Neurochemical Research (2019) 44:1999–2006
https://doi.org/10.1007/s11064-019-02836-y

ORIGINAL PAPER

Genetic Difference of Hypothyroidism-Induced Cognitive Dysfunction in C57BL/6j and 129/Sv Mice

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Received: 1 March 2019 / Revised: 19 June 2019 / Accepted: 21 June 2019 / Published online: 19 July 2019

Abstract

Adult-onset hypothyroidism induces cognitive impairments in learning and memory. Thyroxin (T4) replacement therapy appears to be effective in biochemically restoring euthyroidism, as evidenced by serum T4 and triiodothyronine concentrations within the normal range, although some the patients still exhibit cognitive dysfunctions. Here, we investigated the cognitive functions of propylthiouracil-induced hypothyroid mice in C57BL/6j and 129/Sv strains using the passive avoidance task and the novel object recognition test. Cognitive dysfunctions in hypothyroid mice were found only in the C57BL/6j strain, not in the 129/Sv strain. Further, we found that cholinergic neurons in the basal forebrain increased the membrane potential and input resistance with decreased capacitance, and that they decreased the amplitude and width of action potential in hypothyroid mice in the C57BL/6j strain but not in those in the 129/Sv strain, compared with the controls for each strain. Additionally, the excitability of cholinergic neurons in the basal forebrain was reduced in the hypothyroid mice in the C57BL/6j strain. These results indicated that transgenic mice with the C57BL/6j genetic background are more suitable for revealing the mechanism underlying hypothyroidism-induced cognitive dysfunction, and that the cholinergic basal forebrain may be the appropriate target for treating cognitive dysfunction in adult-onset hypothyroidism.

Keywords Hypothyroidism · Cholinergic neurons · Basal forebrain · C57BL/6j · 129/Sv · Mice

Introduction

Thyroid hormones are important in energy metabolism, growth, and brain development, and they help to maintain normal brain functions. The deficiency of thyroid hormones induced hypothyroidism. The incidence of hypothyroidism is between 0.3 and 3.7% in general people in the USA [1], a prevalence which is two to three times higher in women than in men [2]. Due to the absence of typical symptoms, many people with hypothyroidism go unrecognized and untreated, which results in serious adverse outcomes including the psychiatric disorders and a decline in cognitive functions [1]. However, some hypothyroidism patients who received thyroxine (T4) replacement therapy to the euthyroid status have still shown higher levels of cognitive dysfunction [3–5]. Researchers have sought to clarify the underlying mechanisms in hypothyroidism-related cognitive impairments, but treatment of the cognitive dysfunctions in hypothyroidism is still limited.

Previous research has shown the variation in the learning and memory-processing brain structures in hypothyroid patients through functional magnetic resonance imaging (fMRI). Subjects with hypothyroidism had impaired memory along with decreased hippocampal volume [6] and abnormal fMRI signals in the frontal brain areas [7, 8]. Studies have revealed that thyroid hormone deficiency results in the dysfunction of many neurotransmitters, such as the GABAergic, glutamatergic, and cholinergic systems, in which cholinergic neurons are related to cognitive function [9–11]. In addition, the expression of synaptic proteins, including synaptotagmin 1, Munc-18, and SNARE complex, which are essential

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in mediating the release of neurotransmitters, is affected in propylthiouracil (PTU)-treated rats [12, 13].

To explore the potential mechanism of hypothyroidism-induced deficiencies in learning and memory processing, an effective transgenic animal model of hypothyroidism with cognitive dysfunction is needed. Decades of experimental research have shown different outcomes among various mouse strains and laboratories using several different experimental animal models [14, 15]. Therefore, the strain of the animal may have many different effects on the outcomes of animal model experiments, as indicated by a previous report [16]. Many transgenic mice originate from C57BL/6j or 129/Sv inbred strains. However, few studies have shown considerable strain-related differences in adult-onset hypothyroidism-induced cognitive dysfunction. In this study, we established hypothyroid mice models in both C57BL/6j and 129/Sv strains and compared their cognitive functions using the step-through passive avoidance task and the novel object recognition test. We then tested the electrical properties of cholinergic neurons in the basal forebrain of hypothyroid mice and their euthyroid littermates.

