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Hypothyroidism Induces Interleukin-1-Dependent Autophagy Mechanism as a Key Mediator of Hippocampal Neuronal Apoptosis and Cognitive Decline in Postnatal Rats

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Abstract

Thyroid hormone (TH) is essential for brain development, and hypothyroidism induces cognitive deficits in children and young adults. However, the participating mechanisms remain less explored. Here, we examined the molecular mechanism, hypothesizing the involvement of a deregulated autophagy and apoptosis pathway in hippocampal neurons that regulate cognitive functions. Therefore, we used a rat model of developmental hypothyroidism, generated through methimazole treatment from gestation until young adulthood. We detected that methimazole stimulated the autophagy mechanism, characterized by increased LC3B-II, Beclin-1, ATG7, and ATG5–12 conjugate and decreased p-mTOR/mTOR and p-ULK1/ULK1 autophagy regulators in the hippocampus of developing and young adult rats. This methimazole-induced hippocampal autophagy could be inhibited by thyroxine treatment. Subsequently, probing the upstream mediators of autophagy revealed an increased hippocampal neuroinflammation, marked by upregulated interleukin (IL)-1alpha and beta and activated microglial marker, Iba1, promoting neuronal IL-1 receptor-1 expression. Hence, IL-1R-antagonist (IL-1Ra), which reduced hippocampal neuronal IL-1R1, also inhibited the enhanced autophagy in hypothyroid rats. We then linked these events with hypothyroidism-induced apoptosis and loss of hippocampal neurons, where we observed that like thyroxine, IL-1Ra and autophagy inhibitor, 3-methyladenine, reduced the cleaved caspase-3 and TUNEL-stained apoptotic neurons and enhanced Nissl-stained neuronal count in methimazole-treated rats. We further related these molecular results with cognition through Y-maze and passive avoidance tests, demonstrating an IL-1Ra and 3-methyladenine-mediated improvement in learning-memory performances of the hypothyroid rats. Taken together, our study enlightens the critical role of neuroinflammation-dependent autophagy mechanism in TH-regulated hippocampal functions, disrupted in developmental hypothyroidism.

Keywords Thyroid hormone deficiency · Inflammation · Autophagy · Hippocampus · Memory loss

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Introduction

Thyroid hormones (TH), triiodothyronine (T_3) and thyroxine (T_4), are crucial for development and maturation of the central nervous system, playing a key role in prenatal, postnatal, and adult brain functioning [1, 2]. Conversely, TH deficiency, or hypothyroidism, which is prevalent in severely iodine-deficient population or as an autoimmune response of the body, alters neuronal functions, including cognition [3–6]. The hippocampus is integral to cognitive performances [7], and increased apoptosis and loss of hippocampal neurons and decreased synaptic plasticity appear as key factors for hypothyroidism-induced learning-memory impairments [8, 9]. On the other hand, T4 supplementation improved working memory in terms of cognitive performance scores, particularly during brain development [10, 11]. However, the fundamental mechanisms for hypothyroidism-induced learning-memory deficits and T4-mediated protection at the postnatal stages are less studied.

Autophagy that involves the delivery and degradation or recycling of cytoplasmic material at the lysosomes is important for removing dysfunctional and inessential components from the body. Hence, autophagy has been generally considered to play an intricate balance between cell survival and cell death [12, 13]. At the same time, a dysregulated and uncontrolled autophagy process elicits pathological responses, reported in the brain as well [14, 15]. Particularly, an altered expression of Beclin-1 and microtubule-associated protein 1A/1B-light chain 3 (LC3), which mediate the genesis of autophagosome formation and prompt degradation of autophagic cargo, induces hippocampal neuronal dysfunction. This may also associate with modulated ATG5–12 and ATG7 levels in the hippocampus [16, 17]. Additionally, a deregulated hippocampal autophagy via mammalian target of rapamycin (mTOR) and Unc-51 like autophagy-activating kinase (ULK1) reduced cognitive ability [18].

A distinct link between hippocampal neuronal autophagy and apoptosis-mediated learning and memory decline has been reported, where autophagy protected hippocampal neurons against synaptic impairment and apoptosis [19, 20]. On the contrary, an increased autophagy via reduced p-mTOR/mTOR signaling led to cognitive impairment, involving mitochondrial loss and caspase-3 activation [16]. However, whether TH deficiency affected hippocampal neuronal autophagy and if that correlated with apoptosis and learning-memory loss remain unknown.

