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Neonatal Dexamethasone Treatment Suppresses Hippocampal Estrogen Receptor α Expression in Adolescent Female Rats

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Abstract

Previous studies showed that neonatal dexamethasone treatment (NDT) transiently impaired hippocampal function in male rats. Hippocampal estrogen receptors (ERs) participate in avoidance learning. As previous studies focused on males only, this study was aimed to investigate the NDT effects on the hippocampal function of female rats. Newborn Wistar female rats were subjected to a tapering dose of dexamethasone (0.5 mg, 0.3 mg, and 0.1 mg/kg, subcutaneously) from postnatal days 1 to 3 and were subjected to experiments at the age of 6 weeks (adolescence). Brain slice extracellular recording and the inhibitory avoidance (IA) test were used to evaluate the NDT effects on hippocampal function. The results showed that NDT completely blocked the hippocampal long-term potentiation (LTP) formation and IA learning of adolescents. The expression of hippocampal estrogen receptor alpha (ERα) was attenuated in NDT subjects. Reduced histone acetylation of the ERα gene was found, possibly explaining the reduced hippocampal ERα expression in NDT female rats. Suprafusion of estradiol (E_2) partially restored the hippocampal LTP formation in adolescent NDT female rats. Coadministration of the histone deacetylase inhibitor trichostatin-A restored the hippocampal ERα expression, hippocampal LTP formation, and IA learning in adolescent NDT female rats. Collectively, these results suggested that NDT has an epigenetic modulation effect on the expression of hippocampal ERα, which is responsible for its adverse effect on hippocampal function.

Keywords Neonatal · Dexamethasone · Hippocampus · Estrogen receptor α

Introduction

Synthetic glucocorticoid dexamethasone (DEX) is frequently used to lessen the respiratory distress syndrome in extremely low birth weight (ELBW) infants [1, 2]. Although clinical trials have failed to consistently demonstrate improvement in terms of the mortality or hospitalization duration of ELBW infants, treatments for as long as 42 days are still commonly used [3]. Common side effects of neonatal DEX treatment (NDT) in ELBW infants include hypertension [4], bowel perforation, infection [5], ventricular hypertrophy [6], catabolic changes [7], and alterations in the limbic HPA axis [8–11]. In animal studies, NDT in rats has resulted in kidney damage in adulthood [12]. NDT also increases the susceptibility to experimental autoimmune disease [13] and the risk for pulmonary hypertension in adult rats [14].

Previous studies have also linked NDT to long-term neurological effects, including an increased incidence of cerebral palsy and decreased cerebral volume [15, 16]. The results also suggest that NDT impairs brain development and cognitive function [17–19]. It had been proven that NDT alters hippocampal synaptic plasticity and associative memory in adult male rats [20, 21]. Flagel et al. showed that NDT animals are less active in light and dark environments and exhibit a blunted serum corticosterone response to novel stresses [22, 23]. The hippocampus is involved in spatial memory and is also a critical site of neuronal plasticity for classical conditioning. Little is known about the NDT effects on the hippocampal

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function of females. It is well known that estrogen via estrogen receptors (ERs) plays an important role in neurogenesis, neuroplasticity, and long-term potentiation in the hippocampus. We speculate that NDT might alter ER expression and have long-lasting adverse effects on hippocampal function. Long-term hormone replacement therapy in human females experiencing either surgical or natural menopause protects against both memory loss in both verbal and nonverbal memory tests and attention deficits [24]. Collectively, these results suggested the importance of hippocampal E_2 signaling, which plays an essential role in hippocampal function, particularly memory formation.

Recently, we demonstrated that NDT elevates the long-term potentiation (LTP) response and the phosphorylation level of MAPK in the amygdala of male rats [25]. Collectively, these results imply that NDT has long-lasting adverse effects on the amygdala, which may result in adverse consequences in adolescents. Its detailed mechanism is worthy of further investigation. This study was aimed to investigate the NDT effects on the hippocampal function of female rats.

Material and Methods

Animals

Pregnant female Wistar rats were obtained from BioLASCO (Taiwan). Only pups born on days 22 and 23 of gestation were used. The pups were weaned at 21 days and remained housed with their littermates until experimentation commenced at 6 weeks old. The animals were housed in a temperature (24 °C) controlled animal colony, with continuous access to food and water. The animals were maintained on a 12:12 light-dark cycle with lights on at 07:00. All behavioral procedures took place during the light portion of the animal cycle. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the local Institutional animal Care and Use Committee (IACUC) at the National Taiwan Normal University. All efforts were made to minimize animal suffering and to minimize the number of animals necessary to produce reliable data.

Neonatal Dexamethasone Administration Protocol

From postnatal day (PND) 1 to day 3, the pups were randomly assigned to two groups, the saline-treated group (SAL group) and the neonatal dexamethasone-treated group (NDT group). The pups were gently removed from their home cage and separated from their mother for drug treatment and body weight measurement. All procedures were carried out between 10:00 AM and 2:00 PM and were completed within 10 min. The NDT group pups received a daily subcutaneous injection of DEX from PND 1 to 3. DEX was given in tapering doses of 0.5, 0.3, and 0.1 mg/kg subcutaneously on PND 1, 2, and 3, respectively. The pups in the SAL group received equivalent volumes of subcutaneously injected saline. The tapering doses of DEX were adapted from previous studies that aimed to mimic the clinical application of NDT in preterm infants [21, 25, 26].

