Histone deacetylase inhibition reduces hypothyroidism-induced neurodevelopmental defects in rats

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Abstract

Thyroid hormone (TH) through its receptor (TRα/β) influences spatio-temporal regulation of its target gene repertoire during brain development. Though hypothyroidism in WT rodent models of perinatal hypothyroidism severely impairs neurodevelopment, its effect on TRα/β knockout mice is less severe. An explanation to this paradox is attributed to a possible repressive action of unliganded TRs during development. Since unliganded TRs suppress gene expression through the recruitment of histone deacetylase (HDACs) via co-repressor complexes, we tested whether pharmacological inhibition of HDACs may prevent the effects of hypothyroidism on brain development. Using valproate, an HDAC inhibitor, we show that HDAC inhibition significantly blocks the deleterious effects of hypothyroidism on rat cerebellum, evident by recovery of TH target genes like Bdnf, Pcp2 and Mbp as well as improved dendritic structure of cerebellar Purkinje neurons. Together with this, HDAC inhibition also rescues hypothyroidism-induced motor and cognitive defects. This study therefore provides an insight into the role of HDACs in TH insufficiency during neurodevelopment and their inhibition as a possible therapeutics for treatment.

Introduction

Thyroid hormone receptors (TRs) belong to the steroid family of nuclear receptor and are expressed as two gene products TRα and TRβ (Yen 2001). TRs typically form heterodimers with another member of the nuclear receptor superfamily, RXR, that bind to thyroid hormone response elements (TREs), which are commonly located in the promoter regions of target genes (Yen 2001). A unique characteristic of TR, unlike many other steroid receptors, is the ability to regulate their target gene expression in either presence or absence of their ligand. This bimodal transcriptional regulation is achieved through rapid exchange of co-activator and co-repressor complexes by ligand bound or free TRs (Feng et al. 2001). Co-repressor complexes bound to unliganded TR generally comprise of either nuclear receptor co-repressor (NCoR) or silencing mediator of retinoid and thyroid hormone receptor (SMRT) proteins along with associated histone deacetylase (HDACs) (Shimizu et al. 2015). NCoR and SMRT contain two TR-interaction sequences composed of consensus LXXI/HIXXXI/L sequence that resemble the LXXLL sequence.
sequence of co-activators that enable their interaction with nuclear hormone receptors (Hu & Lazar 1999, Makowski et al. 2003). These respective motifs enable co-repressors and co-activators to interact with similar amino acid residues involved in the formation of the TR ligand-binding pocket. Thyroid hormone (TH) binding to TR causes conformational changes in these and other sites that distinguish co-repressor vs co-activator binding to TR (Hu & Lazar 1999, Makowski et al. 2003).

Co-repressors form a complex with other proteins such as Sin3 and HDACs, particularly HDAC3 (Li et al. 2002). This complex causes histone deacetylation in the chromatin surrounding DNA regions near the TRE. These histone modifications lead to changes in the local chromatin structure that favor chromatin compaction resulting in decreased recruitment of RNA pol II and general transcription factors, which consequently leads to repression of basal transcription. Methyl-CpG-binding proteins can associate with a co-repressor complex containing Sin3 and HDACs histone to further increase basal repression in the absence of TH by increasing DNA-methylation (Rietveld et al. 2002). Triiodothyronine (T_3) binding to TR causes conformational changes in the TR that lead to dissociation of CoRs from TRs and recruitment of co-activators (CoAs) to the TRE-bound TR. Thus, T_3 reverses basal repression and stimulates transcription by recruiting different TR-binding cofactors that increase histone acetylation near the TRE.

Studies in transgenic and knock-in models of dominant negative NCoR mutant mice show that reduced NCoR action led to decreased basal repression and enhanced transcriptional activity in vivo (Astapova & Hollenberg 2013). The latter observation suggests that co-repressors also can modulate T_3-mediated transcriptional activity by competing with co-activators for binding to TR despite having significantly lower binding affinity for ligand-bound TR than for unliganded TR. Similarly, mutant mice lacking interaction of HDAC3 and NCoR also exhibited derepression of several TH-activated genes both in euthyroid and hypothyroid states (You et al. 2010). Moreover, HDAC inhibitors such as trichostatin A and suberanilohydroxamic acid (SAHA/Vorinostat) can relieve basal repression and enhance TH-induced transcription pharmacologically (Kim et al. 2014).

