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Research Article

RESEARCH ARTICLE

Aberrant Monoaminergic System in Thyroid Hormone Receptor-β Deficient Mice as a Model of Attention-Deficit/Hyperactivity Disorder

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Abstract

Background: Thyroid hormone receptors are divided into 2 functional types: TRα and TRβ. Thyroid hormone receptors play pivotal roles in the developing brain, and disruption of thyroid hormone receptors can produce permanent behavioral abnormality in animal models and humans.
Methods: Here we examined behavioral changes, regional monoamine metabolism, and expression of epigenetic modulatory proteins, including acetylated histone H3 and histone deacetylase, in the developing brain of TRα-disrupted ($TR\alpha^{0/0}$) and TRβ-deficient ($TR\beta^{-/-}$) mice. Tissue concentrations of dopamine, serotonin (5-hydroxytryptamine) and their metabolites in the mesocorticolimbic pathway were measured.
Results: $TR\beta^{-/-}$ mice, a model of attention-deficit/hyperactivity disorder, showed significantly high exploratory activity and reduced habituation, whereas $TR\alpha^{0/0}$ mice showed normal exploratory activity. The biochemical profiles of dopamine and 5-hydroxytryptamine showed significantly low dopamine metabolic rates in the caudate putamen and nucleus accumbens and overall low 5-hydroxytryptamine metabolic rates in $TR\beta^{-/-}$ mice, but not in $TR\alpha^{0/0}$ mice. Furthermore, the expression of acetylated histone H3 was low in the dorsal raphe of $TR\beta^{-/-}$ mice, and histone deacetylase 2/3 proteins were widely increased in the mesolimbic system.
Conclusions: These findings suggest that TRβ deficiency causes dysfunction of the monoaminergic system, accompanied by epigenetic disruption during the brain maturation process.

Keywords: ADHD, histone deacetylase, serotonin, dopamine, reward system

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Introduction

Sufficient amounts of thyroid hormones are produced after birth, and the expression of thyroid hormone receptors (TRs) in the brain of rodents has been shown to peak during the neonatal period (Bradley et al., 1992). TRs play multiple roles in the structural and functional development of the immature brain, including neuronal cell maturation, migration, and synaptogenesis as well as glial proliferation/maturation (Cheng et al., 2010). Triiodothyronine functions are mediated by 2 major TR isoforms: TRα and TRβ, which are differentially expressed in cell types and brain regions to control the expression of a wide array of genes during development (Bradley et al., 1992; O'Shea and Williams, 2002). TRα expression is widely distributed in the brain, whereas TRβ transcript expression is more transient and restricted in distribution (Bradley et al., 1989, 1992). Such a spatiotemporal expression of TRs is presumed to have a complicated contribution to the establishment of functions of the mature brain.

Resistance to thyroid hormone is an inherited syndrome caused by a mutated TRβ gene with a reduction or loss of triiodothyronine binding activity or the transcriptional capacity (Hauser et al., 1993; Brucker-Davis et al., 1995). Of note, there is a high proportion (up to 60%) of patients with resistance to thyroid hormone syndrome who have the comorbidity of attention-deficit/hyperactivity disorder (ADHD) (Hauser et al., 1993; Brucker-Davis et al., 1995), although the majority of these patients are heterozygous. On the other hand, TRα gene disruption is rare, and there is quite limited information on its association with mental illness (Schoenmakers et al., 2013).

ADHD is characterized by hyperactivity, impaired sustained attention, impulsivity, and distractibility; it affects 3 to 7% of the school-age population worldwide and is the most diagnosed disorder in children (Weyandt and Dupaul, 2008). Children with ADHD are clinically heterogeneous and are classified into 3 subtypes: predominantly inattentive, predominantly hyperactive-impulsive, and the combined type. Thyroid hormone disruption during pregnancy is presumed to be a risk factor for ADHD, including iodine deficiency (Vermiglio et al., 2004) and exposure to bisphenol A, a food contaminant chemical that acts as a thyroid hormone disruptor (Xu et al., 2007; Harley et al., 2013). The effect of thyroid hormone disruption in the immature brain, however, is not well understood.

Although the causative mechanisms of ADHD remain unclear, a strong genetic component has been indicated in the etiology (Wood and Neale, 2010). A number of genetic association studies of ADHD have identified promising candidate genes related to both dopamine (DA) and 5-hydroxytryptamine (5-HT) systems (Oades, 2008). Genetic liability to ADHD is likely polygenic (Wood and Neale, 2010), but DA and 5-HT systems are believed to be involved in ADHD-like behavioral abnormality, as shown by phenotypic analysis of mice lacking DA transporter (DAT) (Gainetdinov et al., 1998; Zhuang et al., 2001), DA receptor 1 (Xu et al., 1994), or 5-HT receptor 1B (Brunner et al., 1999). Pharmacologic factors impacting DA/5-HT signaling during the neonatal period can also modulate adult monoaminergic function (Yu et al., 2014). For example, neonatal rats with 6-hydroxydopamine lesioning, a representative pharmacological model, exhibit spontaneous but age-limited locomotor hyperactivity (Avale et al., 2004; van der Kooij and Glennon, 2007). Such lesioning causes massive depletion of striatal DA and subsequent serotonergic hyperinnervation during the neonatal period; however, normalization induced by 5-HT depletion can reverse this hyperactivity (Avale et al., 2004). These observations implicate that DA/5-HT imbalance during the brain maturation process can cause the brain dysfunction (Gainetdinov et al., 1999; Avale et al., 2004; Oades, 2008).

