BASIC SCIENCE ARTICLE

Normalizing hyperactivity of the Gunn rat with bilirubin-induced neurological disorders via ketanserin

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BACKGROUND: Severe neonatal hyperbilirubinemia has been known to cause the clinical syndrome of kernicterus and a milder one the syndrome of bilirubin-induced neurologic dysfunction (BIND). BIND clinically manifests itself after the neonatal period as developmental delay, cognitive impairment, and related behavioral and psychiatric disorders. The complete picture of BIND is not clear.
METHODS: The Gunn rat is a mutant strain of the Wistar rat with the BIND phenotype, and it demonstrates abnormal behavior. We investigated serotonergic dysfunction in Gunn rats by pharmacological analyses and ex vivo neurochemical analyses.
RESULTS: Ketanserin, the 5-HT2AR antagonist, normalizes hyperlocomotion of Gunn rats. Both serotonin and its metabolites in the frontal cortex of Gunn rats were higher in concentrations than in control Wistar rats. The 5-HT2AR mRNA expression was downregulated without alteration of the protein abundance in the Gunn rat frontal cortex. The TPH2 protein level in the Gunn rat raphe region was significantly higher than that in the Wistar rat.
CONCLUSIONS: It would be of value to be able to postulate that a therapeutic strategy for BIND disorders would be the restoration of brain regions affected by the serotonergic dysfunction to normal operation to prevent before or to normalize after onset of BIND manifestations.

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IMPACT:

- We demonstrated serotonergic dysregulation underlying hyperlocomotion in Gunn rats. This finding suggests that a therapeutic strategy for bilirubin-induced neurologic dysfunction (BIND) would be the restoration of brain regions affected by the serotonergic dysfunction to normal operation to prevent before or to normalize after the onset of the BIND manifestations.
- Ketanserin normalizes hyperlocomotion of Gunn rats.
- To our knowledge, this is the first study to demonstrate a hyperlocomotion link to serotonergic dysregulation in Gunn rats.

INTRODUCTION

Delivery from in utero low-oxic condition into the oxygen-rich atmosphere lets newborn infants switch their oxygen carrying hemoglobin (Hb) from fetal-type HbF to adult-type HbA, where HbF and HbA are, respectively, hetero-tetramers $\alpha_2\gamma_2$ and $\alpha_2\beta_2$ of α-, β-, and γ-globin-chain subunits.[1,2] While the switching is completed about 1 year after birth, relatively immature livers in infants have to dispose of the neurotoxic bilirubin, a degradation product of hemoglobin, using UDP-glucuronosyltransferase (UGT1A1), a liver enzyme essential for the disposal of bilirubin. Newborn infants having defective UGT1A1 genes are therefore more predisposed to hyperbilirubinemia,[2–5] which is not rare in Asian races.[3,5]

Severe neonatal hyperbilirubinemia has been known to cause the clinical syndrome of kernicterus, as well as the milder syndrome of bilirubin-induced neurologic dysfunction (BIND). BIND clinically manifests itself after the neonatal period in the form of developmental delay, cognitive impairment, disordered executive function, and such behavioral and psychiatric disorders as attention-deficit/hyperactivity disorder (ADHD), schizophrenia, and autism spectrum disorder (ASD).[6–10] In neonatal hyperbilirubinemia, neurotoxic bilirubin in the peripheral blood crosses the blood–brain barrier and binds to such brain regions as the brain stem, basal ganglia, hippocampus, and cerebellum to adversely affect neuro-developmental processes including neurogenesis, myelination, and synaptogenesis during the early developmental stage.[11] Mechanism-based elucidation, however, of the linkage from hyperbilirubinemia in peripheral blood to those disturbances in brain function has still to be carried out.

The Gunn rat is a mutant strain of the Wistar rat but lacking UGT1A1 activity caused by the homozygous defective UGT1A1 gene,[12,13] and hyperbilirubinemia of the rat begins soon after birth and persists for life.[14] The neurological abnormalities and the histopathology of the rat are similar to those of human nuclear jaundice,[15] and the severe clinical symptoms of the rat are extrapyramidal disorders, such as dystonia, seizures, and death.[15]

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Histopathology of the rat showed that it had neuropathies such as cerebellar hypoplasia,[12] cell loss, and gliosis in the auditory nuclei, hippocampus, and basal ganglia.[12] The rat also showed various behavioral abnormalities including deficiency of social behavior,[16] cognitive impairment,[16–19] and hyperactivity in an open field test.[19] Our research group has also observed hyperactivity and increased rearing of the Gunn rat[16,18] and reported a finding relevant to the present study, namely, that the hyperactivity of the rat was not improved by haloperidol but by risperidone injections. While the former is a dopamine D2 receptor (D2R) antagonist,[18] the latter binds about 20 times as strongly to serotonin 2A receptor (5-HT2AR) than to D2R.[20] In the light of this finding, we inferred that correction of the serotonergic dysfunction by blocking 5-HT2AR would have a therapeutic effect on the hyperactive phenotype of the Gunn rat.

We herein report that 9–10-week-old Gunn rats having been subcutaneously (s.c.) injected with ketanserin 30 min before open field tests showed normalized hyperlocomotion and serotonergic dysfunction. Ketanserin is a quinazoline derivative and 5-HT2AR antagonist, and a study of [3H]ketanserin binding showed that after its injection (intravenous (i.v.)), the highest accumulation area was the frontal cortex and striatum in rats.[21] Then, by ex vivo neurochemical analyses, we compared the concentrations of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA; main metabolite of 5-HT) in the frontal cortices and striata of Gunn and control Wistar rats. Both 5-HT and 5-HIAA in the frontal cortex of the former were higher in concentration than in the latter, although in the striatum of the two groups neither of the them were statistically different from each other. The 5-HT2AR mRNA expression and protein abundance were studied, respectively, by real-time PCR (RT-PCR) and western blotting to show down-regulation of the mRNA expression without alteration of the protein abundance in the frontal cortex, but not in the striata, of Gunn rats compared with control. Of note was that serotonergic dysfunction was not found in the striatum but was found in the frontal cortex of the Gunn rat. These results suggest that serotonergic dysfunction (or increase in serotonergic transmission) in the frontal cortex, but not by dopaminergic dysfunction in the striatum, potentially contributes to hyperlocomotion of the Gunn rat.