Perinatal hypothyroidism and thyroid hormone resistance syndrome (RTH) are usually associated with severe CNS dysfunction in humans and are well recapitulated in rodent models (Koibuchi 2009). However, TH are thought to act principally by binding to their nuclear receptors, it is intriguing why TR knockout animals exhibit a much milder phenotype compared to hypothyroid animals (Gothe et al. 1999). These differences in the phenotypes have been attributed to the unliganded TRs and their bound co-repressors in the pathogenesis of hypothyroidism-induced brain abnormalities (Hashimoto et al. 2001, Morte et al. 2002, Venero et al. 2005, Kapoor et al. 2010, Mittag et al. 2010, Bernal & Morte 2013, Shi 2013). Since HDACs are associated with the co-repressors bound unliganded TRs, we hypothesized that inhibiting HDAC activity would block the negative regulation of target genes by unliganded TRs and may restore normal brain development under hypothyroidism. Using rat model of perinatal hypothyroidism, we here demonstrate that administration of the specific HDAC inhibitor, valproate (VPA) (Liu et al. 2007, Sinn et al. 2007), partially restores the levels of TH target gene, improves dendritic arborization of cerebellar Purkinje neurons and rescues hypothyroidism-induced motor and cognitive defects. Thus, we show HDACs as a vital link to understand the molecular basis of hypothyroidism-induced neurodevelopmental abnormalities in mammals.

### Material and methods

#### Hypothyroid model and VPA administration

Hypothyroidism was induced in pregnant Wistar rats by giving methimazole (0.025% w/v) in drinking water from gestational day 6 and continued thereafter till sacrifice of pups at respective time points for tissue collection. For VPA (Sigma) administration, hypothyroid rat pups were intraperitoneally injected 300 mg/kg VPA dissolved in PBS daily from PD4 until sacrifice with euthyroid rats receiving equivalent amount of PBS. This dose of VPA has been described to inhibit HDAC activity in rodent brain in vivo (Liu et al. 2007, Sinn et al. 2007). For hormone measurement, blood was drawn on postnatal day 12 (PD12). Cerebella (in all groups) from five different litters were harvested at PD0, PD8, PD12 and PD24, snap-frozen in liquid nitrogen and stored at −80 °C, and five cerebella were fixed in 4% paraformaldehyde (4% PFA) until further investigation. Total thyroxine (TT_4) and total T_3 (TT_3) was analyzed in the serum of the rat pups by RIA using DPC kits (DPC, New York, NY, USA). Twelve pups from each of the treatment and euthyroid groups were allowed to grow into neonates for behavioral testing. All animal procedures performed were approved by the institutional animal ethics committee as per the international guidelines for animal care and research.
Protein extraction and western blotting

Cerebellar tissues were washed once with PBS, suspended in 10 volumes of lysis buffer containing dithiothreitol (DTT) and protease inhibitors and kept on ice for 10 min. The tissues were then homogenized using a Teflon homogenizer and centrifuged at 12 000 g for 15 min at 4 °C and the supernatant was collected. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Inc.). The cell lysates (50 μg) were subjected to 12% SDS-PAGE and electro-transferred onto nitrocellulose membrane. The membranes were incubated with primary antibodies for myelin basic protein (Mbp, Millipore), brain-derived neurotrophic factor (Bdnf, Santa Cruz), acetyl-histone H3 (Ac-H3) and acetyl-histone H3 (K9) (Ac-H3, Ac-H3-K9, CST) and subsequently with secondary antibodies. Signals were detected using an enhanced chemiluminescence detection system (Amersham Biosciences). The relative expression of each protein was determined by densitometry analysis using LabWorks 4.0 software (Ultra-Violet Products Ltd, Cambridge, UK).

Tissue HDAC activity

Homogenates (25 μg of total protein) from cerebellum were incubated with Fluor-de-Lys substrate in triplicates for 30 min at 37 °C to initiate the HDAC reaction. Fluor-de-Lys Developer was then added and the mixture was incubated for another 10 min at room temperature. Fluorescence intensity was measured by fluorometer Synergy XT with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. HDAC activity was calculated according to manufacturer’s instructions (Enzo Life Sciences, NY 11735, USA).