Emerging evidence has demonstrated that the pathology of developmental mental disorders associates with aberrant epigenetic modifications (Graff et al., 2011). Rats with neonatal hypothyroidism are hyperactive and exhibit low histone acetylation in their developing brain (Lakshmy et al., 1999; Xu et al., 2007). Because thyroid hormone signaling is regulated by an epigenetic mechanism involving histone deacetylase (HDAC) (Ishizuka and Lazar, 2003; Vermeulen et al., 2004; You et al., 2010), a systemic profile of epigenetic states might reflect regional impairment caused by disruption of thyroid hormone signaling in the immature brain. The mammalian HDAC family consists of 4 protein groups: class I (HDAC1, -2, -3 and -8), class II (HDAC4, -5, -6, -7, -9 and -10), class III (sirtuins 1–7), and class IV with HDAC11 as the sole member. HDAC1, -2, and -3 are the major isoforms of class I and are mainly expressed in neurons (Broide et al., 2007). In this study, we selected several candidate regions that have dense monoaminergic projections in the mesocorticolimbic pathway. First, we assessed behavioral abnormalities in both TRα- and TRβ-mutant mice. Next, we measured tissue concentrations of DA, 5-HT, and their metabolites in several regions of developing brain. Then we determined protein expression of class I HDAC isoforms and acetylated histone H3 (acH3).

Methods

Animals

Littermates of $TR\alpha^{0/0}$ $TR\beta^{+/+}$ ($TR\alpha^{0/0}$) mice, $TR\alpha^{+/+}TR\beta^{-/-}$ ($TR\beta^{-/-}$) mice, and wild-type mice (WT) were generated from heterozygous dams and were used in behavioral and neurochemical experiments. $TR\alpha^{0/0}$ mice have a merit to minimalize the dominant negative effect caused by other TR subtypes (O'Shea and Williams, 2002). $TR\alpha^{0/0}$ and $TR\beta^{-/-}$mice were originally generated and propagated in the C57/BL6 background strain by Dr. J. Sumarut et al (Gauthier et al., 2001). Congenic mutants were gifted from Dr. K. Hashizume and backcrossed in the Laboratory Animal Center of Shiga University of Medical Science. It is possible that there is a gender difference due to crosstalk between thyroid hormones, estrogen and androgen; thereby, we used only male mice here. Mice were housed in a temperature-controlled environment with a 12-/12-hour-light–dark cycle and had access to food and water ad libitum. Comparisons were made among siblings within the same littermates. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental protocols were approved by the institutional animal care and use committee of the Shiga University of Medical Science.

Behavioral Examination

Three-month-old male mice were used for an open-field test (OFT). The group compositions were WT (n = 18), $TR\alpha^{0/0}$ (n = 10), and $TR\beta^{-/-}$ (n = 13) mice. Mice were kept in isolation for 4 weeks to acclimate to the test room and received handling until the start of experiment. Mice were individually placed in the center of a circular enclosure (75 cm in diameter). The animals underwent 5 test sessions, each separated by 24 hours to ensure testing occurred at the same time each day. The activity data were collected and analyzed at a sampling rate of 0.1 second for 180 seconds by a video-tracking system (Muromachi Kikai

Co. Ltd., Japan). In general, habituation in novel environments leads to a decline in horizontal exploratory activities over time either within a single test or multiple tests. Hyperactivity with reduced habituation is considered as a behavioral hallmark of ADHD-like abnormality (Zhuang et al., 2001). We thus performed the OFT with short exposures to novel circumstances under 80 lux to measure initial exploratory response without fear-related freezing. All 5 sessions were evaluated by travel distance (centimeters) and spent time (seconds). To discriminate emotional components in behaviors, activities including rearing, defecation/urination, and grooming were also quantified. The OFT arena was divided into 2 zones of equal area: central and peripheral. The total distances traveled as well as the time spent in these zones were measured.

High Performance Liquid Chromatography (HPLC) Analysis

Male mice (n = 6 per group) of 8 weeks of age were used. The brains were quickly removed and frozen with dry ice powder. HPLC analysis of DA and its metabolites in the brain regions was performed as previously described (Ookubo et al., 2008, 2013). The brains were coronally sectioned and then dissected into 7 regions, including the main olfactory bulb, caudate and putamen (CPu), nucleus accumbens (Acb), hippocampus (Hi), anterior cingulate cortex (Cg), amygdala, and dorsal raphe nucleus (DR), using corresponding coronal diagrams of the mouse brain atlas. The brain tissue samples of both hemispheres were sonicated in individual sterile tubes kept on ice using an ultrasonic homogenizer and then centrifuged; the resulting supernatants were placed in ice-cold 0.2 M perchloric acid containing 100 ng/mL isoproterenol as an internal standard. DA, 3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) were quantified by HPLC with an electrochemical detector (Eicom, Japan).

Western Blotting of HDAC, acH3, and Tyrosine Hydroxylase (TH)

Male mice (n = 6 per group) of 8 weeks of age were used. The brains were sectioned and dissected into 7 regions as described above. The frozen brain tissues were homogenized in HEPES-buffered sucrose (0.32 M sucrose containing 4 μg/mL pepstatin, 5 μg/mL aprotinin, 20 μg/mL trypsin inhibitor, 4 μg/mL leupeptin, 0.2 mM phenylmethanesulfonyl fluoride, 2 mM EDTA, 2 mM EGTA, and 20 mM HEPES, pH 7.2) using an ultrasonic homogenizer as previously described (Ookubo et al., 2013). The homogenates were solubilized with LDS sample buffer (Invitrogen). Protein samples (10 μg) were loaded on 4% to 12% Bis-Tris Gel and transferred to polyvinylidene difluoride membranes with a semi-dry blotting system for 1 hour. The polyvinylidene difluoride membranes were incubated for 1 hour at room temperature with phosphate-buffered saline containing 0.1% Tween 20 and 0.5% skim milk, followed by overnight incubation at 4°C with the desired primary antibodies: anti-HDAC1 monoclonal antibody (1:1000, Santa Cruz Biotech), anti-HDAC2 polyclonal antibody (1:1000, Santa Cruz Biotech), anti-HDAC3 polyclonal antibody (1:1000), anti-acH3 (1:1000, Millipore, BRD), or anti-tyrosine hydroxylase (TH) monoclonal antibody (1:2000, Chemicon International Inc.). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (1:4000, Sigma-Aldrich) was used to ensure equal sample loading. Membranes were washed 3 times for 10 minutes at room temperature and incubated with secondary antibodies in phosphate-buffered saline containing 0.1% Tween 20 containing 0.5% skim milk for 1 hour. Immunoreactive bands were visualized by enhanced chemiluminescent autoradiography (ECL Kit, Amersham) with Image Quant LAS-4000 (GE Healthcare). Optical densities were determined using a computerized image analysis system (MultiGauge, Fujifilm, Japan).