METHODS
Experimental animals
Nine-to-ten-week-old male homozygous Gunn rats and male Wistar rats (SLC Inc., Hamamatsu, Japan) were used in this study. The animal facility of Shimane University was under controlled conditions of light (from 7:00 a.m. to 7:00 p.m.), temperature (23 ± 2 °C), and humidity (55 ± 5%). The rats were allowed free access to a standard laboratory diet and tap water. One week before the experiment, each of the rats was separately housed in a cage and handled once a day for 5 days. All experiments were performed with the approval of the Shimane University Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Japan. We used 41 rats (18 Gunn rats and 23 Wistar rats) for behavioral tests, 12 rats (6 Gunn rats and 6 Wistar rats) to perfuse brain tissue for immunohistochemistry (IHC), and 38 rats (19 Gunn rats and 19 Wistar rats) to collect fresh brain tissue for experiments of 5-HT, dopamine (DA), and metabolite assay; real-time PCR; and western blot. According to general model animal studies, significant differences can be detected with $N = 4$–$6<$.[22,23] Thus, we experimented with 5–8 animals per group.

The Gunn rat is a mutant strain that spontaneously appeared in a breeding colony of the Wistar rat. Liver UDP-glucuronosyltransferase (UGT: an enzyme responsible for bilirubin conjugation and excretion) activity of the rat is very low, caused by a single guanosine base deletion in the common-region exon 4

of UGT1A1.[24] The liver function of the Gunn rats we used was also low; total, indirect, and direct bilirubin levels in the sera of the rats were significantly higher than those of control Wistar rats (confirmed by serum biochemical analysis done by Oriental Yeast Co., Ltd., Tokyo, Japan). Other liver enzymes (aspartate amino-transferase, alanine aminotransferase, γ-guanosine triphosphate) were not higher than those of control Wistar rats (Supplementary Table 1).

S.c. injection of ketanserin
Ketanserin (K0051; TCI Chemicals, Tokyo, Japan) was dissolved in vehicle (5% dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO), 5% Tween-80 (Sigma Aldrich, St. Louis, MO) in 0.9% saline) and given at 1 ml by single s.c. injection to the Gunn and Wistar rats 30 min before open field tests.[25] The injected ketanserin dose to a rat was set at 0, 1, and 3 mg/kg. The animals were divided into the following six groups according to their species (Gunn or Wistar) and ketanserin dose (0, 1, and 3 mg/kg): G0, G1, G3, W0, W1, and W3 groups. We used 18 Gunn rats (G0 = 6, G1 = 6, G3 = 6) and 23 Wistar rats (W0 = 11, W1 = 6, W3 = 6) for behavior tests.

Open field test
A rat to be tested was placed in a $42 \times 42 \times 42$ cm plexiglass-made activity chamber under 290 lux illumination[26] (Scanet MV-40; MELQUEST, Toyama, Japan) 10 min before the open field test for accustomization.[18] The chamber has two infrared (IR) motion sensors at 0 and 13 cm height from the chamber base: the one senses horizontal activity of the rat (ambulation), and the other vertical activity (rearing). When the rat crosses either or both of the IR beams, sensor-signal breaks were automatically counted and accumulated at every 1-min interval for a total of 60 min.[16,18] We also counted the number of groomings and total grooming time to assay their stresses.[27]

Brain sampling
The Gunn and Wistar rats were intraperitoneal anesthetized with an anesthetic mixture of medetomidine (Domitor; Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan) at 0.15 mg, midazolam (Dormicum; Astelllas Pharma INC., Tokyo, Japan) at 2 mg, and butorphanol (Vetorphale; Meiji Seika Pharma Co., Ltd., Tokyo, Japan) at 2.5 mg/kg b.w./rat, and saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) was added to adjust the mixture to a volume of 0.5 ml/100 g b.w./rat. The rats were perfused intracardially with ice-cold sterilized saline (about 500 ml/rat), followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) (about 500 ml/rat).

Whole brains were removed immediately after perfusion and were post-fixed with 4% PFA in 0.1 M PB at room temperature (RT) for 4 hr. The brains were immersed in PB containing 10% sucrose at 4 °C overnight and subsequently were immersed in PB containing 20% sucrose at 4 °C for 2 or 3 days. Serial frontal sections of the brains were cut at 30 μm thickness with a freezing microtome (Microm HM 430; Thermo Scientific, Walldorf, Germany). These brain slice samples were used for IHC.

On the other hand, following blood removal, the frontal cortex and striatum were expeditiously isolated from the brain using a rat brain matrix (RBM-4000C; ASI Instruments, Warren, MI) and a rat brain atlas.[28] The frontal cortex in the rat brain extends from approximately +6.12 to +3.00 mm bregma, and the striatum extends from approximately +2.28 to −1.56 mm bregma.[28] These brain samples were stored at −80 °C until the day of analysis for 5-HT, DA, and metabolite assay and real-time PCR and western blotting.

5-HT, DA, and metabolite assay
5-HT, 5-HIAA, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and 3-methoxy-4-hydroxyphenyl acetic acid (HVA) in the frontal cortices and striata of the Gunn and control Wistar rats were quantitatively

analyzed by using a HPLC-ECD (electrochemical detection) system (HTEC-500; Eicom, Kyoto, Japan). Briefly, each brain section was weighed and homogenized in 200 μl of 0.2 M perchloric acid containing isoproterenol hydrochloride (Sigma-Aldrich, Tokyo, Japan) as an internal standard. The tissue homogenates were placed on ice for 30 min and then centrifuged at $15,000 \times g$ for 30 min at 4 °C. Collected supernatants were filtrated by 0.22-μm filter (ATTOPREP MF; ATTO Co., Tokyo, Japan), and the solution acidity was adjusted to pH 3.5 with 1 M sodium acetate. An aliquot of 10 μl sample solution was injected into a C-18 reverse-phase HPLC column (SC-50DS, 3.0×150 mm; Eicom, Kyoto, Japan) of HTEC-500, where the column temperature and detector potential were set at 25 °C and $+750$ mV, respectively. The mobile phase of the column was 0.1 M citric acid and 0.1 M sodium acetate, pH 3.6, containing 14% methanol, 140 mg/l sodium-l-octane sulfonate, and 5 mg/l EDTA, with a flow rate of 420 μl/min. Turnover rates of 5-HT and DA were calculated from concentrations of 5-HT, 5-HIAA, DA, DOPAC, and HVA, respectively.