RNA extraction and real-time PCR

Total RNA was isolated from the cerebella of euthyroid, hypothyroid and VPA-treated hypothyroid pups at PD12 from five litters following the single-step mRNA isolation method using TRI reagent (MRC, Inc., Cincinnati, OH, USA). Total RNA (2 μg) were reverse-transcribed to cDNA using oligo-(deoxythymidine) 16 primers with the Thermoscript RT-PCR kit (Invitrogen Corp.) following the manufacturer’s instructions. Real-time analysis for specific genes (Mbp, Bdnf, and Pcp2) was carried out as per the manufacturer’s instruction (Applied Biosystems) on the ABI Prism 7500 Sequence Detection System (Applied Biosystems) using SYBR Green based chemistry. The expression was calculated by using the 2−ΔΔCT method as fold change with respect to euthyroid rats and normalized with Gapdh.

Golgi staining

Whole cerebella from all the three groups of rats were taken. The brain slices of 3–5 mm were fixed in 10% formalin. These cerebellar slices were immersed in formalin for 2 weeks. These slices were processed in mordant (5% w/v chloral hydrate, 5% w/v potassium dichromate, 10% v/v formalin in ddH2O) in dark conditions at 35 °C for 4 days in an amber glass jar. The chamber was placed under 22–26 in of Hg vacuum condition. After the 4th day the tissue slices were rinsed in 1% silver nitrate 2–3 times and the kept for 4 days, including change of 1% silver nitrate after 48 h. The brain slices were partially hydrated in 70% alcohol. The mid-sagittal sections of 50 μm thickness were cut using vibrating blade microtome (Leica VT1000S) that were completely dehydrated in 100% alcohol. Then sections were mounted on poly-l-lysine (PLL)-coated slides with dibutyl phthalate xylene (DPX). Images were taken from cerebellar lobule 40× using bright field microscope (Olympus IX73).

Behavioral test

The cerebellum is vulnerable to growth restriction and neuronal depletion induced by hypothyroidism during the brain growth spurt of neonatal rats. All behavioral testing was done between 0900 and 1300 h and the test time of day was balanced across experimental groups. For the behavioral study, the pups of hypothyroid group were treated with VPA from PD4 to PD12 and were kept on normal diet and normal water. The behavioral studies were performed on rat pups at PD40 in each euthyroid, hypothyroid and VPA-treated hypothyroid group.

Rotarod test

We performed a rotarod test to examine motor coordination per the protocol validated earlier. Rats were placed on the rotarod drums (IITC Life Science Series 8, part 755), which had an accelerating motor-driven drums in five lanes and the rotation speed was accelerated from 4 to 40 r.p.m. over 180 s in forward mode with a rest period of about 3 min in between trails. The animals were placed on textured drums to avoid slipping. When an animal dropped onto the individual sensing platforms below, test results were recorded. The drop was detected by accurate magnetic switches and the LED display showed all test results for each animal position, including stopping r.p.m., length of test and distance traveled. Vertically sliding acrylic front panels prevented the escape of the animals. Rats from all three groups at PD40 were trained thrice for 4 consecutive days before the final experiment was performed. Each rat was tested four times during each
time point, but the first trial was excluded from analysis as it served the purpose of acclimatizing the rat to the rotarod. The remaining three trials of each rat for 6 days were averaged and statistically analyzed (n = 12 in each group).

**Grip strength test**  Grip strength test was performed after 4 days of training for all the seven rats of each group at PD40. The rats were trained to hold the wire, which was tied on both the ends. The rat’s forepaws were placed on a wire and the latency to fall was recorded for 6 days. The time of holding the wire was recorded in each group (n = 12 in each group).