Results

Behavioral Analysis in the OFT

Locomotor activities were measured in WT, TRα$^{0/0}$, and TRβ$^{-/-}$ mice for 3 minutes per day for 5 days. No impairment of neuromotor performance, pathological stereotypy, or freezing was observed in any groups of congenic-conditioned mice at 3 months of age. WT, TRα$^{0/0}$, and TRβ$^{-/-}$ mice did not exhibit significant differences in mean velocity (WT: 21.2 ± 0.2, TRα$^{0/0}$: 21.4 ± 0.5, TRβ$^{-/-}$: 22.0 ± 0.3, mean ± SEM, cm/s). Nonetheless, total distance travelled during all 5 sessions was significantly larger in TRβ$^{-/-}$ mice than in WT mice, but TRα$^{0/0}$ and WT mice showed similar levels (WT: 1571 ± 62, TRα$^{0/0}$: 1634 ± 105, TRβ$^{-/-}$: 1925 ± 81, mean ± SEM, cm). Then, we focused on less habituation of the exploratory response to novel circumstances as a mark of ADHD-like hyperactivity. Figure 1*a* shows the typical tracks of each mouse strain on the fifth day. TRβ$^{-/-}$ mice showed hyperactivity and tended to avoid making body contact with the wall while running along it. The repetition of, and time spent in, tests reduces the novelty of the testing field. The decline in exploratory activity for each group across time intervals and serial sessions was evaluated for intra- and inter-session habituation, respectively (Figure 1*b*-*c*). To compare inter-session habituation of exploratory activities, the data were binned into 45-second blocks for the statistical analysis of locomotor activity. Compared with WT mice, TRβ$^{-/-}$ mice exhibited significantly prolonged locomotor responses in the middle of a session (Figure 1*b*). TRβ$^{-/-}$ mice also displayed less habituation across the trial series (Figure 1*c*).

TRα$^{0/0}$ mice exhibited a slight delay in entering novel areas from the starting point (a possible tendency of anxiety; data not shown), whereas TRβ$^{-/-}$ mice had shorter latency. This suggests the possibility that TRα$^{0/0}$ mice have an increase in anxiety as reported (Wilcoxon et al., 2007). Thus, we also assessed the impact of emotional factors on the locomotor activities and measured the number of times the animals reared and the ratio of time spent in the center vs the periphery of the open field. The sum of numbers of rearing behavior in all 5 of the sessions did not significantly differ among the groups (WT: 51 ± 0.8, TRα$^{0/0}$: 49 ± 3.3, TRβ$^{-/-}$: 43 ± 0.7 times). The ratios of time spent in the center area vs the periphery also did not differ (WT: 31 ± 3.5%, TRα$^{0/0}$: 36 ± 8.1%, TRβ$^{-/-}$: 31 ± 4.3%, percentage of time spent in the center area). These findings suggest that an emotional component, for example, fear, is not an important contributor to the observed differences in the horizontal exploratory activity. Collectively, TRβ$^{-/-}$ mice displayed hyperactivity and less habituation in an initial response to novel inescapable circumstances, whereas TRα$^{0/0}$ mice did not differ from WT mice.

HPLC Analysis of DA and 5-HT Metabolism in Developing TR Mutant Mice

We examined the levels of DA, 5-HT, and their metabolites in several brain regions richly innervated with DA and 5-HT fibers of 8-week-old mice. The results of the neurochemical analysis of DA and their metabolites are summarized in Table 1. TRβ$^{-/-}$ mice showed significant changes in the CPu, Acb, and DR and high concentrations of tissue DA and low