Body Weight Measurement

The body weights were recorded and compared between the NDT and SAL groups on postnatal days 1, 2, 3, 7, 14, 21, 28, and 42. The pups were gently removed from their home cage and separated from their mother for body weight measurement. All procedures were carried out between 10:00 AM and 2:00 PM and completed within 3 min.

Trichostatin-A Administration

The dosage of trichostatin-A (TSA) chosen was based on the previous study of Zhu [27]. The newborn rats were randomly assigned to four groups: (1) SAL + dimethyl sulfoxide (DMSO) group for rats injected with saline and DMSO (2%; Cell Signaling Tech., USA), (2) SAL + TSA group for rats injected with saline and TSA dissolved in 2% DMSO (1 mg/kg/day; Cell Signaling Tech., USA), (3) NDT + DMSO group for rats injected with DEX and DMSO, and (4) NDT + TSA group for rats that received freshly prepared TSA. Vehicle or TSA was injected immediately after the SAL or DEX injection on P1. Animals were then sacrificed at the age of 2 weeks by decapitation. The hippocampus was dissected out and subjected to western blotting.

E_2 Administration (Electrophysiological Record)

We used the ER agonist 17β-estradiol (E_2) to induce LTP formation. In hippocampal slices perfused with ACSF, we recorded the baseline signal for 20 min, reperfused the slices with 17β-estradiol (0.1 nM) or vehicle (0.1 mM acetone) for 20 min, and then recorded high-frequency electrical stimulation-induced LTP signals [28].

Brain Slice Extracellular Recording

In a separate in vitro experiment, animals were decapitated, and the brains were quickly removed from the skull. Coronal slices were cut to a thickness of 400 μm using a vibratome (MA752 Campden Instruments Ltd., UK). The appropriate slices were placed in a beaker of artificial cerebrospinal fluid (ACSF). The ACSF was bubbled continuously with 95% O_2/5% CO_2 to maintain the pH at 7.3–7.5. The ACSF consisted of 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 2 mM NaH_2PO_4, and 11 mM

glucose. The slices were kept at room temperature for at least 1 h for stabilization before recording. A single slice was then transferred to the recording chamber, where it was held submerged between two nylon nets and maintained at 32 ± 1 °C. The chamber consists of a circular, low-volume (1–1.5 ml) well and was perfused constantly at the rate of 3–4 ml/min. A bipolar stimulating electrode (SNE-2OOX, Kopf Instrument, USA) was used. The field excitatory postsynaptic potential (fEPSP) was recorded extracellularly using glass microelectrodes filled with 3 M NaCl (3–8 MΩ). The stimulus and recording electrodes were placed in their respective Schaffer collaterals. The evoked fEPSP signals were recorded using an Axoclamp 2B amplifier (Axon Instruments, USA). The responses were elicited using low square-wave pulses delivered at 20 s intervals and were filtered at 1 kHz and digitized at 5 kHz (Digidata 1322A; Axon Instruments, USA). The stimulation voltage was adjusted individually for each experiment to produce the fEPSP, which were 30–40% of the maximum possible response. The strength of synaptic transmission was quantified by measuring the initial slope of the fEPSP and analyzed using pCLAMP software (version 10.2; Axon Instruments, USA). LTP was induced by high-frequency stimulation (HFS) at an intensity two times higher than the test pulse intensity (3X 1-s trains of 100 Hz stimuli separated by an intertrain interval of 20 s).

Inhibitory Avoidance Task

A conventional one-way inhibitory avoidance learning task was used to measure retention performance. The process included a training phase and a testing phase. Both phases were conducted between 9:00 AM and 4:00 PM. Before experimentation, the rats were kept in a dim room for 1 h for acclimation. During the training phase, the rat was placed at the far end of the illuminated compartment facing away from the door. As the rat turned around, the door was opened. When the rat entered the dark compartment, the door was closed, and a 0.4-mA/1-s foot-shock was applied [29–31]. The animal was then removed from the dark compartment and returned to its home cage. The retention test was performed 24 h later. The animal was again placed in the illuminated compartment, and the latency to step into the dark compartment was recorded as the measure of retention. The ceiling score was set to 600 s. To avoid a possible confounding effect of estrogen changes during the estrus cycle, the animals were subjected to vaginal smear tests to evaluate their estrous cycle state. The inhibitory avoidance test was conducted during the proestrous stage.

Locomotor Activity Test

NDT may also elicit certain nonspecific effects on locomotor activity during the test session, resulting in a misinterpretation of its blockage effects on inhibitory avoidance learning. We examined the animals' locomotor activity performance to evaluate this possibility. The animals were placed in the center of a Plexiglas testing chamber (square base with 42 cm sides, 36 cm height). The horizontal moving distance was recorded by an EthoVision video tracking system (EthoVision, Noldus, NED). The tests lasted 15 min, which was divided into three blocks of 5 min.

Western Blot Analysis

At the end of the behavioral session, the animals were decapitated, and the brains were quickly removed from the skulls. The tissue in the hippocampal region was dissected and homogenized using a T-PER tissue protein extraction reagent kit (Pierce Biotechnology Inc., USA). The samples were ground in a blender at low power until fully homogenized. The samples were spun down at 13,200×*g* at 4 °C for 10 min. The total protein concentration was determined by a Bio-Rad Bradford protein assay kit (Bio-Rad, Hercules, USA). Each sample with equal 25 μg amounts of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was separated on a PVDF membrane (Merck Millipore, Germany). The blots were blocked in a PBS solution containing 5% nonfat milk and 0.05% Tween 20 for 1 h at room temperature. Primary antibodies were used to detect ERα and ERβ (1:2000; Cell Signaling Tech, USA) and to detect β-actin (1:3000; Gene Tax, Inc., USA). The PVDF membranes were first incubated in primary antibody PBS solution overnight at 4 °C. The membranes were then probed with HRP-conjugated secondary antibodies for 1 h at room temperature. The signals were detected using enhanced chemiluminescence (Merck Millipore, Germany) and analyzed using a LAS3000 digital imaging system (Fujifilm, Japan). The expression levels of hippocampal ERα and ERβ were determined using the expression relative to that of β-actin.

