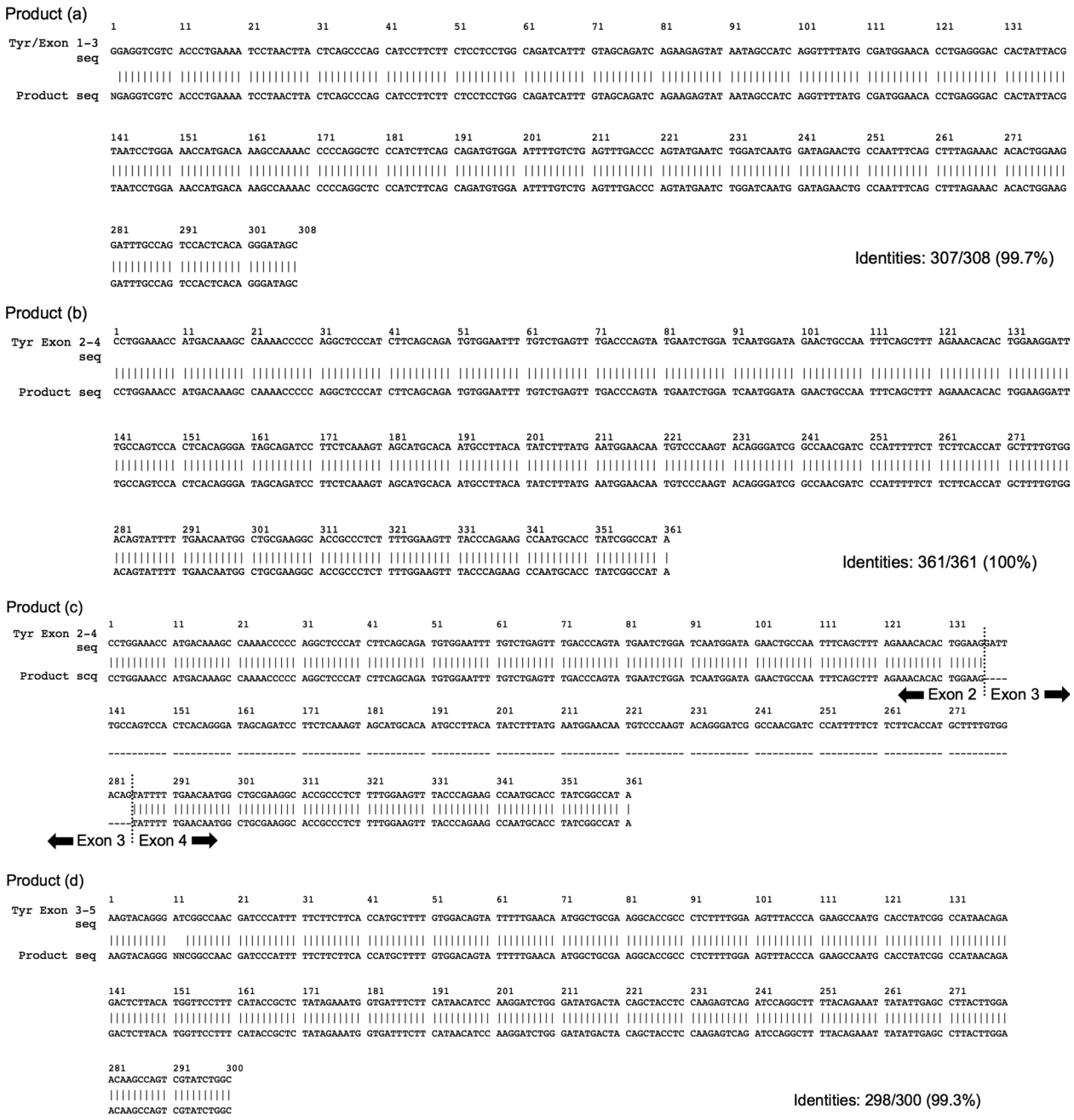

Fig. 2. Nucleotide sequences of the PCR products amplified by primers for the mouse tyrosinase gene (a–d in Fig. 1).