Materials and Methods

Animals

Pathogen-free adult male C57BL/6j and 129/Sv mice (10 weeks of age and 20–25 g in weight at the beginning of the experiment; Sino-British SIPPR/BK Lab Animal Ltd., Shanghai, China) were used in the present study. Ambient room temperature was maintained at 22 ± 1 °C and relative humidity at $60 \pm 5\%$. The 12-h/12-h light/dark cycle (lights on at 07:00) was automatically controlled. Water and food were available ad libitum. All experiments were carried out in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the Anhui Medical University Committee on Animal Care and Use.

Chemicals and Experimental Procedure

PTU was purchased from Sigma-Aldrich, Inc. (Missouri, USA). One hundred and eight mice were assigned to experimental groups (10–12 mice/group for behavioral tests and 5 mice/group for electrophysiological recording), and each mouse was used only once. The mice received the normal husbandry, with fresh wood-chip bedding, freshly prepared PTU or distilled water, and standard chow every week. The bodyweight of each mouse was recorded during the husbandry. Half of the mice were treated with PTU at a concentration of 1 mg/ml, diluted in drinking water, for 6 weeks to obtain the clinical–physiological status of hypothyroidism, according to the previous report. The control (CTRL) mice received distilled water [17].

Step-Through Passive Avoidance Task

The step-through passive avoidance task was conducted over three consecutive days to investigate the memory of mice using a two-compartment box (Med Associates Inc., USA). On the first day, each animal was put into a bright chamber and allowed to habituate to the experimental environment. The training trial was performed 24 h later. Once the mouse entered the dark compartment, a mild foot shock (0.5 mA, 2 s) was delivered through the grid floor, and the mouse was then returned to the home cage. On the third day, for the retention test, the latency of stepping into the dark compartment was recorded over the course of a 180 s retesting period. When the mouse entered the dark chamber, the test session was ended, and the step-though latency was recorded by a computer. If the mouse was kept in the light chamber for 180 s, the step-though latency would be taken down as 180 s. There was no electric shock in the dark compartment during this session [18]. Ten mice of each group were used in this procedure, and there were 40 mice in total.

Novel Object Recognition Test

Novel object recognition was performed using an open field box ($40 \times 40 \times 35$ cm) constructed of white acrylic with a clear acrylic lid (XR-XX117, Shanghai Xinruan Information Technology Co., Ltd.). Each animal was allowed to habituate to the apparatus for 5 min for 6 days before the behavior test. During the training session, the mouse was placed in the center of the box for 10 min with two identical Duplo Lego blocks (A) in the box, positioned 10 cm from the walls. Twenty-four hours after training, the memory test was conducted by putting the same mouse in the box with the familiar block (A) and a novel-shaped block (B); the mouse explored freely for 5 min. A recognition index (novel object preference) calculated for each animal was expressed as $100\% \times$(time unfamiliar)/(time familiar + time unfamiliar). Between trials, the open field box and blocks were cleaned with 10% ethanol solution and air dried [19, 20]. Twelve mice of each group were used in this section, and there were 48 mice in total.

Thyroid-Hormone Assay

To test the thyroid hormones in the PTU-induced hypothyroidism, we measured the levels of circulating triiodothyronine (T3), T4, and thyroid-stimulating hormone (TSH). Blood sampling was performed using cardiac puncture under deep anesthesia (chloral hydrate, 400 mg/kg). For this assay, all of the blood samples were collected in sterilized tubes on ice after the behavioral test or before the electrophysiological recording. The samples were kept on ice for 1 h and centrifuged at 3000×*g* and at 4 °C for 10 min. Serum samples were collected into the sterilized tubes and frozen at − 80 °C until the assay was performed [21]. Serum thyroid hormones were measured with enzyme-specific immunoassay kits (Ruiying Biological Technology, Suzhou, China) following the manufacturers' protocol.