The hippocampus of an immature brain is vulnerable to inflammatory insults, with detrimental consequences on cognitive performances. Owing to the significant expression of receptors for inflammatory mediators in the hippocampus, the pro-inflammatory cytokines negatively influence the normal cellular signaling mechanisms, and influence apoptosis as well [21, 22]. With regard to hypothyroidism, there have been reports on the participation of inflammation in hippocampal neurodegeneration and spatial memory impairments at early ages and even after maturation [23–25]. However, to the best of knowledge, a direct link between the pro-inflammatory cytokines, apoptosis, and learning-memory loss, along with autophagy, particularly at the developmental stages of TH deficiency awaits investigation.

By using a methimazole-induced rat model of perinatal hypothyroidism through maternal exposure [26, 27], we determined the hippocampal autophagy mechanism in the developing brain. Continuing the methimazole treatment post weaning, we also evaluated the effects on autophagy in young adult rats. We measured neuroinflammation and apoptosis and correlated them with autophagy pathway and ultimate hypothyroidism-induced learning-memory impairment. We also assessed the recovery mechanism through T4 treatment, and overall, identified a novel TH deficiency–induced pathway that reduces hippocampal neuronal cell survival and cognitive functions during development and young adulthood.

Materials and Method

Chemicals and Reagents

Methimazole, L-thyroxine (T4), paraformaldehyde, CelLytic™ MT cell lysis reagent, sodium orthovanadate, protease inhibitor cocktail, DL-dithiothreitol (DTT), sodium dodecyl sulphate, Trizma® base, acrylamide, bis-acrylamide, ammonium sulphate, N,N,N′,N′-tetramethylethylenediamine, bovine serum albumin, TWEEN® 20, sodium citrate, picric acid, in situ cell death detection kit fluorescein for terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), cresyl violet acetate, (3-aminopropyl) triethoxysilane, and TRI Reagent® were procured from Sigma-Aldrich Chemicals Co. (St. Louis, MO). 3-Methyladenine (3-MA), ethanol, glycerol, methanol, acetone, acetic acid, xylene, isopropanol, chloroform, Immobilon-P PVDF membrane, and Immobilon Western Chemiluminescent HRP Substrate were purchased from MilliporeSigma (Burlington, MA). Maxima SYBR Green/ROX qPCR master mix (2×), and horse serum were bought from Thermo Fischer Scientific (Waltham, MA). Superscript™ III first-strand synthesis kit and nuclease free water were purchased from Invitrogen (Carlsbad, CA). Sucrose and DPX mountant were purchased from SRL Pvt. Ltd. (Mumbai, India). Prestained protein ladder was bought from Genetix Biotech Asia Pvt. Ltd. (New Delhi, India). VECTASHIELD antifade mounting medium with DAPI was bought from Vector Laboratories (Burlingame, CA). Interleukin-1receptor antagonist (1IL-1Ra) was procured from R&D systems (Minneapolis, MN).

Antibodies

Rabbit polyclonal ATG7 (cat no. 2631) and ATG5 (cat no. 2630) and rabbit monoclonal phospho-ULK1 (Ser757) (cat no. 14202), ULK1 (cat no. 8054), and cleaved caspase-3 (cat no. 9664) antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit monoclonal phospho-mTOR (S2448) (cat no. ab109268) and mTOR (cat no. ab134903) and rabbit polyclonal IL-1α (cat no. ab7632) and IL-1β (cat no. ab9722) antibodies were from Abcam (Cambridge, UK). Mouse monoclonal Iba1 (cat no. MABN92) antibody was from MilliporeSigma. Rabbit polyclonal LC3B (cat no. L7543), Beclin-1 (cat no. PRS3613) and SQSTM1/p62 (cat no. P0067), mouse monoclonal β-tubulin III (cat no. T8578), and horseradish peroxidase (HRP)-conjugated secondary anti-rabbit (cat no. A0545) antibodies were from Sigma-Aldrich Chemical Co. Rabbit polyclonal IL-1R1 (cat no. sc-689) and HRP-conjugated mouse monoclonal β-Actin (cat no. sc-47778) antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX). Alexa Fluor®546 goat anti-rabbit IgG (cat no. A-11010), Alexa Fluor®488 goat anti-rabbit IgG (cat no. A-11008), Alexa Fluor®546 goat anti-mouse IgG (cat no. A-11030), and Alexa Fluor®488 goat anti-mouse IgG (cat no. A-11001) were from Invitrogen.