**Y-maze test**  Neurobehavioral assessment was done by using the Y-maze test. This maze is used to assess the effect of electric shocks on exploratory behavior measuring animal’s avoidance of an arm in which it has previously received an electric shock. The test is used as a measure of short-term memory. The animal that makes an incorrect decision is punished with shocks. The rats were trained for 30 trials each over a period of 4 days before doing the final experiment. The rats were subjected to the avoidance test by being placed in a compartment with light at an intensity of 8 (scale from 0 to 10 (brightest)) in Y-maze compartments (Techno, India). There was a separation of the light compartment from the dark compartment with an automated guillotine door. After an acclimatization period of 30 s, the guillotine door was opened and closed after entry of the rat into the dark compartment. The subject received a low-intensity foot shock (0.5 mA; 10 s) in the dark compartment and infrared sensors monitored the transfer of the animal from one compartment to another. Change in the position of the rat, while changing the current in the arms was taken as a positive response. When rat did not change its position with the change in current, negative response was marked (n = 12 in each group).

**Statistical analysis**

Statistical analysis was performed using the SPSS package (SAS Institute, Inc., Cary, NC, USA). When indicated, the statistical significance of the differences was assessed using ANOVA. The threshold for statistical significance was set at *P* < 0.05.

**Results**

**VPA treatment prevents hypothyroidism-induced HDAC activity in rat cerebellum increases histone acetylation in vivo**

Thyroidal status was confirmed in different experimental groups by measuring serum total T3 and T4 levels (Fig. 1A and B). Circulating levels of TT3 and TT4 in pups born to dams that were maintained on methimazole treatment showed significantly low values compared to untreated age-matched euthyroid vs hypothyroid counterpart are indicated as *P* < 0.05. (E and F) Representative immunoblot of Ac-H3-K9 and acetyl-histone H3-K9 (Ac-H3-K9) levels in euthyroid, hypothyroid and VPA-treated hypothyroid pups at postnatal day 12 (PD12). Results are expressed as means ± S.E.M. of cerebellar tissues of pups from five different dams in each group. Significant differences compared with euthyroid vs hypothyroid counterpart are indicated as *P* < 0.05, and *P* < 0.05 represents significant difference between hypothyroid vs hypothyroid + VPA.
H3K9 acetylation is one of the most common histone modifications found in actively transcribed promoters. Results showed that in euthyroid pups H3K9 acetylation optima is reached at PD8–PD12 stage. Further, hypothyroidism significantly increased total HDAC activity in cerebellar extracts. Our analysis revealed that hypothyroidism significantly decreased histone acetylation (H3K9) in developing cerebellum during different postnatal growth periods (*P < 0.05) (Fig. 1E and F). Next, by administering VPA we found that decrease in both the total and K9-specific acetylation of histone H3 under hypothyroidism, we analyzed H3K9 acetylation using immunoblotting.

HDAC inhibition partially reverses TH-responsive gene repression under hypothyroidism

To test the hypothesis that HDAC inhibition can prevent the effects of hypothyroidism on the developing brain, we investigated the effect of VPA on TH target genes. Using real-time PCR analysis, we found that Bdnf, which is a neurotrophin known to be down regulated under hypothyroidism (Sinha et al. 2009), was partially restored to its euthyroid levels in VPA treated hypothyroid group (Fig. 2A).

Similar trends were observed in the expression profile of Pcp2 and Mbp mRNA in different experimental groups (Fig. 2B and C). Pcp2 has been suggested to play a role in Purkinje cell differentiation and Mbp gene regulate oligodendrocyte maturation (Eylar et al. 1971, Walton et al. 2012). All these genes harbor TREs and are direct targets of TRs in developing cerebellum. Similar to Bdnf, both Pcp2 and Mbp are downregulated under hypothyroidism but showed partial recovery after VPA treatment. Besides its effect on the mRNA levels, the de-repression on Bdnf and Mbp expression upon VPA treatment was also observed at the protein levels (Fig. 2D, E and F).