generally mis-spliced [27], as described above. Furthermore, at least twelve alternatively spliced transcripts of the *Tyr* gene have been confirmed in melanocytes from wild-type mouse skin, which are considered to be variants with loss-of-function [24]. The *Tyr* gene that has been processed normally is translated as a 55-kDa protein, and the mature protein is converted to about 67 to 75 kDa with glycosylation [5, 13]. In the present study, *Tyr* mRNA detected in Str closely corresponded with the sequence of *Mus musculus* tyrosinase mRNA (NM_011661) except for a variant skipping exon 3, and the gene/translation products with an approximately 68-kDa molecular weight possess oxidization properties for L-tyrosine. Meanwhile, another tyrosinase protein signal of 62 kDa was detected only in the Hip of all B10 mice analyzed. Generally in melanocytes, tyrosinase transla-

tional products undergo complex post-translational processing before reaching the melanosomal membrane. In mice, this processing including N-glycosylation arises in at least four of the six available sites for glycolsylation [3, 22], and abnormal glycosylation is involved in the depigmented phenotype, indicating that the acquisition of enzymatic activity is closely associated with the glycosylation state of tyrosinase. The present results may indicate the possibility of distinct glycolsylation among brain regions. In addition, primer pair Exon 1–3 failed to amplify any PCR products from LC, where is the nuclei of origin of the noradrenergic neurons, not dopaminergic. Moreover, *Tyr* mis-spliced transcripts were only found in FC and Str, even though in dopaminergic neurons. These findings indicate the neuron- and/or region-specific expression of *Tyr* gene, but remains unexplained.

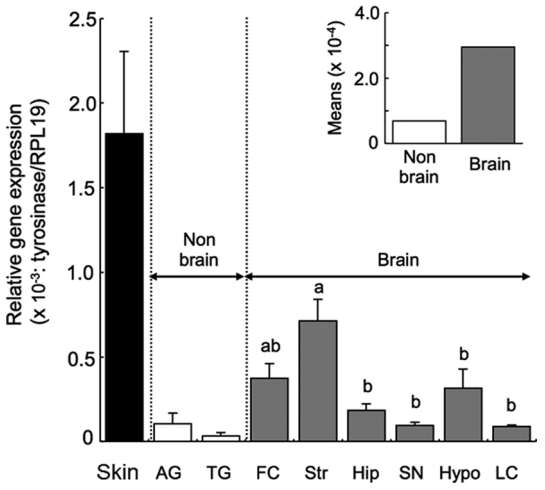

Fig. 3. RT-qPCR analysis of *Tyr* mRNA from specific brain regions of B10 mice. Non-brain areas with biosynthesis pathways leading to the amino acid tyrosine were also examined regarding the expression and compared with the brain. Data are expressed as the mean ± SEM. Different lowercase letters indicate a significant difference between brain regions (n=3, $P<0.05$). AG: adrenal gland, TG: thyroid gland, FC: frontal cortex, Str: striatum, Hip: hippocampus, SN: substantia nigra, Hypo: hypothalamus, LC: locus ceruleus.

The study of the relationship between albino mutation and behavioral traits began approximately 50 years ago. Very earlier finding in relation to behavioral characteristics observed in albino mice was regarded to be photophobia by a lack of retinal pigmentation [7]. However, subsequent works using non-visual learning tasks indicated behavioral differences between albino and non-albino mice, and concluded that the behavioral traits in albino mice might be attributed to an inability to respond efficiently to the environmental changes, rather than photophobia [9,23]. It is well-recognized that L-tyrosine as the start point of the melanin synthetic pathway is also a major source of neurotransmitter catecholamines. In general, catecholaminergic neurons, which include dopaminergic, noradrenergic, and adrenergic neurons, are located in discrete regions of the central nervous system and have an important role in a wide range of brain func-

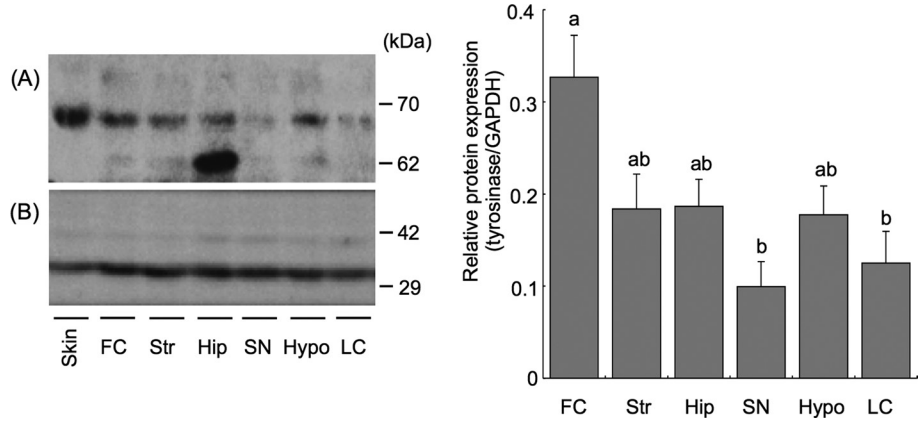

Fig. 4. Western blot analysis of tyrosinase protein from specific brain regions of B10 mice. A single band of tyrosinase (A: approximately 68 kDa) and GAPDH (B) proteins were detected in each case by ImageJ software. Data analyzed are expressed as the mean ± SEM. Different lowercase letters indicate a significant difference between brain regions (n=3, $P<0.05$). FC: frontal cortex, Str: striatum, Hip: hippocampus, SN: substantia nigra, Hypo: hypothalamus, LC: locus ceruleus.