Animal Treatment

All animal experimental procedures were prior approved (Institutional Animal Ethics Committee of CSIR-CDRI (approval no. IAEC/2020/58/RENEW0) and CSIR-IITR (approval no. IITR/IAEC/75/17–53/19)) and conducted as per the guidelines laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. Wistar rat dams were housed under standard conditions of 12 h light/dark at 24 ± 2 °C and 40–60% relative humidity, with ad libitum availability of chow diet and reverse osmosis (R. O.) water. To induce hypothyroidism, methimazole (MZ) (0.025%) in R. O. water [27] was fed to rat dams from gestational day 5 (G05) until weaning, and MZ treatment was continued in the post-weaning rats (mixed gender) until postnatal day (PND) 60. Control rats (CT) received R. O. water. To study the effect of MZ, tissue samples were isolated from the CT and MZ rat sets at PND0, PND08, PND16, PND30, and PND60.

To study the effect of thyroxine supplementation, MZ rats were injected (intraperitoneal route) with T4 (15μg/Kg body weight) or normal saline as the vehicle (V) [26] daily from PND08 to PND16 or PND30 to PND60.

To study whether inflammation and autophagy participated in the hypothyroidism-induced effects, IL-1Ra (5 μl, 350 ng/ml in vehicle, PBS) and autophagy inhibitor 3-MA (5 μl, 300 nM in vehicle, normal saline) respectively were used. They were delivered, once, by the intrahippocampal route, through stereotaxic surgery in MZ rats at PND12 (stereotaxic coordinates: − 2.6 mm from bregma, 2.5 mm (medial-lateral), and 2.5 mm (dorsal-ventral)) [28] or PND50 (stereotaxic coordinates: − 4 mm (anterior-posterior), 2.6 mm (medial-lateral), and 3.5 mm (doral-ventral)) [29] for dissection at PND16 and PND60, respectively, following the protocol described earlier [16, 30]. The corresponding control groups received PBS or normal saline as vehicle (V). Tissue samples were then isolated from the V, MZ, MZ + T4, MZ + IL-1Ra, and MZ + 3-MA rat sets at PND16 and PND60.

Detection of Serum TH Levels

Blood was collected from the heart of anesthetized rats by cardiac puncture, and then centrifuged at 1500*g* for 10 min to isolate serum. Total T3 and T4 levels in the serum were assessed through radio-immuno assay (RIA) method by the Diagnostic Products Corporation kits (DPC, New York, NY) as done before [27].

Western Blotting

Rats were sacrificed and their brain dissected out. The hippocampal tissues were isolated, snap frozen in liquid nitrogen and stored at − 80 °C until analysis. For western blotting, hippocampal tissues were homogenized in lysis buffer containing protease inhibitor cocktail and DTT, and sodium orthovanadate and sodium fluoride were added for phosphorylated proteins. The lysates were centrifuged (4 °C, 20,000*g*, 30 min), and protein content in the supernatant estimated by Bradford assay. Protein samples were prepared and equal amount of protein (30–50 μg) was loaded onto each lane. Samples were then run on SDS-PAGE and transferred onto PVDF membrane as described earlier [31]. Blots were blocked with 5% BSA, probed overnight with LC3B, Beclin-1, ATG7, ATG5–12, p62, p-mTOR, mTOR, p-ULK1, ULK1, IL-1α, IL-1β, and IL-1R1 (1:1000) primary antibodies and HRP-conjugated β-actin antibody (1:5000, 2 h). After washing in TBST, the blots (other than β-actin) were probed with HRP-conjugated secondary antibody (1:5000) for 2 h. The blots were then developed in Amersham Imager 600 system (GE Healthcare; Chicago, IL) using Immobilon Western Chemiluminescent HRP Substrate. The protein bands were quantified by Quantity One software (Bio-Rd laboratories; Hercules, CA).