Electrophysiological Recording

Patch-clamp recording was performed as described previously [22, 23]. In brief, the mice were anaesthetized and perfused transcardially with modified aCSF containing (mM) 213 sucrose, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 3 MgSO4, 2 Na-pyruvate, 1.25 NaH_2PO_4, 0.4 ascorbic acid, and 0.1 $CaCl_2$, and saturated with 95% O_2 and 5% CO_2. Coronal slices of the mouse forebrain (300 µm) were cut on a vibratome (VT 1200 S, Leica) just rostral to the crossing of the anterior commissure and were then transferred to normal aCSF containing (mM) 126 NaCl, 26 $NaHCO_3$, 25 glucose, 2.5 KCl, 2 $CaCl_2$, 1.25 NaH_2PO_4, and 1 $MgSO_4$.

Slices were incubated at 32 °C for at least 30 min and subsequently maintained at room temperature for 30 min before the patch-clamp recording. Whole-cell current-clamp recordings were made using patch electrodes (4–5 MΩ) containing (mM) 120 potassium gluconate, 20 KCl, 10 HEPES, 2 MgATP, 1 MgCl, 0.5 EGTA, 0.5 NaGTP, 0.16 $CaCl_2$, and 0.2 biocytin (pH 7.4, 300–310 mOsm). Voltage signals were recorded with a MultiClamp 700B amplifier and pClamp10.3 software (Axon Instruments). The cells with series resistance changes over 25% were discarded. Eighteen to 20 cholinergic neurons from five mice of each group were recorded during this section, and there were 20 mice in total.

Immunohistochemistry

After the electrophysiological recording, the brain slices were then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2 at 4 °C for 24 h). Slices were then processed for choline acetyltransferase (ChAT) and biocytin immunostaining. The sections were incubated with goat anti-ChAT antibody (1:2000; Millipore) overnight at room temperature, and the sections were then incubated in streptavidin green dye conjugate and Alexa Fluor 594 conjugated anti-goat antibody (1:500; Jackson ImmunoResearch) [24]. Images were analyzed with a laser confocal microscope LSM730 (Carl Zeiss).

Data Analysis

All results were expressed as mean ± standard error of the mean (SEM). Statistical comparisons among groups were performed using the two-way ANOVA followed by the post-hoc Tukey test, with strain and treatment as the main factors. In all cases, $P < 0.05$ was considered significant.

Results

Thyroid Hormone Levels and Bodyweight Changed After the PTU Administration

The average levels of serum T3 ($F_{(1, 7)} = 53.61$, $P < 0.01$) and T4 ($F_{(1, 7)} = 101.10$, $P < 0.01$) were significantly lower in the PTU-treated mice, while the level of serum TSH ($F_{(1, 7)} = 492.00$, $P < 0.01$) was higher in the PTU-induced hypothyroid mice compared to their CTRL ones for both C57BL/6j and 129/Sv (Fig. 1a–c). There were no significant differences of the level of serum T3 ($F_{(1, 7)} = 1.88$, $P = 0.21$), T4 ($F_{(1, 7)} = 0.11$, $P = 0.75$) and TSH ($F_{(1, 7)} = 0.01$, $P = 0.93$) between C57BL/6j and 129/Sv mice. The bodyweight of mice was recorded every week when the mice received the normal husbandry. There was no significant difference in bodyweight among the groups of mice at the beginning of the experiment. The bodyweight of the mice decreased dramatically after the PTU treatment from the first to the third week compared to the CTRL mice within ($F_{(6, 196)} = 59.94$, $P < 0.05$) or across strains ($F_{(3, 196)} = 12.86$, $P < 0.05$, Fig. 1d). From the fourth week, there was no significant difference in bodyweight between PTU-treated mice and their CTRL ones within or across strains in both C57BL/6j and 129/Sv mice ($P > 0.05$, Fig. 1d).

Step-Through Passive Avoidance Task

The step-through passive avoidance task was used to investigate the learning and memory activities of mice in the present study. The experimental timeline of this task is presented in Fig. 2a. There was no significant difference of step-through latency during the training sessions among the different strains and treatments of mice ($F_{(1, 36)} = 0.99$, $P = 0.32$, Fig. 2b). The step-through latency significantly decreased in PTU-induced hypothyroid mice of the C57BL/6j strain compared to the CTRL ones, but it did not significantly decrease in the 129/Sv mice ($F_{(1, 9)} = 43.06$, $P < 0.01$, Fig. 2c).