Chromatin Immunoprecipitation

To determine the NDT effect on the H3K9 acetylation of ERα in the hippocampus, the chromatin immunoprecipitation assay (ChIP) was used. H3K9 antibody (ab4441) was purchased from Abcam (USA). Two-week-old female rats were decapitated, and the hippocampus was dissected out. The selected time point was aimed at minimizing the possible acute effect of NDT on the expression of hippocampal ERα. The relevant primers for the region of the H3K9 binding site are summarized as follows [32].

Forward primer: 5′ AggAAgAAACTCCCCTCAgC 3′;
Reverse primer: 5′ CCTACCTgTggAgCCAgAAA 3′.

The q-PCR conditions were as follows: 95 °C—10 min, followed by 40 cycles of 95 °C—30 s, 53.8 °C—30 s, and 72 °C—30 s.

Statistical Analysis

Data are expressed as the mean ± SEM. Body weight and behavior (inhibitory avoidance) data were analyzed by one-way analysis of variance (ANOVA). Fisher's post hoc tests were performed to determine the source of the significance differences found with ANOVA. LTP statistical analysis was performed using the Mann-Whitney *U* test. Western blot data and ChIP data were analyzed using an unpaired Student's *t* test. Probability levels below 0.05 ($p < 0.05$) were considered significant. All statistical analysis was performed using SPSS version 12.0 (SPSS, Chicago, USA).

Results

Somatic Growth of NDT Pups Was Impaired Temporarily

The results showed that NDT significantly reduced the body weight gain on P2 ($p = 0.004$), P3 ($p = 0.009$), P7 ($p < 0.001$), P14 ($p < 0.001$), P21 ($p < 0.001$), P28 ($p < 0.001$), and P42 ($p < 0.001$) compared with that in the corresponding SAL animals. This result is consistent with the previous finding that NDT has a temporary effect on the somatic growth of pups ($n = 7$ for each group; Fig. 1).

The High-Frequency Stimulation-Induced Hippocampal LTP Was Altered in Adolescent NDT Female Rats

Lin et al. showed that NDT alters the synaptic plasticity in the hippocampus of adolescent male rats [21]. We used extracellular recording to determine whether a similar effect occurred for the hippocampal LTP formation of NDT female rats. Six-week-old animals were subjected to in vitro extracellular recording of the HFS-induced hippocampal LTP formation. NDT was found to result in blockage and attenuation of the hippocampal LTP formation in the 6-week-old animals. The average magnitude of potentiation in the adolescent NDT rats, measured 50 min after a tetanic stimulus, was 99 ± 1% as opposed to 181 ± 22% in the SAL group ($p < 0.01$; $n = 6$ for each group; Fig. 2a). This result suggests that NDT could have a long-lasting effect on the hippocampal plasticity in adolescent NDT female rats.

Inhibitory Avoidance Learning Was Blocked in Adolescent NDT Female Rats

The extracellular recording results clearly showed that NDT attenuated hippocampal LTP formation. We therefore speculate that inhibitory avoidance learning, a hippocampus-dependent task (IA task), may also have been altered. Eighteen animals were randomly distributed into the SAL group or the NDT group. The NDT adolescent rats showed lower retention latencies (median = 4 s) than the SAL rats (median = 88 s, $n = 9$ for each group; Fig. 2b). The results for the total horizontal distance movement did not reveal any significant difference between the SAL and NDT groups (Fig. 2c).

The Expression of Hippocampal Estrogen Receptor Alpha Was Altered in NDT Female Rats

It is well known that estrogen via estrogen receptors (ERs) participates in the neurogenesis and neuroplasticity of the hippocampus [33–35]. We speculated that NDT might alter hippocampal ER expression. We examined the expression of hippocampal ERs using western blotting. The results showed a significant decrease in the hippocampal ERα expression for

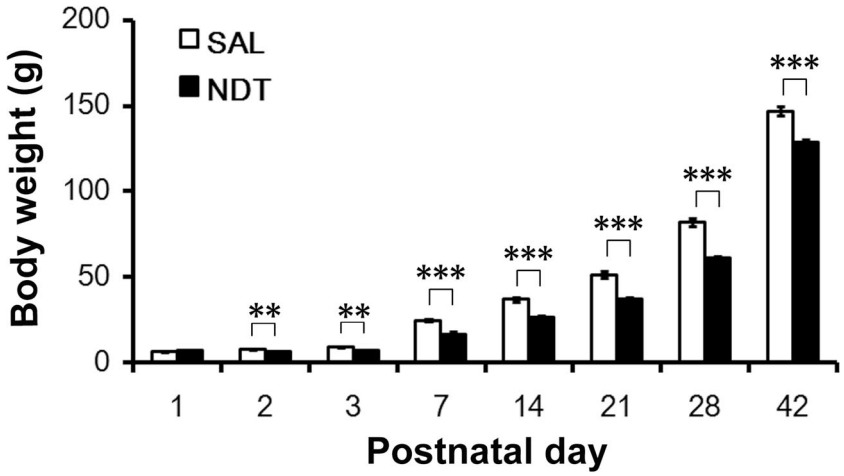

Fig. 1 Neonatal DEX treatment affects the somatic growth of female rats. DEX administration in neonatal animals at P2, P3, P7, P14, P21, P28, and P42 decreased the weight compared with that in the control group, with significant differences between the two groups. Each vertical bar represents the mean ± SEM of each group ($n = 7$ for each group; $^{**}p < 0.01$, $^{***}p < 0.001$)