Real-time PCR

RNA was isolated from the hippocampal tissues of rats using the TRI reagent (TRIzol), and cDNA was synthesized using Superscript™ III First-Strand Synthesis kit as described earlier [32]. Real-time PCR was performed using p62 and GAPDH

primers (Table 1) and SYBR Green dye following 40 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 30 s), and extension (72 °C, 30 s) steps in real-time PCR Instrument (Applied Biosystems; Foster City, CA). Relative mRNA expression was calculated using $RQ = 2^{-\Delta\Delta CT}$ Method.

Cryosectioning

Towards sample isolation for cryosectioning, rats were anesthetized and trans-cardially perfused with 0.2% picric acid in 4% paraformaldehyde, as described earlier [33]. The whole brain was isolated, cryoprotected in 30% sucrose solution and coronal sections were prepared using a cryomicrotome (Leica Biosystems, Wetzlar, Germany) and taken onto silane-coated slides.

Immunofluorescence

The 10-μm cryosections of the brain from age-matched rats were fixed in acetone and antigen retrieved in citrate buffer, as described earlier [34]. Sections were then blocked in horse serum and probed overnight with IL-1α, IL-1β, IL-1R1, Iba1, β-tubulin III, LC3B, Beclin-1, or cleaved caspase-3 (1:300) primary antibodies, followed by Alexa Fluor secondary antibodies (1:600) for 2 h. The sections were then mounted in mounting medium containing DAPI, and the dentate gyrus region of the hippocampus that plays a key role in memory formation [35] was photographed using a fluorescent microscope (Nikon Instruments Inc.; Minato, Tokyo, Japan). Images from different slides were captured with the same software and constant settings, and processed using the ImageJ software (Wayne Rasband, NIH).

TUNEL Assay

TUNEL assay was performed to detect apoptosis, following the protocol described earlier [33]. The 10-μm cryosections of the brain were fixed in acetone, antigen retrieved in citrate buffer and probed with TdT and fluorescein-labeled dUTP at 37 °C for 2–4 h, while using DNAse I-incubated sections (data not shown) as positive control [36]. Sections were then blocked with horse serum, probed overnight with β-tubulin III antibody (1:300), followed by secondary antibody, and mounted with antifade mounting media containing DAPI.

Table 1 Primer sequences for qPCR

Primer	Sequence
p62	F 5′-CCTTTGGCCACCTCTCTG-3′
	R 5′-AGGACGTGGGCTCCAGTT-3′
GAPDH	F 5′-TGGGAAGCTGGTCATCAAC-3′
	R 5′-GCATCACCCCATTTGATGTT-3′

Photomicrographs of the dentate gyrus region of hippocampus were captured under × 10 objective in fluorescent microscope (Nikon Instruments Inc.; Minato, Tokyo, Japan), and images were processed using the ImageJ software (Wayne Rasband, NIH). The % apoptotic index was calculated as number of TUNEL positive nuclei per100 nuclei in β-tubulin III expressing neurons.

Nissl Staining

The 30-μm cryosections of the brain were taken and Nissl staining performed, following the protocol described earlier [37]. Sections were kept in chloroform-ethanol (1:1) mixture for 2–3 h and then in 100% ethanol for 2–3 min. Sections were then stained in cresyl violet (0.1%) solution for 10–15 min, rinsed in water and serially dehydrated in 50, 75, 95, and 100% ethanol. The sections were cleared in xylene, mounted in DPX mounting media and photomicrographs of the dentate gyrus region of hippocampus were captured under × 10 objectives using a Leica DMi1 microscope (Leica Microsystems). The images were exported to the ImageJ software and the number of neurons counted manually from five different random fields per section using Cell counter plugin.

Y-Maze and Passive Avoidance Tests

A Y-maze test was performed to check the learning-memory performance of rats, as described earlier [38]. The Y-maze apparatus consists of three arms, two of which were dark and had foot shock (1–5 mA) (unsafe arms), while the third one had a light cue and was without foot shock (safe arm). On day 1, during learning trials (thirty trials), rats were trained to identify the safe arm, and then memory was checked 24 h, 48 h, and 7 days post learning trials. The apparatus was cleaned between trials. Movement of rats to the unsafe arm was counted as error, and % memory was calculated as $(E_{training} - E_{test}) \times 100/E_{training}$.