Novel Object Recognition Test

The novel object recognition test is another behavioral assay to investigate learning and memory in rodents. The procedure for this test in the current study is shown in Fig. 3a. The

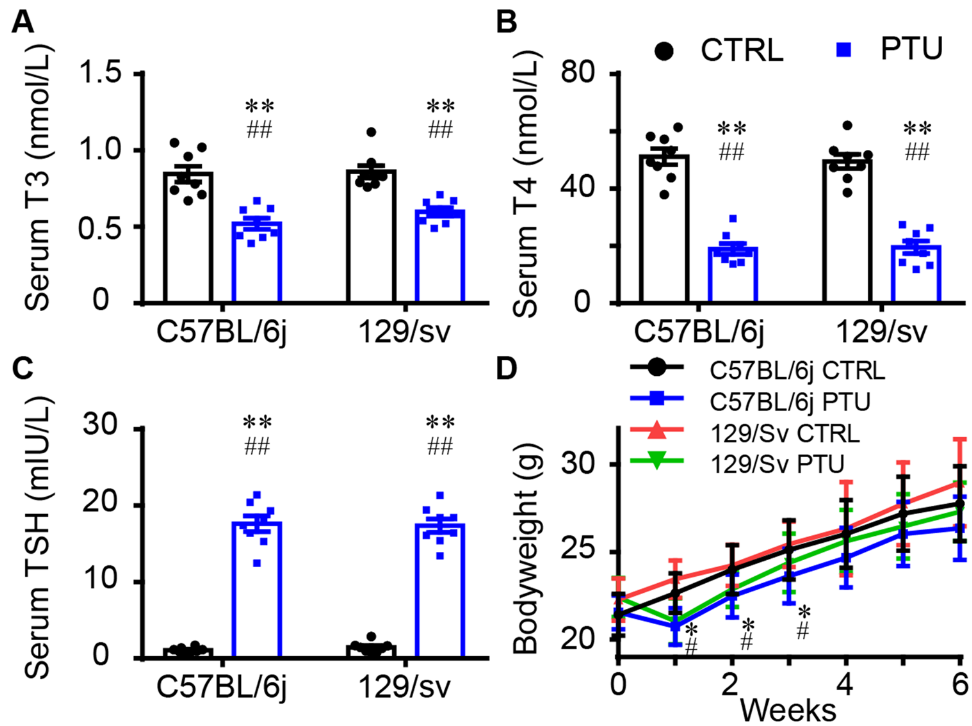

Fig. 1 Thyroid hormone levels and bodyweight changed after the PTU-administration in C57BL/6j and 129/Sv mice. The serum T3 (**a**), T4 (**b**), and TSH (**c**) altered in the PTU-treated C57BL/6j and 129/Sv mice. **d** The bodyweight changes of PTU-treated and CTRL mice in both the C57BL/6j and 129/Sv strains during the experiment. *$P<0.05$, **$P<0.01$ compared to CTRL mice within the same strain, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared to CTRL mice across the strains; $n=8$

recognition index of C57BL/6j mice decreased dramatically under the influence of PTU-induced hypothyroidism compared to their euthyroid littermates ($F_{(1, 11)}=16.40$, $P<0.01$, Fig. 3b). However, there was no significant difference in the novel object preference between the hypothyroid mice and their CTRL ones in the 129/Sv strain.