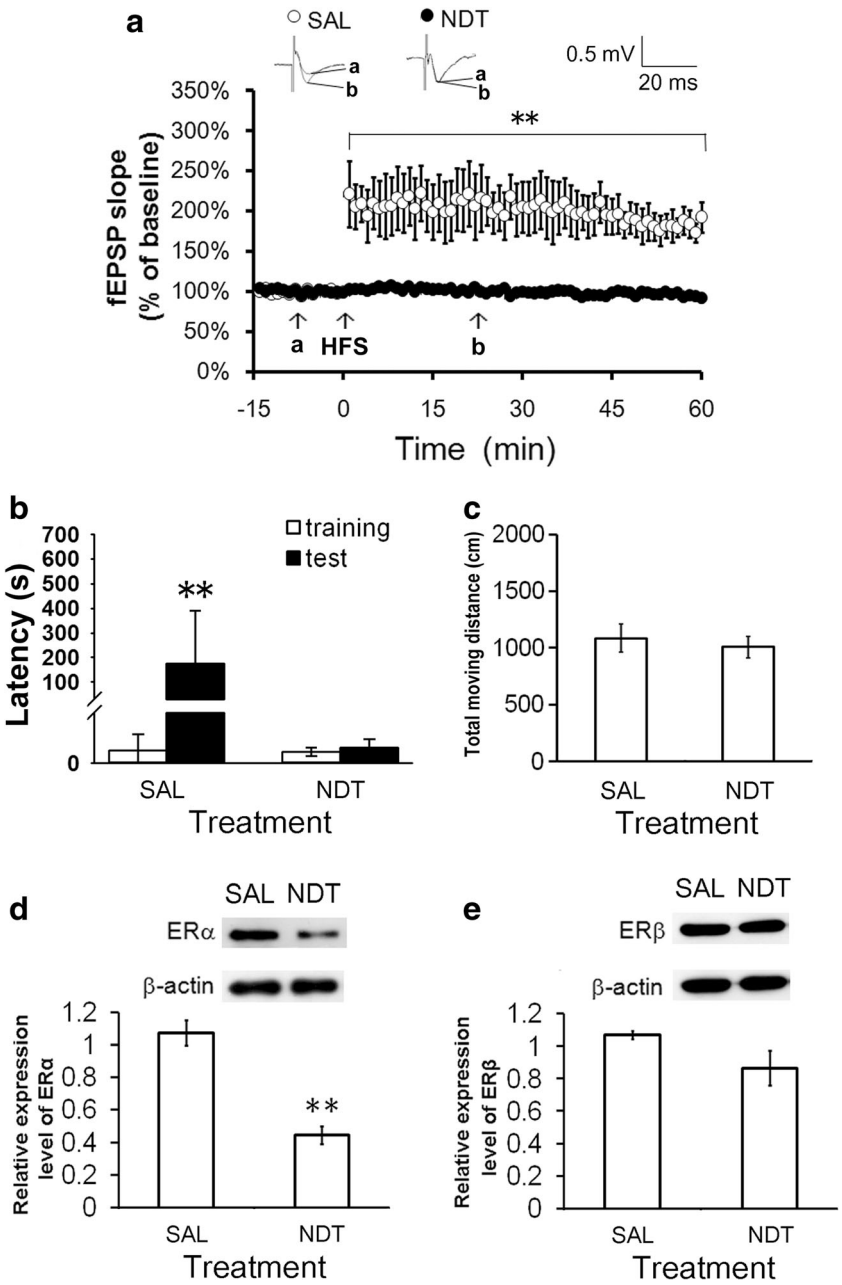

Fig. 2 Neonatal DEX treatment impaired hippocampal LTP formation and blocked inhibitory avoidance learning, and the ERα expression was decreased in adolescent female rats. **a** High-frequency stimulation-induced hippocampal LTP was completely blocked in adolescent female rats. a and b each independently represent high-frequency stimulation (HFS) for 10 min before induction and 20 min after induction, respectively. Typical traces are displayed in the upper panel of each figure. Each vertical bar represents the mean ± SEM for each group ($n = 6$ for each group; $^{**}p < 0.01$). **b** NDT adolescent rats showed lower retention latencies than those of the corresponding SAL rats ($n = 9$ for each group; $^{**}p < 0.01$). **c** There was no significant difference in locomotor activity between either adolescent group ($n = 9$ for each group). **d** The hippocampal ERα expression levels were significantly decreased at P42 compared with those in the corresponding SAL groups ($n = 3$ for each group; $^{**}p < 0.01$). **e** No significant difference in hippocampal ERβ expression was found on P42 ($n = 3$ for each group) among the NDT and SAL groups. Each vertical bar represents the mean ± SEM for each group

all NDT groups compared with that in the corresponding SAL groups on P42 ($p = 0.001$; Fig. 2d), but not in ERβ expression. No difference in the expression of hippocampal ERβ was found among the groups ($p = 0.089$; Fig. 2e). This result suggests long-term alteration of the expression of hippocampal ERα after NDT in female rats.