Real-time PCR
Total RNA was extracted and reverse-transcribed by using, respectively, a Nucleospin RNA Plus XS and a PrimeScript RT-PCR Kit (Takara Bio Inc., Kusatsu, Japan), followed by quantitative PCR using a qPCR reagent (TB Green Fast qPCR MiX, RR430S; Takara Bio Inc., Kusatsu, Japan) and a thermal cycler (Thermal Cycler Dice Real Time System II, TP-900; Takara Bio Inc., Kusatsu, Japan). Quantification was done by using the Ct method as described in the manufacturer's protocol (Takara Bio Inc., Kusatsu, Japan). Data from all groups were normalized to β-actin. Results were compared with the control group to be expressed as fold change ± SEM. The primers were chosen to span an intron to avoid the detection of any contamination of rat genomic DNA.[29]

 5-HT2A receptor (5-HT2AR) primer
 Forward: 5-TACGCAGGCCGAAGGACGAT-3,
 Reverse: 5-GGGCACCACATTACAACAAACAGG-3
 5-HT2C receptor (5-HT2CR) primer
 Forward: 5-CACACCGAGGAGGAACTGGCTAATA-3,
 Reverse: 5-TCTGGGACGCTTTTCTTTCTTCTTT3
 β-actin primer
 Forward: 5-GCGCAAGTACTCTGTGTGGA-3,
 Revers: 5-ACATCTGCTGGAAGGTGGAC-3.

Western blot
We used standard western blot analysis techniques as described previously.[23] Frontal cortices and striata of the Gunn and control Wistar rats were, respectively, homogenized in RIPA buffer (FUJIFILM Wako Pure Chemical, Osaka, Japan) containing 10 μg/ml protease inhibitor cocktail (FUJIFILM Wako Pure Chemical, Osaka, Japan), 8 μg/ml calpain inhibitor I (Sigma-Aldrich, Inc., St. Louis, MO), 8 μg/ml calpain inhibitor II (Sigma-Aldrich, Inc., St. Louis, MO), and 0.1 mg/ml benzamidine (Sigma-Aldrich, Inc., St. Louis, MO). Brain homogenates were centrifuged ($1000 \times g$ for 10 min at 4 °C), supernatants containing soluble brain proteins were carefully separated from the pellets, and total proteins in the supernatants were quantitated by bicinchoninic acid method. Each protein solution was stored at -80 °C until use.

 Brain protein solutions were mixed with EzApply (ATTO Co., Tokyo, Japan), a sample preparation buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and an aliquot of 10 μg proteins was loaded on each lane of a 10–20% gradient polyacrylamide gel to be submitted for SDS-PAGE. Protein bands separated in the gradient gel were electroblotted to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Watford, UK). The membrane was blocked for 1 h at 4 °C in EzBlock Chemi (ATTO Co., Tokyo, Japan) followed by immunostaining overnight in solution with the following primary antibodies: rabbit anti-tryptophan hydroxylase isoform 2 (TPH2) antibody (1:1000; Abcam plc., Cambridge, UK), rabbit anti-5HT2AR antibody (1:100; Abcam,

Tokyo, Japan), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; 1:2000; Proteintech, Chicago), and mouse anti-β-actin antibody (1:2000; Abcam, Tokyo, Japan). The nitrocellulose membrane was subsequently treated in a blocking solution containing horseradish peroxidase-conjugated secondary antibodies against anti-rabbit IgG or anti-mouse IgG (Amersham, Arlington Heights, IL). Secondary antibodies on the membrane were visualized by peroxidase-catalyzed chemiluminescence with Immunostar Zeta (Wako, Tokyo, Japan) according to the manufacturer's protocol, and their images were captured by charge-coupled device (CCD) camera (ImageQuant LAS-4000; FUJIFILM, Tokyo, Japan). Captured 16-bit digital images were processed by Image J (NIH; Bethesda, MD), and pixel values of each protein band were normalized by an average of those of the β-actin or GAPDH band in the same lane.

Immunohistochemistry
We used standard two-step indirect IHC procedures that use an avidin-biotinylated peroxidase complex (ABC) method, as described previously.[30] Free-floating brain sections, of which endogenous peroxidase had beforehand been inactivated by incubation in 1% H_2O_2 for 30 min at room temperature (RT), were preincubated for 1 h at RT with 0.1 M PB containing 0.2% Triton X and 1.5% house serum followed by immunoreaction overnight at RT with a goat primary anti-TPH2 antibody (Ab) (1:2000; Abcam plc., Cambridge, UK) or a mouse anti-tyrosine hydroxylase (anti-TH) Ab (1:5000; Immunostar Inc., Hudson, WI) and by a secondary Ab at RT for 1 h with a biotinylated secondary anti-goat Ab or anti-mouse Ab (1:500; standard ABC kit, Vector Laboratories, Burlingame, CA). The tissue sections were incubated for 1 h at RT in phosphate-buffered saline (PBS) solution containing avidin–biotin peroxidase complex, to finally form complexes of TPH2 or TH, primary Ab, secondary Ab and avidin-biotin peroxidase on the tissue sections. After incubation of the sections for 10 min at RT with a substrate working solution, 3-amino-9-ethylcarbazole (Vector Laboratories, Burlingame, CA) or 3,3'-diaminobenzidine (R&D Systems Inc., Minneapolis, MN), the complexes on the tissue sections were visualized by colored deposit of the substrates. After rinsing with water or PBS, the tissue sections were mounted onto gelatin-coated slides and then coverslips (Matsunami Glass, Osaka, Japan) with mounting medium. Finally, the sections were examined under a light microscope (Keyence BZ-X700, Osaka, Japan). The selected slices were located between approximately -7.32 and -8.28 mm bregma. These slices clearly contained the dorsal raphe nucleus (DRN), posterodorsal raphe nucleus (PDR), and median raphe nucleus (MnR) regions. All images were captured with Keyence BZ-X700 using a ×20 objective lens, and a total of 10 images per rat were captured.

Statistical analysis
Statistical analysis of the data was carried out using the SPSS software (IBM SPSS Statistics for Windows, Version 22.0; IBM Corp., Armonk, NY). The data are presented as mean ± SEM. Comparisons of two groups were performed by analysis of variance, followed by the appropriate multiple Student's t test or Mann–Whitney U test on the basis of the F-test or Dunnett's test. In all cases, differences were considered significant at $P < 0.05$.