Figure 2

(A, B and C) RNA was isolated from cerebellar tissues of rat pups under different treatment groups. Effect of hypothyroidism and VPA treatment on Bdnf, Pcp2 and Mbp transcription. (A, B and C) Real-time PCR analysis of Bdnf (A), Mbp (B) and Pcp2 (C) messenger RNA (mRNA) levels at PD12 cerebellar tissue. Decrease was found to be significant under hypothyroid state compared to euthyroid state (*P < 0.05) and increased on VPA treatment compared to hypothyroid state (*P < 0.05). Real-time PCR analysis only shows partial recovery of transcript expression on VPA treatment. Gapdh mRNA was used as an internal control. (D) Representative immunoblot of Mbp and Bdnf in euthyroid, hypothyroid and VPA treated hypothyroid pups at PD12 respectively. (E and F) Representative immunoblot and densitometry analysis of protein levels of Mbp and Bdnf normalized with β actin. Significant differences compared with age-matched euthyroid vs hypothyroid counterpart are indicated as *P < 0.05, and **P < 0.05 represent significant difference between age matched hypothyroid vs hypothyroid + VPA.
HDAC inhibition by VPA restores neuronal architecture in hypothyroid cerebellum

Stunted dendritic growth of Purkinje neurons is a hallmark of hypothyroid cerebellum (Fauquier et al. 2014). This is due to loss of neurotrophins like BDNF under hypothyroidism (Neveu & Arenas 1996). Since the transcript levels of Bdnf were partially recovered by VPA treatment, the effect of HDAC inhibition on Purkinje cell number and dendritic arborization under hypothyroidism was obvious. Using Golgi stained sections from different experimental groups, we found that VPA partly reversed the stunting of dendritic arborization in Purkinje neurons in hypothyroidism (Fig. 3A and B). Thus, collectively, these results extended the effect of HDAC inhibition in de-repressing gene expression and restoration of neuronal differentiation under hypothyroidism.

VPA partially recovers hypothyroidism-induced motor and cognitive defects

We next checked if normalization of molecular events by HDAC inhibition under hypothyroidism also translated to correction in phenotypic and cognitive performance. To do this we performed a series of neurobehavioral evaluations in euthyroid, hypothyroid and VPA-treated hypothyroid rats. Results obtained after the analysis revealed that hypothyroid animals displayed severe defects in neuromuscular and motor functions attributed to cerebellar derangements as assessed by rotarod and grip strength analysis (Fig. 4A and B). Surprisingly, these defects were significantly corrected on VPA administration to hypothyroid pups during early postnatal period (Fig. 4A and B). Results from neurobehavioral assessment such as the Y-maze test (Fig. 4C) also suggest that the compromised function of other brain regions, including neocortex and hippocampus, may also be corrected by HDAC inhibition.

These results thus collectively support our hypothesis and put forth a direct involvement of HDACs during brain development under hypothyroidism.

Discussion

Nuclear receptors including TRs in the absence of TH represses gene expression through recruiting HDACs via co-repressor complexes (Feng et al. 2001). HDACs and nuclear co-repressors are expressed during brain development and are modulated by TH status (Martinez de Arrieta et al. 2000, Potter et al. 2002, Shen et al. 2005, MacDonald & Roskams 2008, Montgomery et al. 2009). Hypothyroid rat cerebellum is characterized by typical developmental abnormalities like delayed neuronal migration, stunted dendritic arborization and diminished myelination.
Each group. Significant differences compared with age-matched euthyroid in all three groups (euthyroid, hypothyroid and hypothyroid VPA. (B) Histogram representing time of holding the string (grip strength) at PD40. Results are expressed as means ± S.E.M. of values from 12 pups in each group. Significant differences compared with age-matched euthyroid counterpart are indicated as *P<0.05 and †P<0.05 represent significant difference between age matched hypothyroid vs hypothyroid + VPA. (C) Histogram representing average value of positive response in Y-maze test for all three groups (euthyroid, hypothyroid and hypothyroid + VPA-treated) at PD40. Results are expressed as means ± S.E.M. of values from 12 pups in each group. Significant differences compared with age-matched euthyroid vs hypothyroid counterpart are indicated as *P<0.05 and †P<0.05 represent significant difference between age matched hypothyroid vs hypothyroid + VPA.