The Blockage of Hippocampal LTP Formation by NDT in Adolescent Female Rats Was Partially Restored by Suprafusion of E_2

To determine whether the LTP blockade effect of NDT could be reversed by supplementation with an estrogen agonist, E_2 suprafusion was performed 20 min before HFS and continued for 80 min. Our results indicate that the E_2 suprafusion of the hippocampus inhibited LTP formation in the SAL rat brain slices (20 min after HFS: SAL + vehicle 280 ± 48%; SAL + E_2 79 ± 6%; $p < 0.01$, $n = 6$ for each group; Fig. 3a). The E_2 suprafusion partially restored hippocampal LTP formation in the NDT rat brain slice (20 min after HFS: NDT + vehicle 96 ± 3%; NDT + E_2 157 ± 19%; $p < 0.05$, $n = 6$ for each group; Fig. 3b). This result implied that NDT may alter estrogen-related signaling, a change that is responsible for the blocking effect of NDT on hippocampal LTP formation.

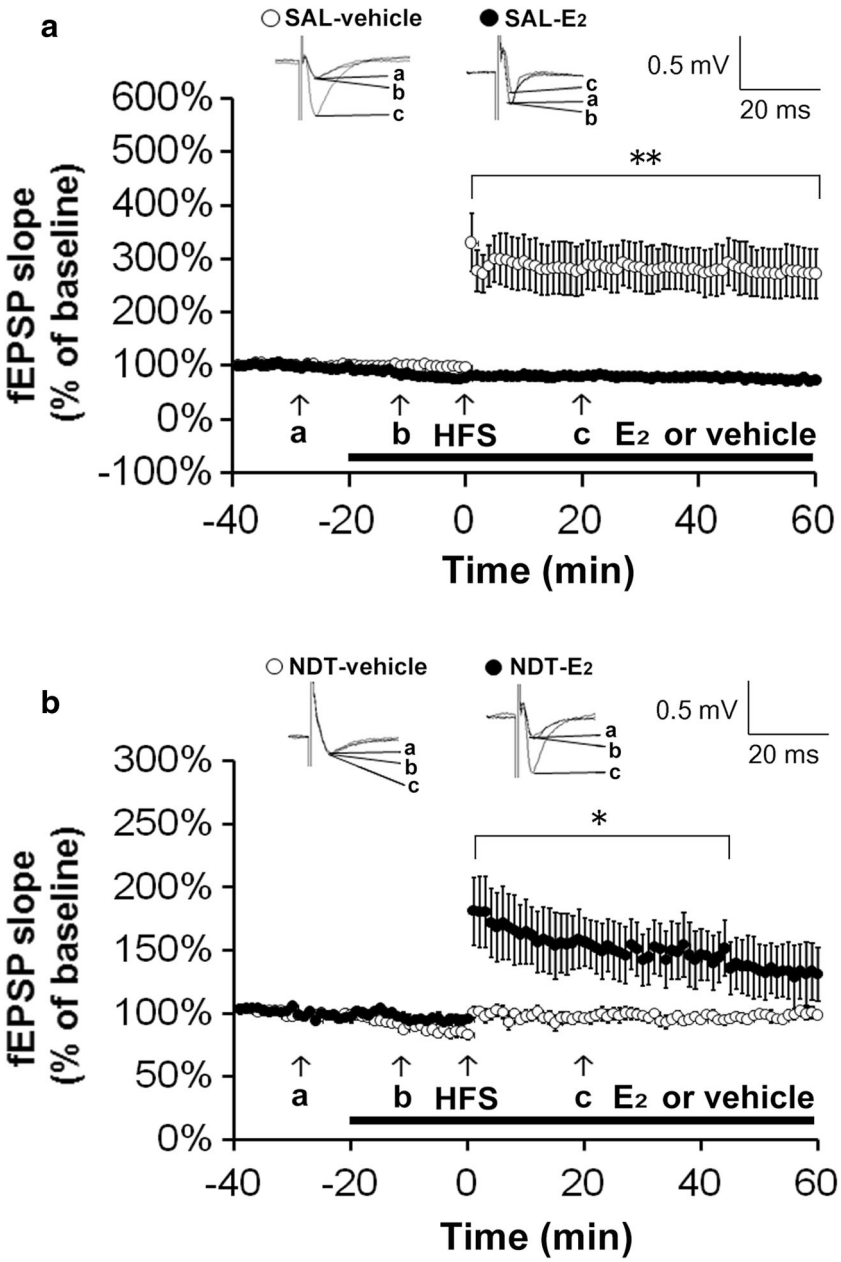

Fig. 3 Suprafusion of estradiol partially restored the hippocampal LTP formation in adolescent NDT female rats. **a** Preadministration of E_2 before HFS blocked the hippocampal LTP formation in the SAL group of female rats. **b** The hippocampal LTP was partially restored in NDT animals after pretreatment with E_2. Typical traces are displayed in the upper panel of each figure; a, b, and c respectively represent 10 min before estradiol administration, 10 min before HFS, and 20 min after induction. Each vertical bar represents the mean ± SEM ($n = 6$ for each group; $^*p < 0.05$; $^{**}p < 0.01$)

The Acetylation of H3K9 Was Significantly Decreased in the Hippocampus of NDT Female Rats

The hippocampal ERα expression was attenuated in adolescent NDT female rats. Since the subjects only received DEX injection from postnatal day 1 to day 3, we speculated that an epigenetic mechanism might be involved. A chromatin immunoprecipitation assay was used to evaluate the histone acetylation in the SAL and NDT groups at the age of 2 weeks. The results showed that the histone acetylation of H3K9 was significantly reduced in the NDT animals (% of SAL) ($p = 0.008$; $n = 3$ for each group; Fig. 4a). No significant difference in the expression of β-actin was found between the NDT and SAL groups (% of SAL) ($p = 0.422$; $n = 3$ for each group; Fig. 4b).