Electrophysiological Recording

In the present study, patch-clamp recordings were performed in cholinergic neurons of both CTRL and PTU-induced hypothyroid mice, and the cell type of recorded neurons was confirmed by double immunostaining of ChAT and biocytin (Fig. 4a). The typical traces of action potential (AP) during a series of depolarizing current pulses delivered to the cholinergic neurons are shown in Fig. 4b. The evoked typical traces of AP in the cholinergic neurons from PTU-treated and CTRL mice are shown in Fig. 4c. PTU-induced hypothyroid cholinergic neurons in the basal forebrain presented higher input resistance ($F_{(1, 68)}=17.35$, $P<0.01$, Fig. 4d), lower membrane capacitance ($F_{(1, 68)}=19.24$, $P<0.01$, Fig. 4e), and more depolarized resting potential ($F_{(1, 68)}=32.58$, $P<0.01$, Fig. 4d) than CTRL ones in C57BL/6j mice, but this did not occur in 129/Sv mice. When compared to the CTRL ones, the PTU-induced hypothyroid cholinergic neurons had an elevated average threshold, while the amplitude ($F_{(1, 68)}=15.03$, $P<0.01$, Fig. 4g), depolarization rate, and half-width ($F_{(1, 68)}=27.02$, $P<0.01$, Fig. 4h) of the AP decreased significantly in C57BL/6j mice. However, the number of APs elicited by a given voltage slope decreased only in the PTU-induced hypothyroid C57BL/6j mice compared with their CTRL ones, but not in the PTU-induced hypothyroid 129/Sv mice (Fig. 4i). These results indicate that the thyroid hormone deficiency altered the basic membrane properties, AP waveforms, and intrinsic membrane excitability of cholinergic neurons in C57BL/6j mice.

Discussion

The thyroid hormones exhibit a wide range of physiologic functions throughout the body, a deficiency of which causes hypothyroidism. For unknown reasons, hypothyroidism occurs more frequently in women than in men [25]. To simplify the complex influences of hypothyroidism, only the male mice from two different mouse strains were used in the present study. In this study, the effects of adult-onset hypothyroidism on cognitive functions and the electrical characteristics of cholinergic neurons in the basal forebrain were investigated in two different mouse strains. The decreased step-through latency and recognition index were observed in PTU-induced hypothyroid C57BL/6j mice, but not in the 129/Sv strain, and the variance of cholinergic neurons may contribute to the cognitive deficits of hypothyroid adult C57BL/6j mice, but not to those of the 129/Sv mice.

Thyroid hormones play an important role in the brain's maturation and the maintenance of its physiological functions [26], and the deficiency of thyroid hormones during