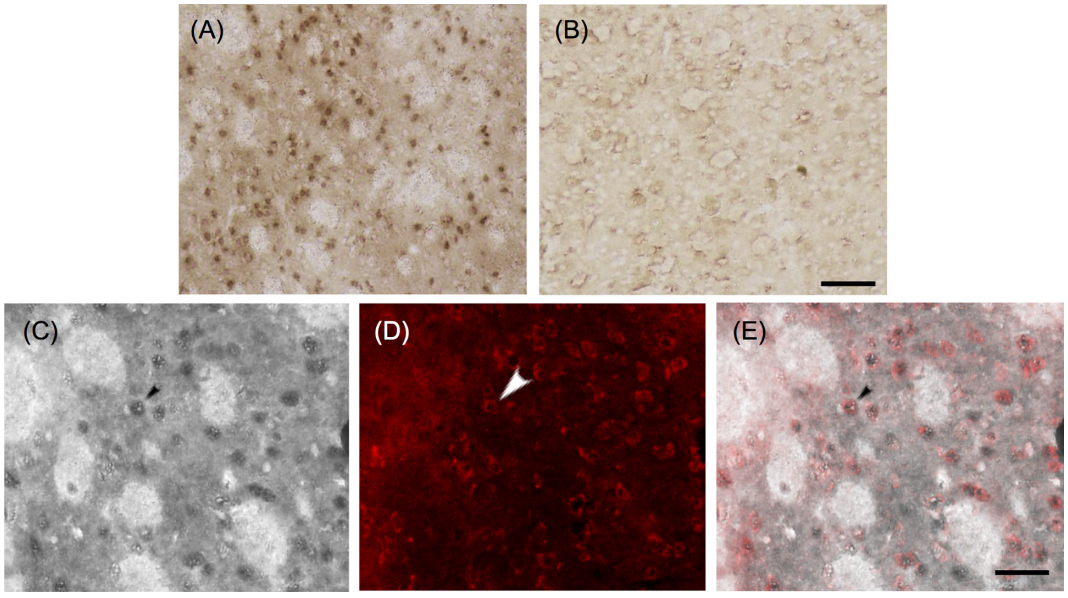

Fig. 5. DOPA (3,4-dihydroxyphenylalanine) reactions in specimens of the striatum from B10 (A) and B10-c (B) mice (Bar, 100 μm), and Immunohistochemical staining with anti-tyrosinase antibody after DOPA reaction in the striatum from B10 mice (C: DOPA reaction, D: anti-tyrosinase antibody, E: Merged C and D). Arrowheads indicate a typical cell with colocalization of melanin pigments produced by the DOPA reaction and tyrosinase protein (Bar, 50 μm).

tions, such as locomotion, sleep, memory, and learning. Particularly, the motility is tightly governed by the substantia nigro-striatum dopaminergic system [1]. The present results provided the potential possibility that the different behavioral traits observed in previous reports between non-albino and albino strains is attributed to the amount of catecholamines produced and/or released into the synaptic cleft, which was influences by the presence of tyrosinase.

As well as melanin pigments produced in melanocytes of the peripheral organs, neuromelanin, which is thought to be an inert cellular by-product, is produced from L-tyrosine in specific catecholaminergic neurons, noradrenergic and dopaminergic but not adrenergic [2, 28]. In mice with albinism, it is likely that much of the L-tyrosine is introduced by tyrosine hydroxylase (TH) into the catecholaminergic biosynthesis pathway, but fails to progress toward neuromelanin because of the lack of tyrosinase, except for a simple autoxidation pathway in both colored and albino mice. TH, the enzyme catalyzing the primary reaction in catecholamine synthesis, can convert L-tyrosine to L-DOPA, which is the sole precursor of the neurotransmitter catecholamines. Although an albino line of the TH-null embryo could not be rescued unless pregnant dams received supplementation with catecholamine precursors, catecholamines were readily detected in embryos or pups from a pigmented line of TH-null mice [26]. These findings indicate and support the hypothesis that tyrosinase participates in the oxidation of L-tyrosine in place of TH or in coordination with TH [20]. In consequence, the amount of catecholamines produced in albino mice is higher than that of colored mice, which are eventual phenotypes reflected by the brain function, but it remains unknown.

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