Coadministration of the HDAC Inhibitor Trichostatin-A Restored Somatic Growth and Hippocampal ERα Expression in NDT Female Rats

We coadministered the histone deacetylase (HDAC) inhibitor trichostatin-A (TSA) to determine whether histone acetylation is involved in the NDT effect on somatic growth and hippocampal ERα expression. Additional animals were assigned to four groups: SAL + vehicle, NDT + vehicle, SAL + TSA, and NDT + TSA. The SAL and NDT groups were injected subcutaneously as described before. The results showed that the somatic growth was restored to the normal range in the NDT + TSA pups (Fig. 5a), the hippocampal ERα expression was compared between the NDT + TSA and NDT + vehicle

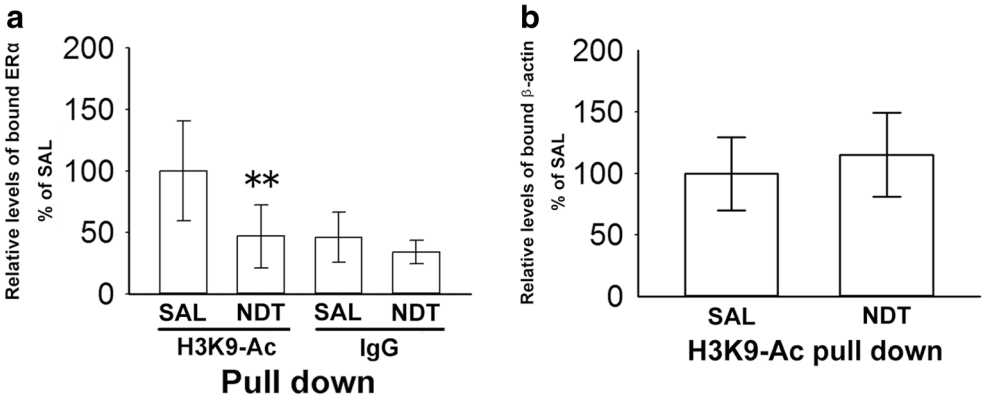

Fig. 4 Decreased hippocampal histone acetylation of the ERα gene in NDT female rats. **a** Compared to the SAL group, the NDT group showed a reduction in histone acetylation of the ERα gene ($n = 3$ for each group; $^{**}p < 0.01$). **b** No differences were found in either the IgG expression or the β-actin expression between the NDT and SAL groups ($n = 3$ for each group)

groups ($p = 0.081$), there was a difference in the expression between the SAL + vehicle and NDT + vehicle groups ($p = 0.024$), and there was no difference in the expression between the SAL + vehicle and NDT + TSA groups ($p = 0.236$) ($n = 3$ for each group; Fig. 5b). Coadministration of TSA did not show any significant effect on the expression of hippocampal ERβ among the groups ($n = 4$ for each group; Fig. 5c).

The Hippocampal LTP Formation in Adolescent NDT Female Rats Was Partially Restored by the HDAC Inhibitor Trichostatin-A

Since both somatic growth and hippocampal ERα expression were restored after TSA administration, we further used extracellular recording to evaluate the possible rescue effects of