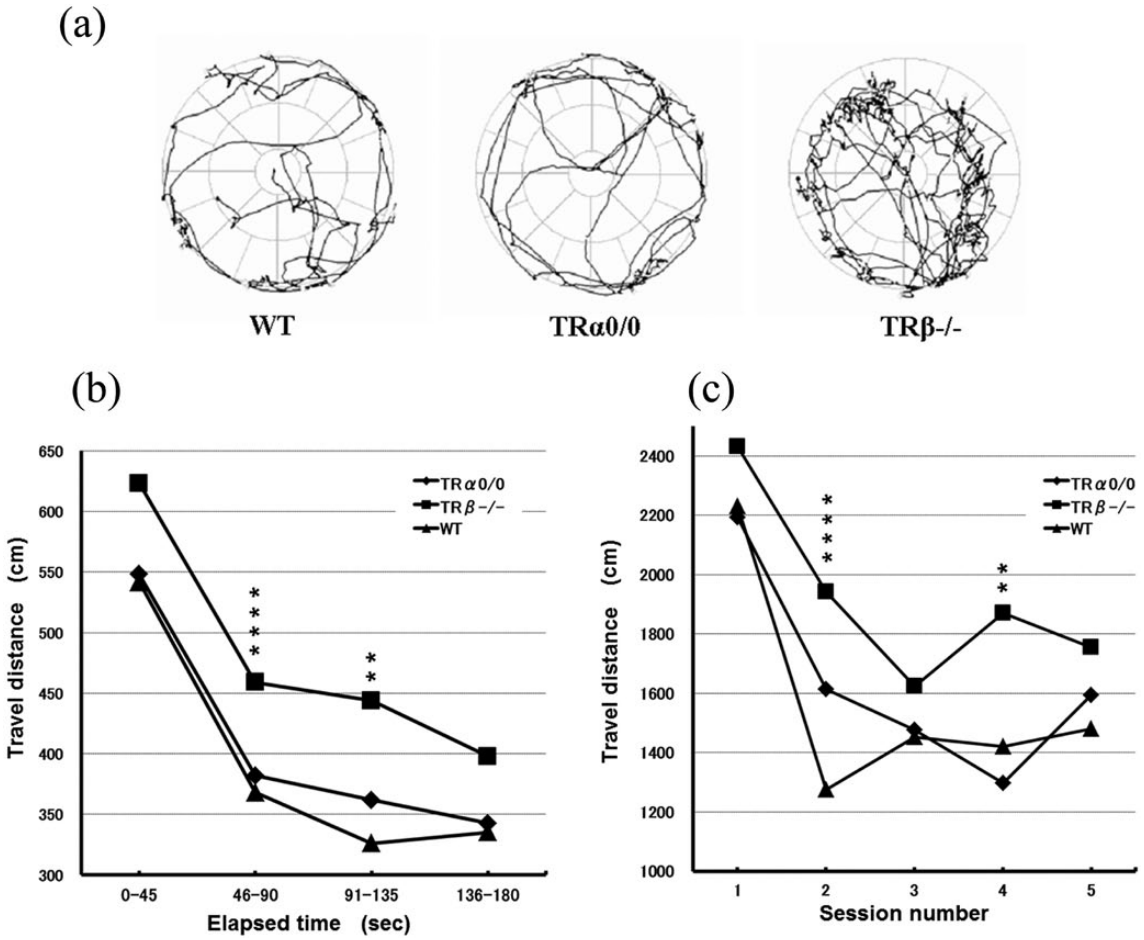

Figure 1. Open field analysis of expiratory activity in mice of TRα$^{0/0}$, TRβ$^{-/-}$, and wild-type (WT) mice at 3 months of age. Figure 1*a* shows typical tracks of each mice strain for 3 minutes on the fifth day of testing. TRβ$^{-/-}$ mice exhibited hyperactivity. Figure 1*b* displays average distances of ambulation every 45-second bin for 3 minutes. Compared with WT mice, TRβ$^{-/-}$ mice showed significant increased activity in the second and third quarter of a session. Each mouse strain nearly reached a steady state within 3 minutes of the start of an open-field test (OFT). Figure 1*c* displays average distances of ambulation in serial trials. TRβ$^{-/-}$ mice displays to retain significant higher levels of activities in the second and fourth series of testing. Asterisks indicate significant differences from WT mice following ANOVA and Tukey's HSD post hoc tests. Error bars represent ± SEM (**$P < .01$, ****$P < .0001$).

DA turnovers (Figure 2). Although TRβ$^{-/-}$ mice showed slightly high DOPAC/HVA concentrations in these regions, the DA metabolic ratios significantly decreased, because of remarkably high tissue content of DA. Conversely, in the Cg of TRβ$^{-/-}$ mice, the HVA level significantly decreased, but the ratio of HVA to DA appeared to be normal because of low tissue DA content. Similarly, the DA concentration in TRα$^{0/0}$ mice showed an increase in the CPu, Acb, and DR but a decrease in the Cg. However, these changes did not reach statistical significance in TRα$^{0/0}$ mice.

The HPLC analysis of 5-HT and its metabolites is graphically shown in Figure 3. In the amygdala and DR, 5-HT concentration significantly increased in TRβ$^{-/-}$ mice but not in TRα$^{0/0}$ mice. In the Cg, CPu, Acb, and Hi, tissue 5-HIAA levels in TRβ$^{-/-}$ mice significantly decreased, while those in TRα$^{0/0}$ mice were almost normal. Despite such differences in detail, the 5-HIAA/5-HT ratio, a 5-HT turnover index, was significantly lower in all brain regions of TRβ$^{-/-}$ mice compared with WT mice, in which the ratios ranged from 0.42 ± 0.06 (Cg) to 1.04 ± 0.11 (Hi). The 5-HIAA/5-HT ratios of TRα$^{0/0}$ mice remained at normal levels in all the brain regions. These findings suggest that 5-HT function is severely altered in TRβ$^{-/-}$ mice, but not in TRα$^{0/0}$ mice, at 8 weeks of age.

Protein Expression of TH in the CPu and Acb

The CPu and Acb receive dopaminergic projections most prominently in the brain. Tissue protein levels of TH, the rate-limiting enzyme in DA synthesis, can be affected by changes in synaptic modification such as dopaminergic hyperinnervation. TH expression remained unchanged in the CPu, whereas it was significantly elevated in the Acb of both TRα$^{0/0}$ and TRβ$^{-/-}$ mice (Figure 4). It raised the possibility that TR dysfunction could cause minor structural changes in the Acb (supplementary Figure S1). However, TH expressions almost equally increased in TRα$^{0/0}$ and TRβ$^{-/-}$ mice. Accordingly, the difference in DA metabolism between TRα$^{0/0}$ and TRβ$^{-/-}$ mice may imply the involvement of other regulatory mechanisms.