RESULTS
Single s.c. injection of ketanserin improved hyperlocomotion in the Gunn rat
Figure 1 compares locomotion data of the Gunn (denoted as (j/j)) and control Wistar (($+/+$)) rats in the open field test, where we noticed that the Gunn rats without ketanserin treatment (i.e., G0 groups) showed more beam-break counts of ambulation (Fig. 1a, b) and higher rates of rearing (Fig. 1c) and grooming (Fig. 1d), like agitation, than the control W0 groups. Comparing those

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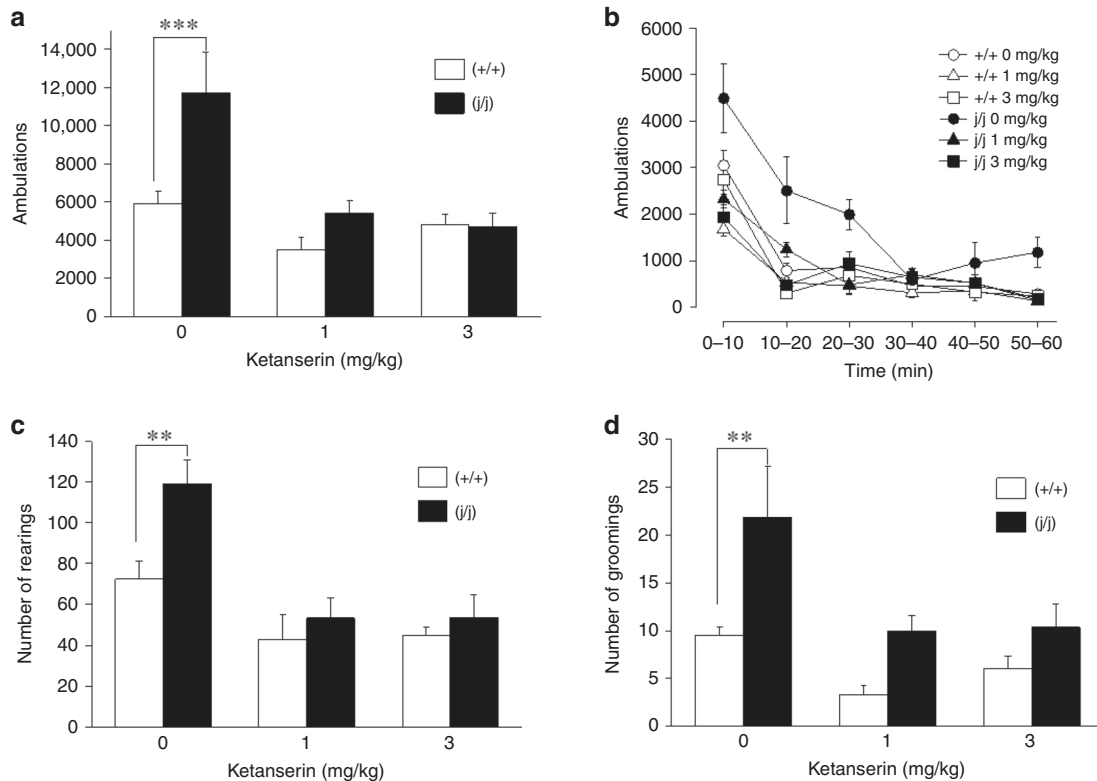
Fig. 1 Ketanserin treatment normalized abnormal locomotor activity in Gunn rats. Locomotor activity was assayed by open field test. Total beam-break counts of ambulations (**a**), the number of rearings (**c**), and the number of groomings (**d**) during 60 min in open field test. Compared with the vehicle (ketanserin 0 mg/kg) treated Wistar rat group, the vehicle-treated Gunn rat group displayed increasing ambulations, number of rearings, and number of groomings (Ambulations: ***$P = 0.00032$, Number of rearings: **$P = 0.006$, Number of groomings: **$P = 0.0013$). The ketanserin (1 mg/kg, 3 mg/kg) treated Wistar and Gunn rat groups were not significantly different compared with the vehicle-treated Wistar rat group. **b** Ambulation during 10 min. Each bar represents 6 subjects in each group ($N = 6–11$ each). Gunn rat: black bar or symbol (j/j), Wistar rat: white bar or symbol (+/+). All data indicate mean ± SEM. Statistical analysis of the data was performed by Dunnett's test (compared with the vehicle-treated Wistar rat group). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

locomotion data of the G1 and G3 groups with those of the W1 and W3 groups in Fig. 1a–d, we further noticed that ketanserin treatment 30 min before the open field test caused both G1 and G3 group rats not to be statistically different from the respective W1 and W3 control groups. Neither Gunn nor control Wistar rats showed any head twitching, a hallucination-like behavior presumed in rodent models of schizophrenia.

Augmentation of 5-HT transmission and downregulation of 5-HT2A/C receptor mRNA in the Gunn rat frontal cortex
Considering the effect of ketanserin treatment on Gunn rat hyperlocomotion together with 5-HT2 receptor distribution in the rat forebrain,[31] we measured the concentrations of neurotransmitters and their metabolites in the frontal cortex and striatum of the Gunn rat without ketanserin treatment by ex vivo neurochemical analyses to determine whether or not 5-HT system dysfunction existed that could be subdued by ketanserin binding to 5-HT2Rs. Figure 2 shows the concentrations in the frontal cortices and striata of the (j/j) and control (+/+) rats of 5-HT, 5-HIAA, DA, DOPAC, and HVA and turnovers of 5-HT and DA, respectively, calculated by the ratios of 5-HIAA/5-HT and (DOPAC + HVA)/DA.

Figure 2a, b show that both 5-HT and 5-HTIAA concentrations were significantly higher in the frontal cortex but not in the striatum of the (j/j) rat compared to the control (+/+) rat, and Fig. 2c shows that 5-HT turnover did not differ significantly both in the frontal cortices and striata between the two rats. Figure 2d–g, on the other hand, show that DA and its metabolite concentrations in the frontal cortices and striata of the (j/j) and control (+/+) rats did not significantly differ from each other.