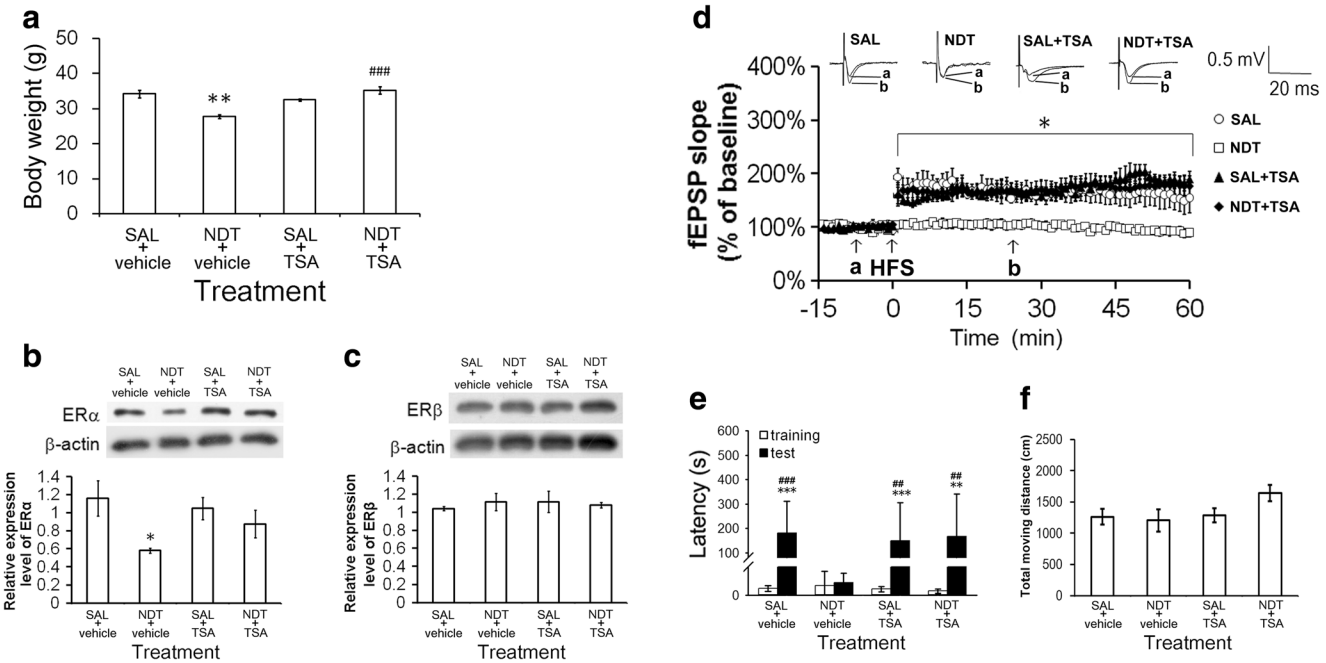

Fig. 5 HDAC inhibitor treatment affects the somatic growth, hippocampal estrogen receptor expression, and inhibitory avoidance learning in NDT female rats. **a** Neonatal DEX and TSA treatment in 2-week-old female rats changed the body weight ($n = 4$ for each group; $^{**}p < 0.01$ compared with the SAL + vehicle group; $^{###}p < 0.001$ compared with the NDT + vehicle group). **b** Coadministration of the HDAC inhibitor TSA restored the hippocampal ERα expression in the NDT animals ($n = 3$ for each group; $^{*}p < 0.05$). **c** No significant difference was found for the hippocampal ERβ expression after TSA administration. Each vertical bar represents the mean ± SEM for each group ($n = 4$ for each group). **d** High-frequency stimulation-induced hippocampal LTP was fully restored in 6-week-old NDT animals after TSA administration. Typical traces are displayed in the upper panel of the figure. Arrows a and b each independently represent HFS 10 min before induction and 20 min after induction. Each vertical bar represents the mean ± SEM for each group (NDT + TSA $n = 6$; SAL+TSA $n = 6$; SAL $n = 6$; NDT $n = 4$; $^{*}p < 0.05$). **e** No significant difference in the retention latencies was found among the SAL and SAL + TSA groups. The inhibitory avoidance learning was fully restored after TSA administration in the adolescent female rats (SAL group $n = 7$; NDT group $n = 7$; SAL + TSA group $n = 8$; NDT + TSA group $n = 6$) ($^{**}p < 0.01$; $^{***}p < 0.001$ compared with the corresponding training session; $^{##}p < 0.01$; $^{###}p < 0.001$ compared with the testing session of the NDT group). **f** The total horizontal distance movement did not differ significantly between the SAL, NDT, SAL + TSA, and NDT + TSA groups (SAL group $n = 7$; NDT group $n = 7$; SAL+TSA group $n = 8$; NDT + TSA group $n = 6$)

TSA on the hippocampal LTP formation. TSA was freshly prepared and dissolved in 2% DMSO. Animals received tapering doses of DEX by injection from PND 1–3 as usual, and vehicle or TSA was injected immediately after the DEX injection on PND 1. The animals were subjected to in vitro extracellular recording of the HFS-induced hippocampal LTP at the age of 6 weeks. Our results indicate the TSA partially restored hippocampal LTP formation in the NDT rat brain slices (measured 40 min after the tetanic stimulus: NDT + TSA 179 ± 21%; SAL + TSA 171 ± 11%; NDT 91 ± 5%; SAL group 164 ± 19%; $p < 0.05$, NDT + TSA $n = 6$; SAL + TSA $n = 6$; SAL $n = 6$; NDT $n = 4$) (Fig. 5d). This result implied that NDT may alter the HDAC-related signaling, a change that is responsible for the blocking effect of NDT on hippocampal LTP formation.

The HDAC Inhibitor Trichostatin-A Restores Inhibitory Avoidance Learning in Adolescent NDT Female Rats

The administration of TSA partially restored hippocampal LTP formation in NDT animals. We therefore further evaluated whether the inhibitory avoidance learning could also be restored. Twenty-eight animals were randomly divided into four groups: the SAL group ($n = 7$), the NDT group ($n = 7$), the SAL + TSA group ($n = 8$), and the NDT + TSA ($n = 6$) group. For the SAL group, SAL + TSA group, and NDT + TSA group, the training and test results were significantly different. No significant difference in the retention latencies was found between the SAL group, SAL + TSA group, and NDT + TSA group of adolescent female rats (Fig. 5e). The SAL, SAL + TSA, and NDT + TSA groups had significant differences between the training and test results (SAL group $p < 0.001$; SAL + TSA group $p < 0.001$; NDT + TSA group $p = 0.002$; Fig. 5d). The NDT adolescent female rats showed lower retention latencies than the SAL rats, SAL + TSA rats, and NDT + TSA rats (SAL group $p < 0.001$; SAL + TSA group $p = 0.001$; NDT + TSA group $p = 0.005$; Fig. 5e). The results for the total horizontal distance movement did not reveal any significant difference between the SAL, NDT, SAL + TSA, and NDT + TSA groups (Fig. 5f).

Discussion

The results obtained from the present study were consistent with the previous finding that NDT impaired hippocampal function in NDT adolescent male rats [21]. We have proven that the estrogen-related pathway plays an essential role in the adverse effect of NDT in female rats. The results showed that both inhibitory avoidance learning and hippocampal LTP formation were impaired in adolescent NDT female rats. In addition, the hippocampal expression of ERα but not ERβ was also attenuated. The hippocampal LTP formation deficit could be rescued by acute administration of estradiol (E_2). We also demonstrated that the histone acetylation of the ERα gene was altered, possibly explaining the decrease in hippocampal ERα expression. Coadministration of the histone deacetylase inhibitor trichostatin-A restored the somatic growth, hippocampal ERα expression, inhibitory avoidance learning, and hippocampal LTP formation in adolescent NDT female rats.