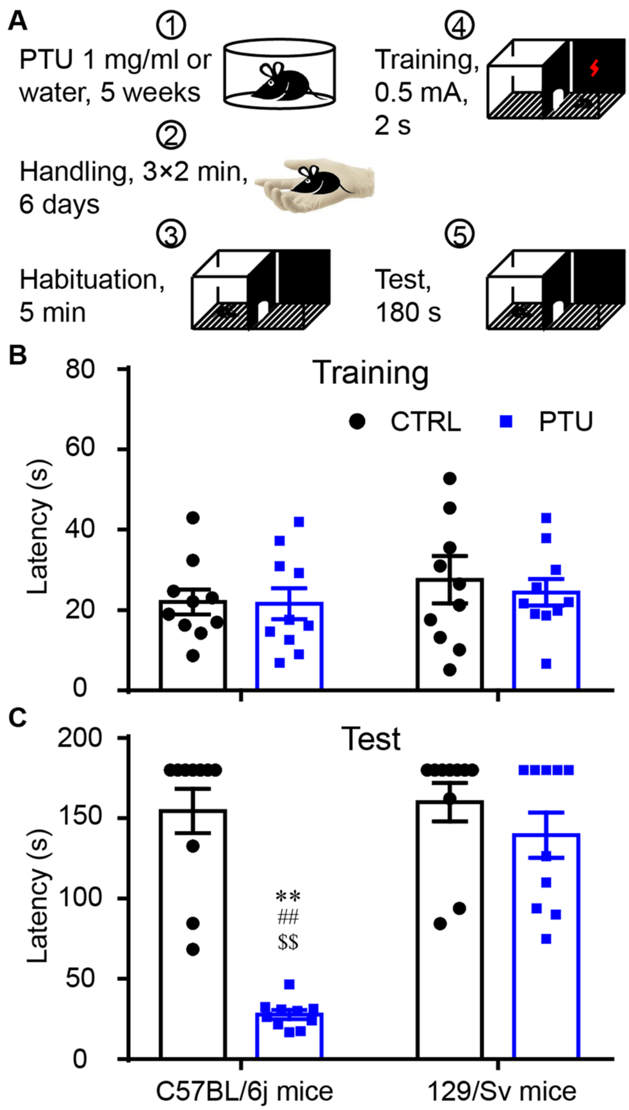

Fig. 2 Adult-onset hypothyroidism reduced step-through latency in C57BL/6j mice but not in 129/Sv mice. **a** Experimental timeline of step-through passive avoidance task. **b** During the training session, there was no significant difference among strain and treatment. **c** During the test, the latency for the step-through passive avoidance task decreased in PTU-induced hypothyroid mice of the C57BL/6j strain, but not in mice of the 129/Sv strain. $^{**}P<0.01$ compared to CTRL mice within the same strain, $^{\#\#}P<0.01$ compared to CTRL mice across the strains, $^{\$}P<0.05$ compared to PTU mice across the strains; $n=10$

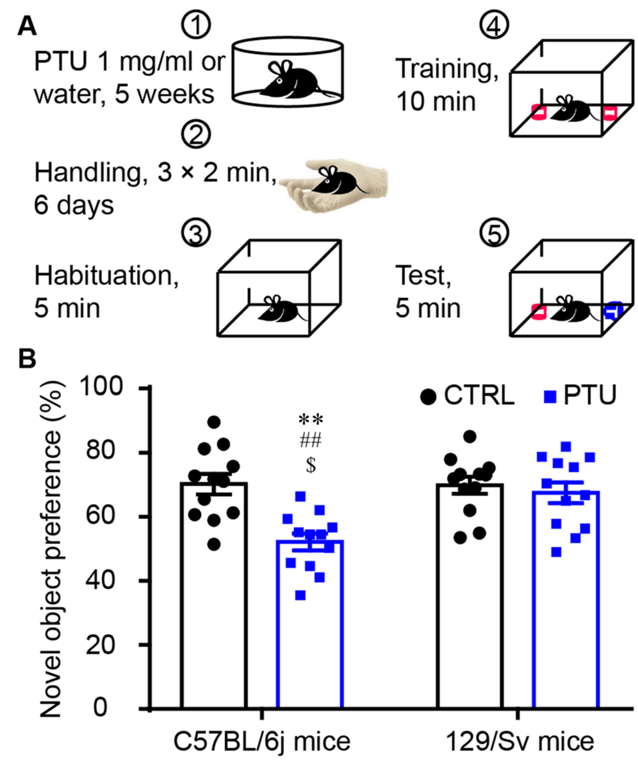

Fig. 3 Adult-onset hypothyroidism decreased novel object preference ratio in C57BL/6j mice but not in 129/Sv mice. **a** Experimental timeline of the novel object recognition test. **b** The novel object preference ratio decreased significantly in PTU-induced hypothyroid mice of the C57BL/6j strain, but not in mice of the 129/Sv strain. $^{**}P<0.01$ compared to CTRL mice within the same strain, $^{\#\#}P<0.01$ compared to CTRL mice across the strains, $^{\$\$}P<0.01$ compared to PTU mice across the strains; $n=12$

development and adulthood results in an abnormal nervous system, including a decreased number of neurons and altered axonal and dendritic branching in the brain [27]. Cholinergic neurons in the basal forebrain, which project to the cerebral cortex and modulate its synaptic plasticity, are involved in learning and memory [28], while the brain's acetylcholine content is regulated by thyroid hormones. In neonatal thyroid deficient rats, ChAT activity in the basal forebrain decreased persistently in comparison with the controls, but not in the cerebral cortex and hippocampus [29]. The activity of acetylcholinesterase varied significantly in PTU-induced hypothyroid rats [30–32]. The present study's data showed the variation of the electrophysiological characteristics of cholinergic neurons in PTU-treated mice, which may contribute to the cognitive impairments of hypothyroidism. Due to the compact size of the neurons in hypothyroidism status [33, 34], which may result in the increased input resistance and decreased membrane capacitance in hypothyroid mice. In addition, the AP characteristics of cholinergic neurons were altered during thyroid hormone deficiency in C57BL/6j mice, which may be affected by the low levels of voltage-dependent sodium channels in hypothyroid animals [35, 36]. Furthermore, the excitability of hypothyroid cholinergic neurons was decreased in PTU-induced hypothyroid C57BL/6j mice, which was similar to previous findings from hippocampal pyramidal neurons from hypothyroid rats [37]. However, there was no significant difference in the electrical properties of the cholinergic neurons in hypothyroid 129/Sv mice compared with their controls. It appears that the cholinergic neurons in 129/Sv mice are resistant to thyroid