Protein Expressions of acH3 and Class I HDAC Isoforms

Expression of each protein was determined in mice at 8 weeks of age. TRβ$^{-/-}$ mice showed restricted change of histone acetylation (Figure 5). Notably, there was a significant decrease of acH3 in the serotonergic DR of TRβ$^{-/-}$ mice but not in TRα$^{0/0}$ mice (data not shown). HDAC1 expression tended to increase in the main

Table 1. HPLC Analysis for the Tissue Concentrations of DA, Its Metabolites, and Their Ratios to DA in TRα$^{0/0}$, TRβ$^{-/-}$ and WT Mice at 8 Weeks of Age.

DA	WT	TRα$^{0/0}$	TRβ$^{-/-}$
mOb	0.371 ± 0.08	0.354 ± 0.048	0.231 ± 0.029
Cg	0.186 ± 0.077	0.119 ± 0.024	0.090 ± 0.016
Cpu	10.859 ± 1.880	14.224 ± 1.488	16.261 ± 1.198*
Acb	2.742 ± 0.697	4.458 ± 0.769	6.207 ± 0.688**
Hi	0.094 ± 0.018	0.306 ± 0.113	0.331 ± 0.145
Amy	0.574 ± 0.098	0.783 ± 0.175	0.725 ± 0.147
DR	0.181 ± 0.027	0.340 ± 0.066	0.312 ± 0.048
DOPAC	WT	TRα$^{0/0}$	TRβ$^{-/-}$
mOb	0.171 ± 0.016	0.196 ± 0.032	0.147 ± 0.011
Cg	0.203 ± 0.049	0.136 ± 0.019	0.118 ± 0.023
Cpu	0.666 ± 0.054	0.759 ± 0.065	0.812 ± 0.055
Acb	0.469 ± 0.048	0.466 ± 0.043	0.604 ± 0.060
Hi	0.146 ± 0.011	0.173 ± 0.012	0.176 ± 0.014
Amy	0.247 ± 0.023	0.230 ± 0.017	0.248 ± 0.015
DR	0.235 ± 0.028	0.254 ± 0.041	0.259 ± 0.020
HVA	WT	TRα$^{0/0}$	TRβ$^{-/-}$
mOb	0.489 ± 0.073	0.463 ± 0.056	0.330 ± 0.024
Cg	0.392 ± 0.058	0.332 ± 0.046	0.225 ± 0.009**
Cpu	1.540 ± 0.109	1.705 ± 0.146	1.637 ± 0.102
Acb	0.930 ± 0.077	0.918 ± 0.066	1.100 ± 0.087
Hi	0.369 ± 0.035	0.407 ± 0.031	0.387 ± 0.030
Amy	0.521 ± 0.045	0.526 ± 0.047	0.494 ± 0.040
DR	0.477 ± 0.049	0.572 ± 0.051	0.534 ± 0.032
DOPAC/DA	WT	TRα$^{0/0}$	TRβ$^{-/-}$
mOb	0.532 ± 0.067	0.619 ± 0.181	0.674 ± 0.094
Cg	1.286 ± 0.321	1.298 ± 0.236	1.329 ± 0.109
Cpu	0.065 ± 0.003	0.055 ± 0.002*	0.054 ± 0.003*
Acb	0.154 ± 0.016	0.146 ± 0.051	0.115 ± 0.009*
Hi	1.841 ± 0.189	1.370 ± 0.259	1.588 ± 0.331
Amy	0.453 ± 0.048	0.387 ± 0.073	0.409 ± 0.067
DR	1.394 ± 0.147	0.793 ± 0.094**	0.873 ± 0.086**
HVA/DA	WT	TRα$^{0/0}$	TRβ$^{-/-}$
mOb	1.472 ± 0.215	1.474 ± 0.391	1.474 ± 0.088
Cg	2.747 ± 0.638	3.072 ± 0.482	2.717 ± 0.320
Cpu	0.152 ± 0.009	0.123 ± 0.005*	0.107 ± 0.008**
Acb	0.302 ± 0.042	0.310 ± 0.129	0.207 ± 0.022*
Hi	4.927 ± 0.763	3.220 ± 0.607	3.547 ± 0.734
Amy	0.987 ± 0.119	0.815 ± 0.125	0.763 ± 0.085
DR	2.998 ± 0.515	1.850 ± 0.254*	1.813 ± 0.178*

Mean concentrations and SDs are expressed in μg/g tissue weight. Asterisks indicate significant differences from wild-type (WT) mice following ANOVA and Tukey's HSD post hoc tests. Error bars represent ± SEM (*$P < .05$, **$P < .01$).

olfactory bulb, Cg, Acb, and Hi of TRβ$^{-/-}$ mice. The HDAC2 and HDAC3 expressions had a similar profile and displayed significant increases in most brain regions. HDAC2/3 expression was higher overall than HDAC1 expression. In particular, the expression of HDAC2/HDAC3, but not HDAC1, significantly increased in the CPu of TRβ$^{-/-}$ mice. Meanwhile, modifications of the histone acetylation and HDAC1-3 protein levels were unlikely associated with each other in the corresponding region, presuming disrupted epigenetic equilibrium between histone acetylation and HDAC proteins.

Discussion

Some kinds of TRβ transgenic mice have been demonstrated to be hyperactive phenotypes characterized by less habituation or a paradoxical response to methylphenidate treatment (O'Shea and Williams, 2002; Siesser et al., 2006). In contrast, TRα$^{0/0}$ mice show mild hypoactivity and an increase in anxiety (Wilcoxon et al., 2007). Similarly, our results in the OFT demonstrated that TRβ$^{-/-}$ mice showed high exploratory activity with reduced habituation, while TRα$^{0/0}$ mice showed normal exploratory activity and a possible increase in anxiety. In biochemical profiles in the candidate regions for the behavioral abnormality, the TRβ deficiency caused both DA and 5-HT dysfunction and epigenetic disruption during the growth period.