Ketanserin is a high-affinity antagonist toward 5-HT2 receptor families (5-HT2Rs) in rodent brain, having a subtype-dependent inhibition constant K_i toward 5-HT2 receptor subtypes A (5-HT2AR) and C (5-HT2CR).[32] In order to determine whether either or both subtypes are involved in the ketanserin effect on Gunn rat hyperlocomotion, we compared the RT-PCR 5-HT2AR and 5-HT2CR expression levels in the frontal cortices and striata of the Gunn and control Wistar rats. As shown in Fig. 3a, 5-HT2AR expression was lower in the frontal cortex of the (j/j) rat, but not in the striatum, than that in the control (+/+) rat. Figure 3b illustrates that the 5-HT2CR expression was not significantly different in the two rats. We also investigated the 5HT2AR protein expression in the Gunn rat frontal cortex and striatum, and there was no significant difference compared with the control (+/+) rat (Supplementary Fig. 2).

Augmentation of TPH2 in raphe region of Gunn rat
Serotonergic neurons project from the raphe nucleus to the forebrain in rat brains,[33] and one of their marker proteins is TPH2, an enzyme involved in 5-HT synthesis in the brain. Figure 4 compares western blots (Fig. 4h) and IHC images (Fig. 4a, b–g) of TPH2 protein in the raphe region of the Gunn and control Wistar rats. The lower panel in Fig. 4h shows CCD-camera-captured 16-bit digital images of TPH2 and GAPDH western-blotted bands of the (+/+) and (j/j) rat samples, and the rectangular shapes surrounding the respective bands were registered in Image J as "regions of interest" (ROIs). Image J analyzed the respective ROIs and reported integrated densities of the TPH2 and GAPDH bands of the (+/+) and (j/j) rats, followed by normalization of the former by the latter in the respective lanes. The lower bar graph in Fig. 4h shows

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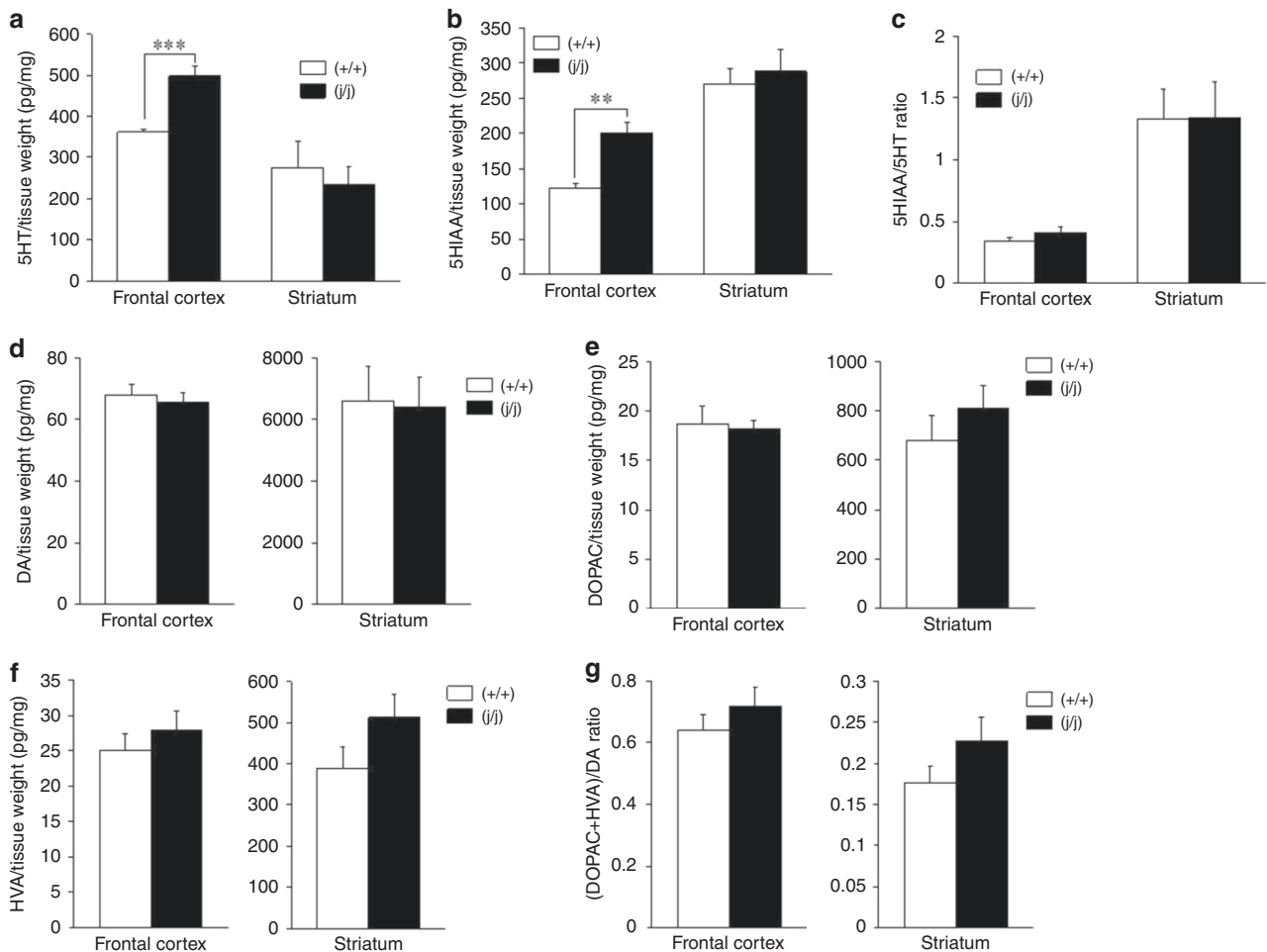
Fig. 2 Increased concentrations of 5-HT and 5HIAA in the Gunn rat frontal cortex. These figures show the concentrations of 5-HT, DA, and their metabolites in the frontal cortex and striatum as assayed by ex vivo neurochemical analysis ($N = 6–8$ each). **a** 5-HT: 5-hydroxytryptamine, **b** 5HIAA: 5-hydroxyindole acetic acid, **c** 5HIAA/5-HT ratio, **d** DA: dopamine, **e** DOPAC: 3,4-dihydroxyphenylacetic acid, **f** HVA: 3-methoxy-4-hydroxyphenyl acetic acid, **g** (DOPAC+HVA)/DA ratio. Gunn rats showed significantly higher concentrations of 5-HT (***$P = 0.0014$) and 5HIAA (**$P = 0.0026$) in the frontal cortex than Wistar rats. DA and its metabolites were not significantly different between Gunn rats and Wistar rats. Gunn rat: black bar (j/j), Wistar rat: white bar (+/+). All data indicate mean ± SEM. Statistical analysis of the data was performed by multiple Student's t test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

normalized TPH2 integrated densities that show about 2.3 times augmentation in the (j/j) samples over the (+/+) ones. Comparison of TPH2-stained IHC images of the DRN (Fig. 4b, c), PDR (Fig. 4d, e), and MnR (Fig. 4f, g), where the left and right columns indicate the (+/+) and (j/j) sample images, respectively, proved that TPH2-positive cells in the (j/j) raphe regions showed no obviously different distributions from the (+/+) regions.