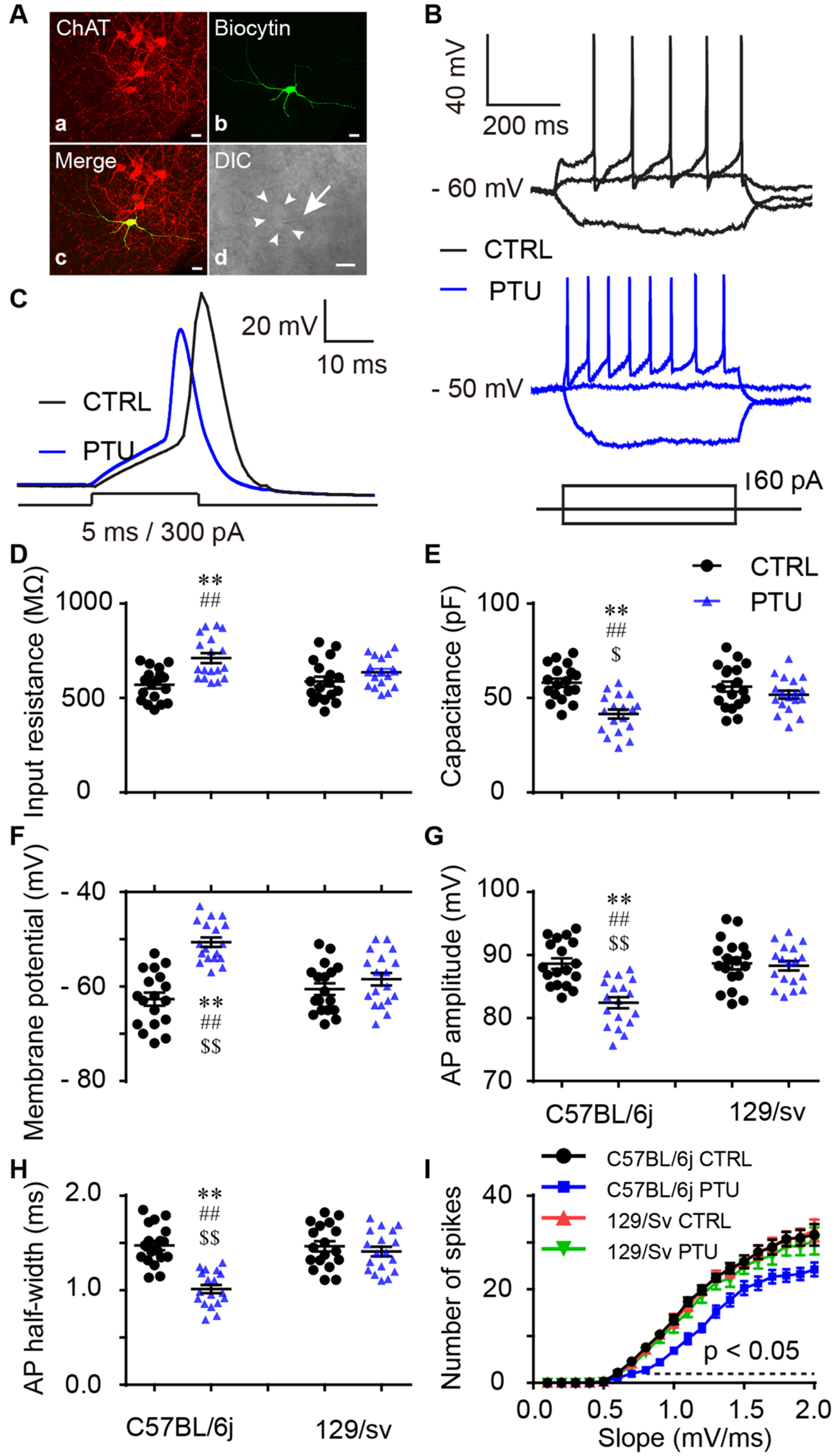

Fig. 4 Thyroid hormone deficiency affects the intrinsic excitability of cholinergic neurons in C57BL/6j mice but not in 129/Sv mice. **a** A typical section for patch-clamp electrophysiology stained against ChAT in the basal forebrain, showing the cholinergic neurons (*a*), the recorded neuron stained against biocytin (*b*) and the merged figure (*c*), and the patch pipette (white arrow) and the recorded neuron in phase contrast (*d*). Scale bar: 20 µm. **b** Example of current-clamp traces of APs in response to the 5 ms depolarizing current pulse (300 pA) recorded in cholinergic neurons from CTRL and PTU-induced hypothyroid C57BL/6j mice. **c** Representative traces of single spikes elicited by brief threshold depolarizing current pulses (5 ms) at ~ 70 mV from CTRL and hypothyroid cholinergic neurons in C57BL/6j mice. Cholinergic neurons in the basal forebrain of PTU-treated mice presented higher input resistance (**d**), lower membrane capacitance (**e**), and more depolarized resting potential (**f**) in C57BL/6j mice, but not in 129/Sv mice, compared with their CTRL. The AP amplitude (**g**) and AP half-width (**h**) changed under the hypothyroid state in PTU-treated C57BL/6j mice, but not in PTU-treated 129/Sv mice. **i** The mean number of APs elicited by the current steps to the values given on the x-axis in PTU-treated C57BL/6j mice, but not in PTU-treated 129/Sv mice. $^{**}P<0.01$ compared to CTRL mice within the same strain, $^{\#\#}P<0.01$ compared to CTRL mice across the strains, $^{\$}P<0.05$, $^{\$\$}P<0.01$ compared to PTU mice across the strain; $n=18$–20 cells from five mice

hormone deficiency, which needs to be studied further in order to solve this mystery.