In genetic models of ADHD, increased striatal DA turnover has often been observed. DAT knockout/knockdown mice have the highest degree of validity as an ADHD model with striatal hyperdopaminergic characteristics (Zhuang et al., 2001; van der Kooij and Glennon, 2007). In DAT knockout/knockdown mice, DA clearance from the synaptic cleft is greatly reduced, resulting in a prolonged and high concentration of extracellular DA, a high

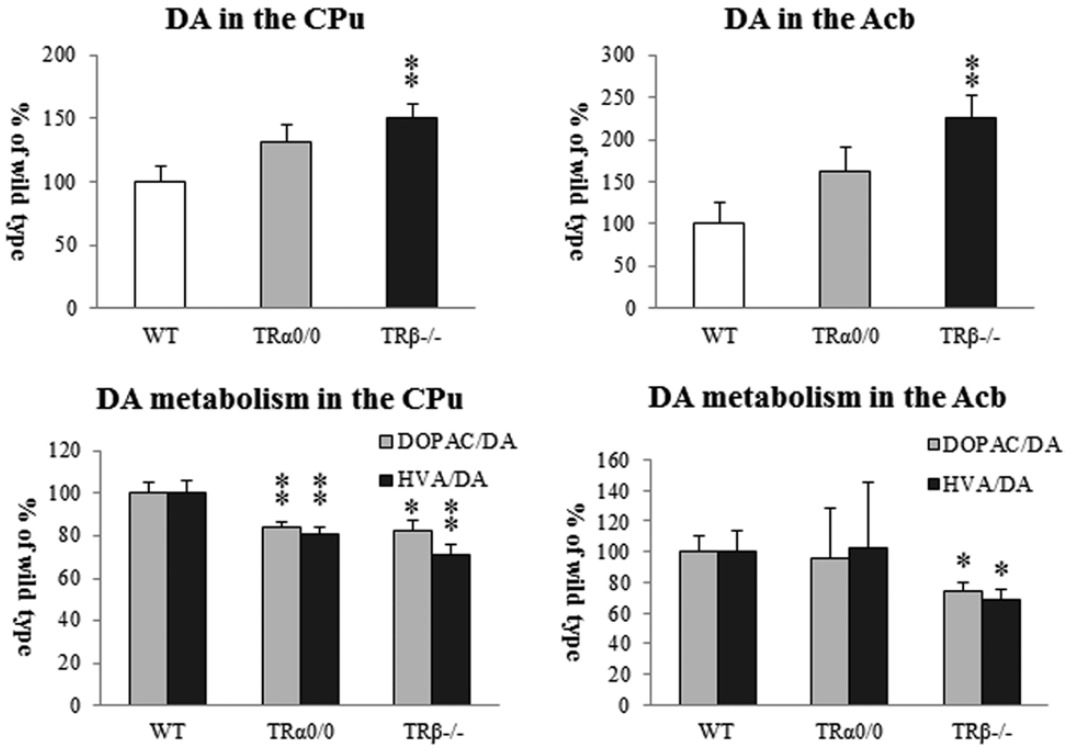

Figure 2. The high performance liquid chromatography (HPLC) analysis for dopamine (DA) contents and DA turnovers in the caudate and putamen (CPu) and nucleus accumbens (Acb) in TRα$^{0/0}$, TRβ$^{-/-}$, and wild-type (WT) mice at 8 weeks of age. Data were normalized for each protein level relative to WT mice. Asterisks indicate significant differences from WT mice following ANOVA and Tukey's HSD post hoc tests. Error bars represent ± SEM (*$P < .05$, **$P < .01$).