While TPH2 is one of the serotonergic neuron markers, TH itself is one of the dopaminergic neuron markers in the central nervous system. Supplementary Fig. 1 shows TH-stained IHC images of the ventral tegmental area (VTA) and substantia nigra, a compact part (SNc) of the (+/+) and (j/j) samples, from which we can see that the dopaminergic neurons in VTA and SNc of the (j/j) rat had no clear differences in distribution from those in the control (+/+) rat. Dopaminergic neurons project from VTA to the frontal cortex and from nigra to the striatum in rat brain,[34] and Supplementary Fig. 1 suggests that no abnormalities were found in the upstream regions of the dopaminergic system in the Gunn rat brain.

DISCUSSION
To our knowledge, this is the first study to demonstrate a hyperactive link to serotonergic dysfunction in Gunn rats. As for

nuclear jaundice in humans, unconjugated bilirubin deposited in basal ganglia, in specific globus pallidus and subthalamic nucleus, as well as in other nerve nuclei has not been revealed.[35]

In summary, 9–10-week-old Gunn rats having been s.c. injected with ketanserin 30 min before open field tests normalized their hyperlocomotion and serotonergic dysfunction. Ketanserin is a quinazoline derivative and 5-HT2AR antagonist, and we compared the concentrations of 5-HT and 5-HIAA in the frontal cortices and striata of Gunn and control Wistar rats by ex vivo neurochemical analyses. In the frontal cortex of Gunn rats, both 5-HT and 5-HIAA were higher in concentrations than in control Wistar rats, but in striata of the two rat types they were not statistically different from each other. The 5-HT2AR mRNA expression and protein abundance were studied by RT-PCR and western blotting, respectively, to reveal the downregulation of the mRNA expression without alteration of the protein abundance in the frontal cortex, but not in the striatum, of the Gunn rats compared with the control Wistar rats. Of note was that serotonergic dysfunction was not found in the striatum, but it was observed in the frontal cortex of the Gunn rats. On the other hand, our study showed that the Gunn rat dopaminergic system was intact.

Figure 1 shows that Gunn rats displayed hyperactivity, frequent grooming and rearing during open field tests, behaviors that

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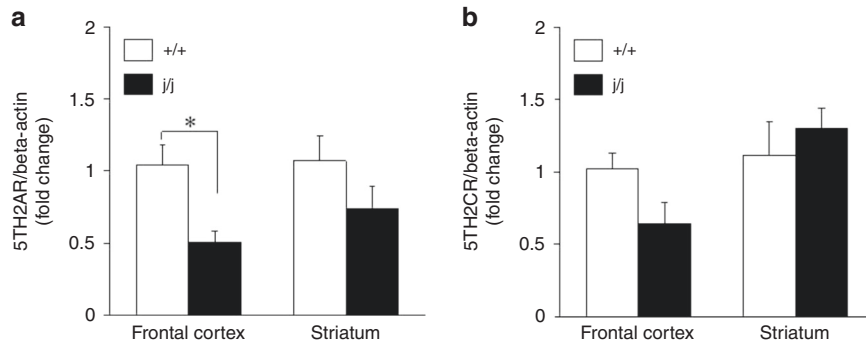
Fig. 3 Reduction of 5-HT2AR mRNA expression in the Gunn rat frontal cortex. Expression levels of 5-HT2AR- and 5-HT2C-mRNA were assayed by reverse transcription PCR (RT-PCR) using total RNA from the frontal cortex and striatum ($N = 6$ each). **a** Decreased expression of 5TH2AR mRNA in the Gunn rat frontal cortex compared with Wistar rat (*$P = 0.015$). **b** On the other hand, expression of 5TH2CR mRNA was not significantly different in the frontal cortex or striatum. Gunn rat: black bar (j/j), Wistar rat: white bar (+/+). All data indicate mean ± SEM. Statistical analysis of the data was performed by multiple Student's t test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

seemed agitation-like. These abnormal behaviors in Gunn rats were ameliorated by 5-HT receptor antagonist, ketanserin, s.c. injection. 5-HT is associated with anxiety, agitation, mood, and aggression, and it is considered to be a main target among neurotransmitters regarding neuropsychiatric disorders.[36] Especially, the pre-frontal cortex (PFC), which projects serotonergic neurons from median raphe nuclei, controls emotion and cognition. PFC has a rich expression of 5-HT2AR, and 5-HT works to increase the postsynaptic current and inhibition of GABAA receptor via its receptors.[37,38] It has also been reported that 5HT2AR antagonists improve hyperactivity and agitation-like behavior in such pharmacological rodent models.[39,40] In vivo binding study of [3H]ketanserin showed that after [3H]ketanserin injection (i.v.), the highest labeling was found in the frontal cortex in rats.[21] In our study, we found hyperlocomotion together with frontal cortical abnormal serotonergic transmission in Gunn rats. It is speculated that the effect of ketanserin on the Gunn rat abnormal behaviors may be due to the result of inhibiting the excess 5-HT transmission via the 5HT2A receptor in the frontal cortex.

Figure 4, on the other hand, shows augmentation of TPH2 protein without clear differences in distribution of TPH2-positive serotonergic cells in the raphe region of the Gunn rat. It is well known that, while cell bodies of most serotonergic neurons are found largely within the boundaries of the raphe nuclei, their axonal projections innervate nearly every area of the central nervous system.[33] It seems plausible that increased 5-HT production in the raphe region of the Gunn rat, as shown in Fig. 4, led to hyper serotonergic transmission in the frontal cortex of Gunn rats, as shown in Fig. 2. The downregulation of 5-HT2AR mRNA, as shown in Fig. 3, might be caused by the hyper serotonergic transmission in the Gunn rat frontal cortex.