A separate set of rats was made to undergo passive avoidance test for learning-memory, following an earlier protocol with slight modification [38]. Rats were placed in a gated light-dark chamber, where they were subjected to acquisition in the light chamber (120 s) followed by a foot shock (0.5 mA, 10 s) in the dark chamber. Shock-free retention trials (R1, R2, and R3) after 24 h, 48 h, and 72 h post acquisition were performed and transfer latency time (TLT) was recorded as the time needed by rats to move from light to dark chamber. The chambers were cleaned between trials. Higher TLT indicated better learning-memory ability.

Statistics

Statistical analysis was performed using GraphPad Prism version 6.01 GraphPad Software (La Jolla, CA). Unpaired

Student's *t* test was performed for analyzing two groups. For analyzing more than two groups, one-way ANOVA was performed, followed by the Tukey's post hoc multiple-comparisons test. For analyzing more than two groups and more than one parameter, two-way ANOVA followed by the Tukey's post hoc multiple-comparisons test was carried out.

Results

Effect of MZ Treatment on Hippocampal LC3B and p-mTOR/mTOR in Postnatal Rats

We treated rats with MZ from G05 to PND60 and assessed serum T3 and T4 levels at PND0, PND08, PND16, PND30 and PND60. We detected an MZ-mediated reduction in serum T3 and T4 in the postnatal rats, at almost all the ages (Table 2). We then investigated the effect of MZ on hippocampal autophagy, and detected an increased LC3B-II level (Fig. 1a). We also observed an MZ-mediated decrease in autophagy regulator, p-mTOR/mTOR (Fig. 1b). Our data here indicate a TH deficiency–induced hippocampal autophagy in the postnatal rats.

Effect of TH Deficiency and T4-Supplementation on Hippocampal Autophagy in Postnatal Rats

To further validate autophagy, we examined the effect of MZ on other important autophagy proteins (besides LC3B), viz. Beclin-1, ATG7, and ATG5–12, and then checked whether T4 treatment had any impact on the hippocampal autophagy. We found that MZ caused an increase in the hippocampal Beclin-1, ATG7, and ATG5–12 levels (Fig. 2a), and T4 treatment not only induced recovery in serum T3 and T4 levels (Table 3) but also reduced the MZ-induced autophagy markers, viz. LC3B-II, Beclin-1, ATG7, and ATG5–12 at both PND16 and PND60, signifying developmental and young adult stages respectively (Fig. 2a). Corroborating the changes in autophagy regulation (as seen in Fig. 1b, showing decreased p-mTOR/mTOR), we observed a reduction in hippocampal p-ULK1/ULK1 in MZ-treated rats and T4-mediated recovery in p-mTOR/mTOR and p-ULK1/ULK1 (Fig. 2b). We next examined the effect of MZ and MZ + T4 on autophagic flux by determining p62 that undergoes degradation in the autophagosomes [39]. We detected an MZ-mediated decrease in hippocampal p62 protein level and its T4-mediated recovery (Fig. 2c). However, we observed an unchanged hippocampal p62 mRNA level following MZ treatment (Fig. 2d), suggesting autophagic degradation rather than a reduced p62 expression for causing P62 protein loss. Thus, our results suggest a hypothyroidism-induced hippocampal autophagy and autophagic flux, and their T4-mediated protection at the postnatal stages.

Table 2 Serum T3 and T4 levels

	CT		MZ	
	T3 (nmol/l)	T4 (nmol/l)	T3 (nmol/l)	T4 (nmol/l)
PND0	1.02 ± 0.09	23.99 ± 1.21	0.52 ± 0.13	06.32 ± 0.58**
PND08	1.20 ± 0.11	31.14 ± 6.62	0.71 ± 0.09	08.84 ± 3.27***
PND16	1.41 ± 0.12	46.92 ± 7.06	0.85 ± 0.09*	06.43 ± 0.92***
PND30	1.56 ± 0.19	55.46 ± 3.49	0.92 ± 0.16**	07.34 ± 1.69***
PND60	2.03 ± 0.26	66.60 ± 2.22	0.90 ± 0.02***	06.82 ± 0.48***

*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ compared with CT of respective age

Effect of TH Deficiency and T4-Supplementation on Hippocampal Neuroinflammation and Its Link with Autophagy in Postnatal Hypothyroid Rats

We then explored the mechanism promoting hippocampal autophagy in the hypothyroid postnatal rats.