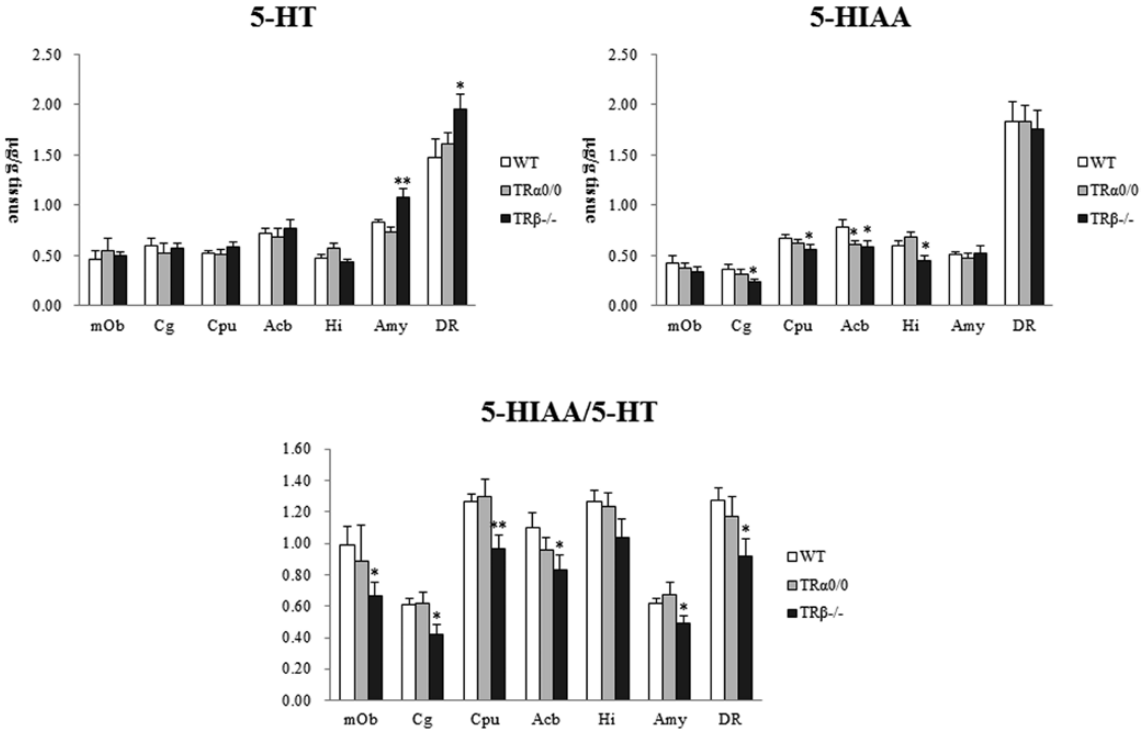

Figure 3. The high performance liquid chromatography (HPLC) analysis for tissue concentrations of 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and 5-HT metabolism ratios in TRα$^{0/0}$, TRβ$^{-/-}$, and wild-type (WT) mice at 8 weeks of age. The 5-HT turnovers in most brain regions of TRβ$^{-/-}$ mice were significant lower than those in WT mice. Mean concentrations and standard deviations are expressed in μg/g tissue weight. Asterisks indicate significant differences from WT mice following ANOVA and Tukey's HSD post hoc tests. Error bars represent ± SEM (*$P < .05$, **$P < .01$).

concentration of tissue DOPAC, downregulation of DA receptors, and compensatory low levels of tissue DA stores and TH expression (Gainetdinov et al., 1998; Zhuang et al., 2001). Spontaneous locomotor hyperactivity under normal circumstances is related to increased dopaminergic tone in the CPu, which is a consequence of increased DA release/turnover (Gainetdinov et al.,

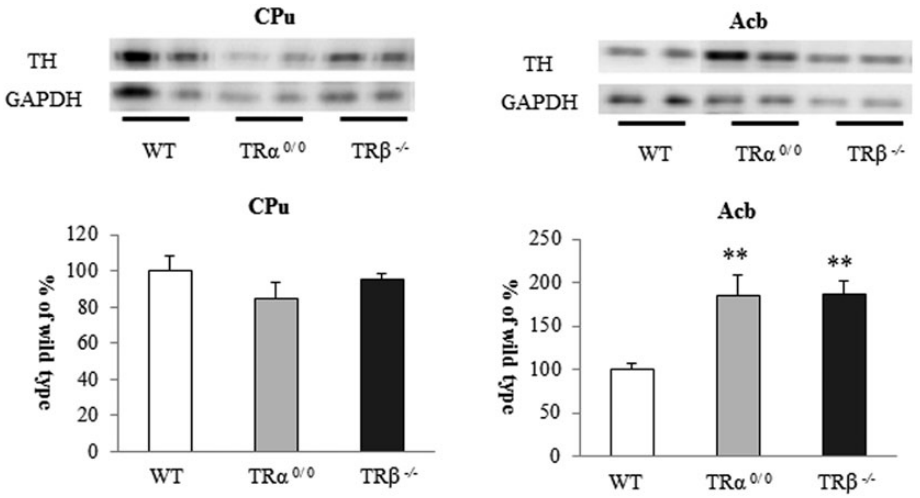

Figure 4. The expression of tyrosine hydroxylase (TH) proteins in the caudate and putamen (CPu) and nucleus accumbens (Acb) in TR$\alpha^{0/0}$, TR$\beta^{-/-}$, and wild-type (WT) mice at 8 weeks of age. Lanes in the upper part of the panels show a part of the blots of Western analysis. Asterisks indicate significant differences from WT mice following ANOVA and Tukey's HSD post hoc tests. Error bars represent ± SEM (**P < .01).