The locomotor circuitry in vertebrate animals is well understood.[41] The basal ganglia and striatal projection neurons of the direct pathway initiate locomotor activity. The indirect pathway suppressed movement via globus pallidus externa and the subthalamic nucleus. The output from the basal ganglia activates the mesencephalic locomotor region, which in turn impinges on the lateral paragigantocellular nucleus that controls the spinal cords. The basal ganglia receive input from the cortex, thalamus, and SNc. Serotonergic neurons are located in the brainstem and innervate several levels of the locomotor circuitry. The rostral group of 5-HT nuclei largely project to the forebrain, and the caudal group of 5-HT nuclei largely project to the spinal cord. Moreover, the 5-HT nuclei also project to the brainstem (midbrain, pons, and medulla).[42] The dopaminergic neurons in SNc also contribute to locomotor activity. The neurons project from SNc to the basal ganglia and brainstem.[43] Based on these findings, other

brain regions (i.e., thalamus, basal ganglia, brainstem, etc.) are also considered to be important for locomotion, so it may be worth investigating these brain regions in Gunn rats.

Previous studies have shown some abnormal behaviors of Gunn rats, such as hyperactivity,[19,44] increased aggression,[16] and attention and learning impairments.[45] Gunn rats after sulfa injection show a clinical course and neuropathological findings similar to bilirubin encephalopathy, such as nuclear jaundice.[15] Severe neurological abnormalities of Gunn rats after sulfa injection are clinical symptoms such as opisthotonos, seizures, ataxia, and dystonia,[15] and characteristic neuropathological findings are cell death or gliosis in auditory nuclei, oculomotor nuclei, cerebellum, hippocampus, and basal ganglia.[46] As for neurotransmitters of Gunn rats, one study reported elevated catecholamine levels in basal ganglia and cerebellum.[45] Our group reported that some cognitive impairment of Gunn rats was likely to be caused by highly activated microglia.[17,47–49] Recently, interneuronal loss in the Gunn rat hippocampus was reported.[30] While those studies suggested associations of central neural dysfunction with the abnormal behaviors of Gunn rats, their mechanisms have not yet been sufficiently revealed.

The results suggest the presence of agitation underlying the hyperactivity of Gunn rats. The relationship between agitation and 5-HT is widely known as "serotonin syndrome." Serotonin syndrome is an adverse phenomenon that is caused by an excess of 5-HT secreted by psychoactive drugs such as selective 5-HT reuptake inhibitors,[50] and its clinical symptoms are remarkable agitation, psychomotor excitation, confusion, autonomic symptoms, etc. Agitation is one main psychological symptom that also accompanies schizophrenia, neurodevelopmental disorder, and dementia, not just serotonin syndrome. Patients with prolonged agitation feel strong suffering, and treatment of agitation has profound significance in the clinical picture.

Neonatal jaundice, including physiological jaundice, occurs in about 90% of Japanese, and we have not recognized the clinical risk of neonatal jaundice since phototherapy became an established therapy for pathological jaundice. However, together with results in the progress of neonatal care, several pediatricians have suggested to the rediscovery of the problems concerning neonatal jaundice particularly in premature babies. Recent studies have suggested that there is a significant correlation between neonatal jaundice and neurodevelopmental disorders.[8,51] One epidemiological study conducted in Denmark for about 10 years reported a significantly increased risk for infantile autism in infants who showed hyperbilirubinemia after birth.[7] The mechanisms associating neonatal jaundice and neurodevelopmental disorders remain poorly understood, so this clinical problem has been left largely unexplored.

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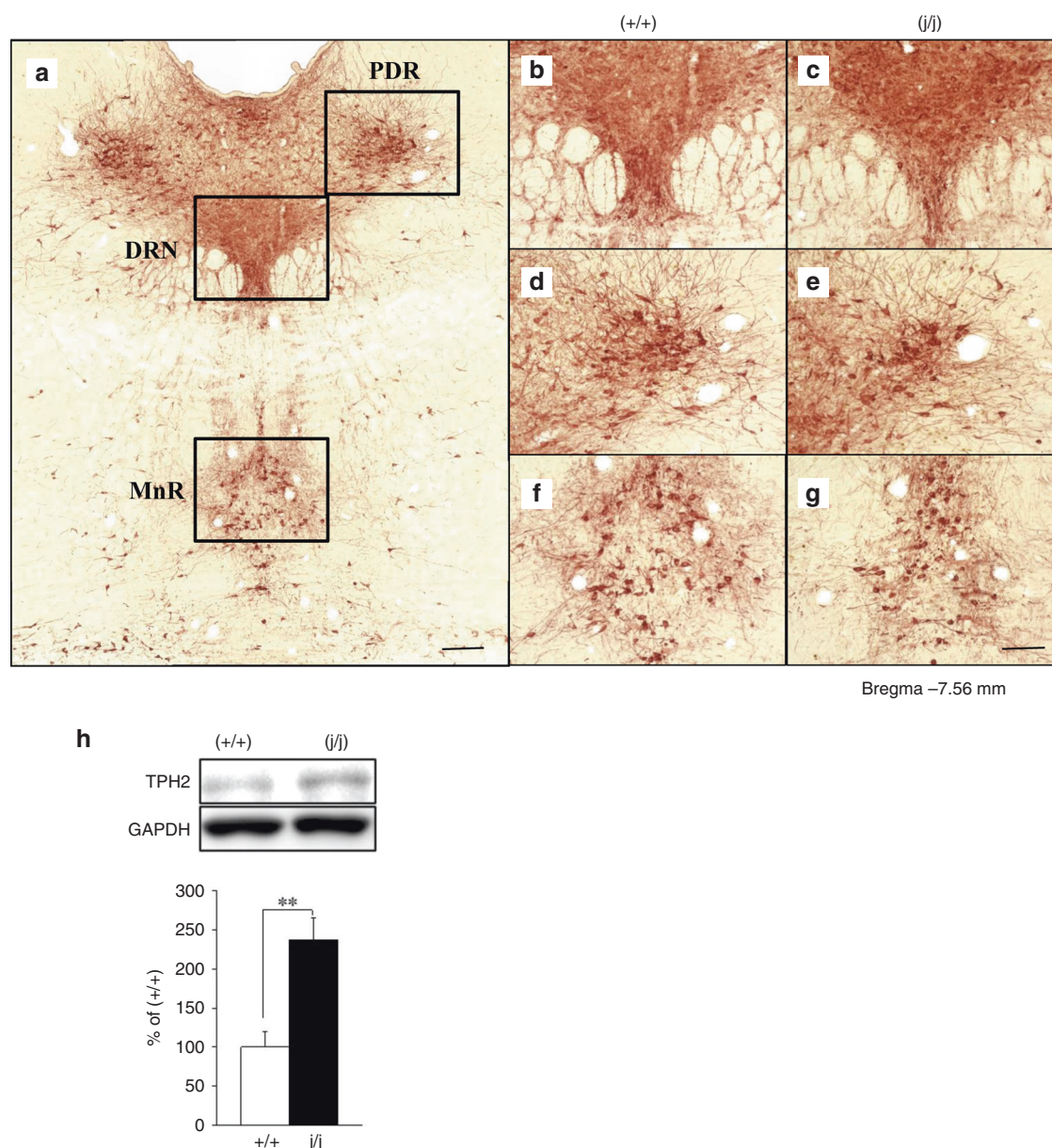
Fig. 4 Immunohistochemistry and western blotting for TPH2 in the raphe region. TPH2-positive cells mainly expressed in three parts of raphe nucleus; DRN dorsal raphe nucleus, PDR posterodorsal raphe nucleus, MnR median raphe nucleus. **a** Whole image of raphe region. Scale bars = 200 μm. **b–g** Micrographs of TPH2-immune stained cells in DRV (**b, c**), PDR (**d, e**), and MnR (**f, g**) regions from Wistar rats (left column) and Gunn rats (right column). Scale bars = 100 μm. Serotonergic neuronal marker tryptophan hydroxylase 2 (TPH2) protein in raphe region were assayed by western blotting and immunohistochemistry (N = 6 each). **h** TPH2 western blot analysis of the raphe region. The upper four panels show western blots of TPH2 proteins together with GAPDH as controls in the raphe region of Wistar and Gunn samples. TPH2- and GAPDH-specific bands were at 56k and 38k, respectively, on SDS-PAGE gel. The TPH2 levels were normalized by GAPDH level. Western blot analysis also showed significantly higher TPH2 protein levels in the Gunn rat raphe region compared with Wistar rat (**P = 0.0029). Gunn rat: black bar (j/j), Wistar rat: white bar (+/+). All data indicate mean ± SEM. Statistical analysis of the data was performed by multiple Student's t test or Student's t test. **$P < 0.01$.