(Neveu & Arenas 1996). The key reasons for these abnormalities are the reduced expression of some master regulatory genes, including neurotrophins such as Bdnf, and arborization cues like Pcp2 and Mbp that harbor TREs at their promoters. Neurotrophins are signaling proteins that regulate neuronal survival and differentiation during development and play important roles in organizing the cellular networks required for normal brain function (Lindholm et al. 1997). They are implicated in regulating synaptogenesis, axonal and dendritic growth. Bdnf expression is upregulated by TH and the possible functional effects of TH activation of BDNF during brain development have been assessed in vivo. BDNF knockout mice have been created and exhibit delayed migration of granule cells and deficits in Purkinje cell dendritic arborization reminiscent of the hypothyroid cerebellum (Neveu & Arenas 1996). Unliganded TRs under hypothyroidism bind to these TREs and downregulate the expression of these genes by a mechanism involving promoter hyperacetylation (Garcia-Villalba et al. 1997, Lakshmy et al. 1999, Sui & Li 2010). We here asked whether deacetylation of histones associated with aberrant recruitment of co-repressors by TRs under hypothyroidism underlies pathological phenotype during neuronal development. In the present study, we provide direct evidence in vivo to show that perinatal hypothyroidism increases HDAC activity in developing cerebellum and inhibiting the HDAC during development maintains the hyper-acetylated nucleosomal histones under hypothyroidism. Hyperacetylation of histones reactivates TH target genes including neuropeptides such as Bdnf and partially ameliorated the abnormalities associated with developmental hypothyroidism. These findings provide in vivo evidence to illustrate the direct role of HDACs in the pathological manifestation of hypothyroidism during brain development.

We used VPA to inhibit HDACs and observed its effect on the development of rat cerebellum under hypothyroidism. VPA is a well-established inhibitor of Class-I HDAC, causing histone hyperacetylation (Gottlicher et al. 2001, Phiel et al. 2001). Acetylation or deacetylation of histone N-terminal tails alter the interaction between histones and DNA molecules in chromatin (Hsieh & Gage 2005) and this remodeling of chromatin has been identified as a key epigenetic mechanism of gene regulation. VPA is in clinical use as an anticonvulsant and mood-stabilizing drug, primarily in the treatment of epilepsy, bipolar disorder and prevention of migraine headaches. However, VPA has been shown to induce autism-like symptoms in some animal models (Wagner et al. 2006, Kim et al. 2013), the dose and time of VPA intervention used in these studies were different than that used in our study and therefore the differential response of VPA in study models would need further evaluation.

In this study we primarily focused on the regulation of only characterized primary TH targets such as Bdnf (Koibuchi et al. 1999), Pcp2 (Anderson et al. 1997) and Mbp (Farsetti et al. 1992). However, improvement in neuronal maturation and resultant neurobehavioral indices after VPA treatment likely suggest a more complex gene regulation and not limited to the three genes focused in the study. Future studies aiming towards a more global gene expression analysis should help to clarify this issue. Also clarifying the role of HDACs in regulating other
neuronal process in different areas of developing brain including visual and auditory regions would be very informative. Another intriguing question that arises from our study regards the reason for a partial rescue effect of VPA in our system. This may be attributed to either narrow specificity of VPA in targeting only a specific class of HDACs or participation of perhaps other epigenetic mechanism such as DNA methylation and miRNAs involved in unliganded TR action.

Since TH deficiency during pregnancy and birth is still a major health concern globally, our results raise an intriguing possibility as to whether HDAC inhibitors that are widely used in treating other neurobehavioral disorders (Sinn et al. 2007, Fischer et al. 2010, Graff et al. 2012) could be used to treat developmental hypothyroidism? In this regard, it has been recently suggested that HDAC inhibitors may be useful in treating hypothyroidism caused by a TRx1 mutation (Kim et al. 2014). Mutations of the thyroid hormone receptor a gene (THRA) cause hypothyroidism in patients with growth and developmental retardation and skeletal dysplasia (Bochukova et al. 2012). Genetic evidence indicates that the dominant negative activity of TRx1 mutants underlies pathological manifestations and is thought to involve weak dissociation of NCoR-HDAC complex from the mutant TR (Bochukova et al. 2012).
Thus, we here present a model (Fig. 5) in which HDAC inhibition can override the repressive action of unliganded TRs and release the transcriptional inhibition on TH target genes, resulting in restored neuronal development.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
P K carried out the experiment work as part of his PhD thesis work. V M designed the experimental work. M C and P K reestablished the animal model and carried out the additional experiments. R A S and M M G conceived the hypothesis and wrote the manuscript.

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