The hippocampus is another critical nucleus for memory processing, while thyroid hormone deficiency may cause the neuronal morphological changes [38] or the expression of synaptic proteins' variance in the hippocampus [12], which may contribute to cognitive impairment in hypothyroid status. In addition, the hippocampus exhibits the ability to make new neurons throughout adult life, which plays an important role in learning and memory [39]. Adult neurogenesis is regulated by thyroid hormones. Both the survival and the neuronal differentiation of adult dentate granule cell progenitors decreased significantly in hypothyroid rats by increasing the apoptosis of the newborn neurons, which can be rescued through T3/T4 replacement treatment [38, 40]. These results indicated that thyroid hormone is a critical epigenetic signal in adult hippocampal neurogenesis, which may be involved in the cognitive dysfunction of hypothyroidism. The genetic influence on adult hippocampal neurogenesis has revealed that the basal level of neurogenesis is different among the mouse strains, in which the baseline level of neurogenesis in C57BL/6j mice is higher than in the other mouse strains, including 129/Sv mice [41]. Thyroid hormone deficiency may have diverse effects on adult hippocampal neurogenesis in the C57BL/6j and 129/Sv mouse strains, which caused the different outcomes of cognitive changes under hypothyroid status [42]. Furthermore, the oxidative stress is increased in patients with hypothyroidism [43], which is harmful for some essential enzymes and cytoskeletal proteins in the brain, may also result in cognitive impairments associated with hypothyroidism.

Taken together, the cognitive dysfunctions were observed in PTU-induced hypothyroid C57BL/6j mice, but not in the 129/Sv strain. Our findings suggest that mice with the C57BL/6j genetic background may fare much better than 129/Sv transgenic mice at elucidating the mechanism of hypothyroidism-induced neuropsychiatric dysfunction.

Acknowledgements This work was supported by National Natural Science Foundation of China (81272152, 81401100). The Key Program in the Youth Elite Support Plan in Universities of Anhui Province (gxyqZD2018020).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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