ADHD is a common psychiatric disorder characterized by hyperlocomotion that affects 8–12% of children worldwide.[52] The pathogenesis of ADHD is multifactorial, such as genetic and environmental factors as well as others not yet identified. Previous genetic studies concerning ADHD have focused mainly on the dysfunction of the dopaminergic system and discussed the defective function of DA and noradrenaline, so a central nervous system stimulant is a typical drug for ADHD. However, previous studies have suggested the important role of the 5-HT system in ADHD.[53] Children diagnosed with ADHD and suffering from

hyperlocomotion, and particularly with a history of BIND, may receive benefits from treatment by 5HT2A antagonist.

Other significant behavioral alterations in Gunn rats were social deficits as reported in our previous studies, which were demonstrated by social interaction tests.[16] Social deficits are one of the characteristic symptoms of ASD. Recent studies suggested a relationship between ASD and 5-HT system dysfunction such as high levels of 5-HT in the brain in ASD model mice.[54,55] We can find a similarity between ASD and BIND in certain aspects of their clinical symptoms and pathological background. From the above,

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5HT2A antagonist treatment may be effective for the treatment of social deficits with a history of BIND.

The use of ketanserin has also been reported for the treatment of neurological disorder (Tourette's syndrome) in children (9–16 years of age).[56] The report showed that ketanserin was given at an initial dose of 20 mg/day to 7 children with Tourette's syndrome. Six children showed dramatic improvement within a few days. Total disappearance of tics was obtained with doses up to 240 mg/day (mean = 120 mg/day). The report also showed that ketanserin was stopped in 1 child after 4 months because of orthostatic hypotension. The case report suggests that ketanserin may be given to children as long as blood pressure is controlled.

We examined the cause of hyperactivity in Gunn rats showing congenital hyperbilirubinemia, and our data demonstrate that hyperactivities in Gunn rat result in serotonergic dysfunction mainly in PFC. Our findings may help in the prevention and treatment of neurodevelopmental disorders with a post-birth history of hyperbilirubinemia.

In terms of psychiatric objectives, the pathophysiological mechanism of neuropsychiatric disorders, such as schizophrenia and neurodevelopmental disorder, has yet to be defined. Previous studies suggested that there are many clinical cases of neuropsychiatric disorders complicated by idiopathic unconjugated hyperbilirubinemia (Gilbert's syndrome (GS)).[10,57] In particular, schizophrenia accompanied by GS (20.6%) is significantly increased compared with other mental disorders (mood disorders: 2.8%, neurosis: 4.2%).[10] These data indicate that we can discuss these issues in relation to the role of hyperbilirubinemia in the pathophysiological mechanism of schizophrenia. In this study, we demonstrated the effect of ketanserin on hyperactivity in model rats of congenital hyperbilirubinemia. It is expected that 5-HT2AR antagonists will treat clinical symptoms observed in schizophrenia, and most notably when complicated by hyperbilirubinemia.

Our study has some limitations. (1) We did not experiment with the use of ketanserin by local injection approach. Therefore, it is unclear whether the frontal cortex is a responsible focus for the behavioral abnormality in Gunn rats. (2) The molecular mechanism by which hyperbilirubinemia adversely affects the serotonergic system has not been clarified. Further studies will lead to understanding of the basic mechanism of the neurological dysfunction triggered by hyperbilirubinemia.

Our results suggest that higher serotonergic transmission voided the downregulation of 5-HT2AR mRNA expression and left the 5-HT2AR protein abundance unchanged in the frontal cortex of the Gunn rat, from which then developed the hyperactive phenotype of the rat. Although our experimental observations were limited to the frontal cortices and striata of 9–10-week-old Gunn and Wistar rats, it would be of value to be able to postulate that a therapeutic strategy for the BIND disorders would include restoration of the brain regions affected by serotonergic dysfunction to normal operation to prevent before or to normalize after onset of the BIND manifestations.

AUTHOR CONTRIBUTIONS
Conceived and designed the experiments: S.M. and A.O.-N. Performed the experiments: S.M., K.T., M.F., R.A., T.T., and A.O.-N. Discussed the data: T.T., K.O., M.H., S.H., R.W., T.M., and M.I. Wrote the paper: all authors contributed. A.O.-N. supervised this study.

ADDITIONAL INFORMATION

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