It is well known that via ERs, estrogen participates in the neurogenesis and neuroplasticity of the hippocampus [33–35]. Both ERα and ERβ contribute to adult neurogenesis in the hippocampus via the conventional nucleus pathway. Administration of E_2 increases the synaptic density in the hippocampal CA1 region of ovariectomized female rats [33, 36]. In addition to the conventional nucleus pathway, E_2 also exerts an effect via membrane-based signaling pathways. Previous results demonstrated that membrane signaling pathways involving estrogen impact neuronal excitability, signal transduction, cell death, neurotransmitter release, and gene expression in the hypothalamus and hippocampus and contribute to the control of physiological and behavioral functions [33, 34, 37]. It has been shown that E_2 may modulate a host of signal transduction pathways, including mobilization of calcium and increased phospholipase and protein kinase activity via G-protein coupled mechanisms [38], and enhance memory consolidation in the dorsal hippocampus via activation of the ERK signaling pathway [39], which is important for the formation of hippocampal LTP and may account for the rescue effect of E_2 on the NDT-induced hippocampal LTP deficit. In the present study, suprafusion of E_2 restored hippocampal LTP formation in NDT rats, supporting this hypothesis. Since E_2 was only suprafused in the brain slice, it is unlikely that the acute rescue effect occurs via the secondary effects of E_2.

We would like to mention that there was an opposite effect of E_2 administration on the hippocampal LTP formation of the SAL group, in which the hippocampal LTP formation was significantly suppressed by E_2. In this study, the animals were sacrificed during the proestrous phase, which coincides with the highest level of circulating estrogen. Previous studies have shown that E_2 alters the hippocampal LTP in female rats depending on the state of their estrous cycle. E_2 increased the LTP in slices from diestrus rats but decreased the LTP in slices from proestrous rats [40]. This finding is consistent with the present results showing that E_2 suppressed the LTP of SAL female rats (Fig. 3a).

Previous studies have found that in human cancer cell experiments, glucocorticoids could inhibit ERα activity [41]. In the present study, we did not apply DEX during the behavioral experiment and/or extracellular recording. The NDT animals only received DEX treatment from PND 1–3. It is unlikely that an acute effect of DEX administration occured here. Our hypothesis is based on the previous observation that a decrease in estradiol and reduced expression of ERα relative to ERβ during menopause would be

predicted to act in concert to decrease transcriptional processes that normally help preserve cognitive function. These results are consistent with the mounting evidence suggesting that increased ERα expression is associated with improved learning and memory. Treatments to alter the expression of ERs within the hippocampus could provide an alternative to hormone replacement in preserving cognitive function [42]. We speculate that the expression of hippocampal ERα will be restored as age increases. In addition, other signaling pathways, such as glutamatergic transmission, are also essential for inhibitory avoidance learning and become dominant with age.

The key issue raised in this study relates to the mechanism by which NDT alters the hippocampal ERα expression. We speculate that an epigenetic mechanism might be involved. Previous studies showed that the acetylation of H3K9 plays an essential role in modulating the expression of ERα. Therefore, we focused our study on evaluating the acetylation of H3K9. Our results revealed that a change in H3K9 acetylation in the hippocampus of NDT female rats and cotreatment with the HDAC inhibitor TSA restore the hippocampal ERα expression to normal levels. These results reveal that an epigenetic mechanism is involved in the suppressive effect of NDT on hippocampal ERα expression. In this study, we only evaluated an HDAC inhibitor; even though we observed a significant rescue effect in the NDT female rats, the effect of TSA on acetylation was not determined. Therefore, we cannot exclude the possible involvement of other kinds of epigenetic modulations, such as DNA methylation. Subsequent experiments using cotreatment with DNMT inhibitors and/or measurements of the acetylation of H3K9 might be helpful for determining the mechanism in detail.

A novel form of estrogen receptor, GPR30, acting in an ionotropic receptor manner, might also be altered in NDT animals. It should also be mentioned that we only focused on the hippocampal ERα expression. We cannot exclude the possible involvement of GPR30 in the TSA rescue effect on adolescent NDT female rats. Our previous results demonstrated that depression-like behavior was increased in NDT adult male rats. NDT might also affect the ERα expression in other brain regions, such as the amygdala or medial prefrontal cortex, and might be responsible for the long-term adverse effect of NDT. Accordingly, other tests, such as immunohistochemical and western blot assays, of those regions are required to test this hypothesis.

In conclusion, we demonstrated that NDT has a long-term adverse effect on hippocampal function. This effect may occur via the altered hippocampal ERα expression. Acute administration of E_2 or its analogs may have a therapeutic effect on the NDT-induced cognitive deficit in female patients receiving NDT.

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Author Contributions KTL and YLY participated in the conception and design. CYC contributed to the electrophysiological experiments. HFC, MWYC, JLC, and JMJL participated in the ChIP experiment. HFC collected and assembled the data. HFC, MWYC, KTL, and YLY analyzed and interpreted the data. KTL and YLY wrote the manuscript and provided financial support. All authors corrected and approved the manuscript.

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Compliance with Ethical Standards

All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the local Institutional animal Care and Use Committee (IACUC) at the National Taiwan Normal University.

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

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