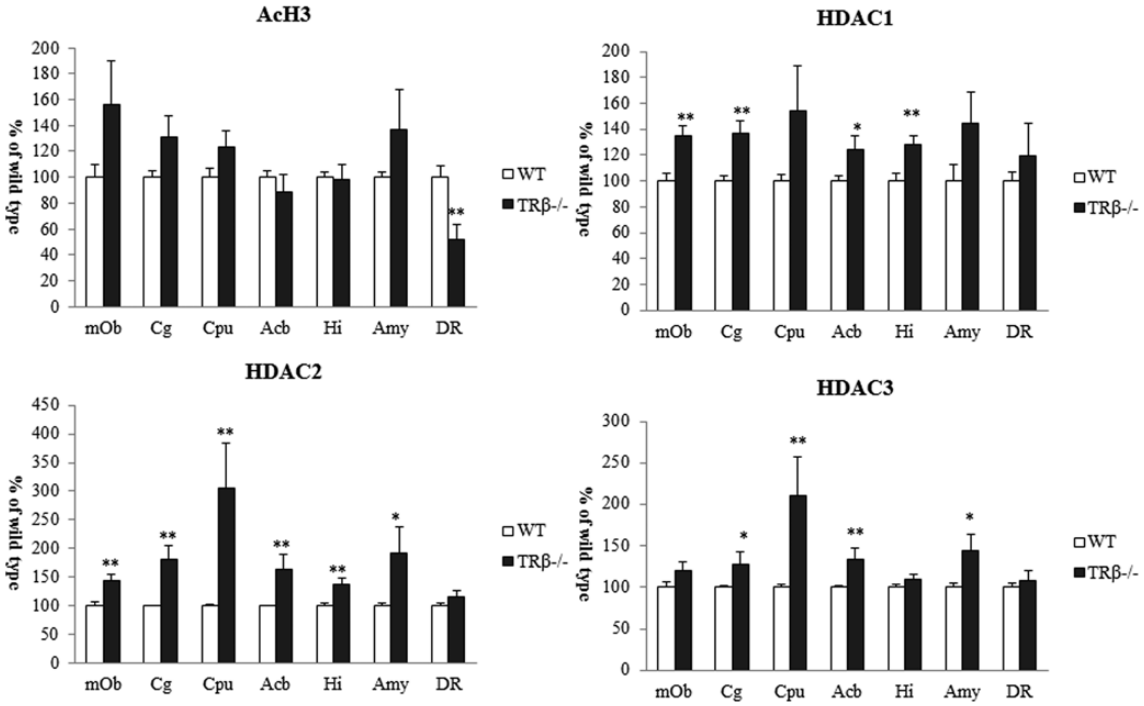

Figure 5. The expression of acetylated histone H3 (acH3), histone deacetylase (HDAC)1, HDAC2, and HADC3 proteins in TR$\beta^{-/-}$ and wild-type (WT) mice at 8 weeks of age. Asterisks indicate significant differences from WT mice following ANOVA and Tukey's HSD post hoc tests. Error bars represent ± SEM (*P < .05, **P < .01).

1998). On the other hand, ADHD-like abnormalities can also be observed in hypodopaminergic models (van der Kooij and Glennon, 2007). Mice lacking a DA receptor 1-signaling molecule such as DARPP-32 and p35 show a low DOPAC/DA ratio but compensatory high levels of tissue DA stores and TH expression (Drerup et al., 2010; Krapacher et al., 2010; Napolitano et al., 2010). In the present study, TR$\beta^{-/-}$ mice appeared to be a model of the striatal hypodopaminergic state, because significantly low DA turnover and a high level of tissue DA content were observed in the CPu and Acb of TR$\beta^{-/-}$ mice at 8 weeks of age. However, no significant differences in monoaminergic metabolism were observed among the groups of adult mice of the same strains (M. Ookubo and H. Kanai, unpublished observations).

Dramatic cytoarchitectural maturation occurs within the first month after birth in the normal CPu; although thyroid hormone deficiency does not entirely prevent the development of the CPu, this deficiency leads to a fairly extensive though critically incomplete degree of maturation, including a decrease in the number of synaptic contacts (Lu and Brown, 1977a, 1977b). The CPu has the highest expression of both TRα and TRβ in rats throughout the prenatal and neonatal periods but shows less expression of TRβ by adulthood (Bradley et al., 1989, 1992). In addition, thyroid hormone dependency of neurite outgrowth for 5-HT and noradrenaline- and DA-containing neurons has been reported in an ex vivo study (Granholm et al., 1984). Therefore, TR mutant mice might develop neural disorganization due to a

distinct growth inhibition in the basal ganglia, although detailed information on the synaptic functions has yet to be determined.

We also found that TRβ$^{-/-}$ mice, but not TR$^{0/0}$ mice, exhibited a normal or high 5-HT content and overall low serotonergic metabolism in the mesolimbic regions. In addition, the serotonergic DR of TRβ$^{-/-}$ mice showed a significantly decrease in histone acetylation. Similarly, hyperactive phenotypes like ADHD with low 5-HT metabolism have been found in mice lacking various genes (Chiavegatto et al., 2001; Hashimoto et al., 2001; Trent et al., 2012). Delay aversion and impulsive choices like ADHD symptoms are associated with dysfunction in the dopaminergic reward signal, and this reward processing is anatomically and physiologically related to the 5-HT system (Winstanley et al., 2006; Miyazaki et al., 2012; Nakamura, 2013). In animal models, systemic 5-HT depletion causes not only this inhibitory-based executive deficit but also exploratory hyperactivity (Breese et al., 1975; Dringenberg et al., 1995; Mobini et al., 2000). Thus, the hyposerotonergic state in the reward system might contribute to hyperactivity in TRβ$^{-/-}$ mice.

Lastly, we are the first study to demonstrate epigenetic alterations in TRβ$^{-/-}$ mice; this could be said to be unique characteristics of this ADHD model. The low acH3 in the serotonergic DR and the high expression of HDAC2/3 proteins in the mesolimbic DA system may underlie the functional changes in monoaminergic tone. In contrast, the changes in HDAC expression levels in TR$^{0/0}$ mice were mostly smaller than those in TRβ$^{-/-}$ mice (M. Ookubo and H. Kanai, unpublished observations). Sustained changes in HDAC activity and histone modification can stably modify cell activity ex vivo (Kanai et al., 2004). Recently, epigenetic changes in a specific brain region have been implicated in persistent behavioral changes, including drug dependence and memory formation (Morris et al., 2010; Malvaez et al., 2011; Ookubo et al., 2013). Indeed, manipulation of the expression of HDAC1, -2, and -3 differentially modulates synaptic plasticity such as memory facilitation in the Hi of mice (Akhtar et al., 2009; Morris et al., 2010; McQuown and Wood, 2011).

Among HDAC isoforms, HDAC3 is the most abundant isoform in neurons (Broide et al., 2007) and forms a tight complex with NcoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptor); this complex binds unliganded TR (Ishizuka and Lazar, 2003; Vermeulen et al., 2004; You et al., 2010). According to transfection studies, knockdown of HDAC3 or prevention of the interaction between NcoR and HDAC3 markedly decreases the magnitude of gene repression by TR, while knockdown of HDAC1 or HDAC2 has more modest and partly nonspecific effects (Ishizuka and Lazar, 2003). Conversely, class II HDAC isoforms are solely inactive but can bind to HDAC3/NcoR complexes and facilitate the action of HDAC3 (Yang and Gregoire, 2005). Although further research is needed to elucidate various roles of HDAC isoforms in the developing brain, our results show the possibility that disruption of epigenetic modulators also contributes to develop behavioral abnormality.

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Statement of Interest

None.

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