It has been reported that the inflammatory cytokine, IL-1, participates in hypothyroidism-induced hippocampal dysfunction, studied in adult rats [23–25]. Consistent with the observation, we detected an MZ-induced hippocampal IL-1α (Fig. 3a) and IL-1β (Fig. 3b) in the PND16 and PND60 rats. We also detected a T4-mediated reduction in the MZ-induced IL-1α (Fig. 3a) and IL-1β (Fig. 3b). Activated microglia mediate neuroinflammatory responses [40, 41], and hence, we examined whether MZ affected the microglial activation marker, Iba1. MZ appeared to enhance the hippocampal Iba1 and also caused an increased co-immunolocalization of IL-1α (Fig. 3c) and IL-1β (Fig. 3d) with Iba1, which could be reduced by T4 (Fig. 3c, d). IL-R1 transmits IL-1-induced neuroinflammatory signals [42, 43], and we examined the effect of MZ on hippocampal IL-1R1. We found that MZ upregulated IL-1R1 levels in the hippocampus (Fig. 4a), and also appeared to enhance the co-immunolabeling of IL-1R1 with neuronal marker, β-tubulin III (Fig. 4b), which could be reduced by T4 (Fig. 4a, b). Thus, our results appear to indicate hypothyroidism-induced hippocampal neuroinflammation and T4-mediated protection at the postnatal stages.

We then investigated whether this increased IL-1 participated in autophagy mechanism, for which we carried out intrahippocampal infusion of IL-1Ra in the MZ-treated rats. We found that IL-1Ra, which reduced IL-1R1 **(Suppl. 1)**, downregulated hippocampal LC3B-II, Beclin-1, ATG7, and ATG5–12 (Fig. 5a) and increased the p62 (Fig. 5a), p-mTOR/mTOR, and p-ULK1/ULK1 levels (Fig. 5b) in MZ-treated rats, indicating an IL-1-dependent hippocampal autophagy in the postnatal hypothyroid rats.

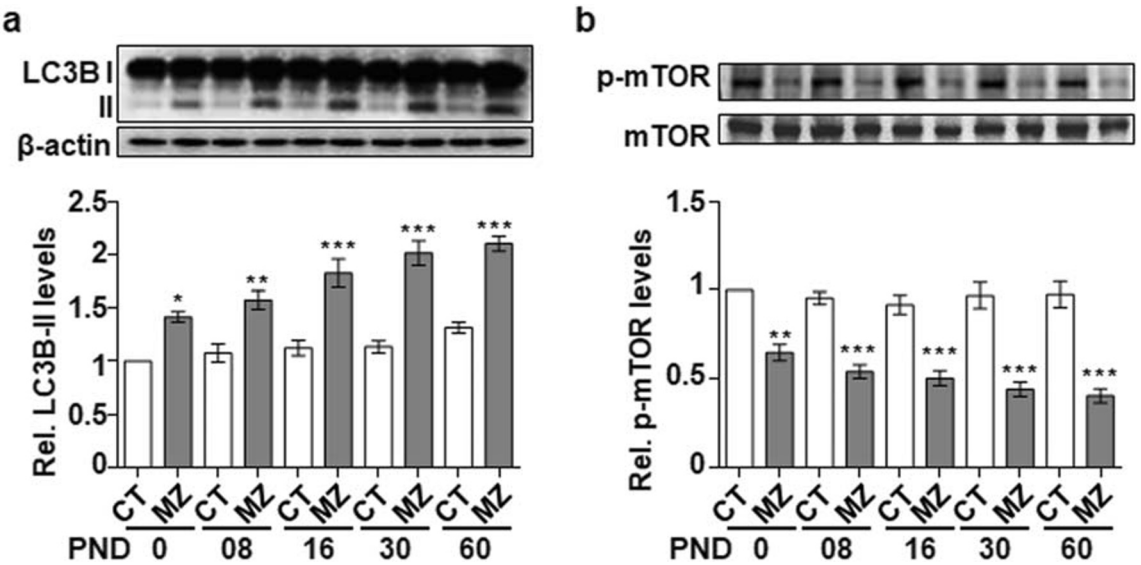

Fig. 1 Hypothyroidism induces hippocampal autophagy in postnatal rats. Hippocampal tissues of PND0, PND08, PND16, PND30, and PND60 rats from control (CT) and methimazole (MZ) sets were isolated. **a**, **b** Representative western blots and densitometry relative to CT-PND0 of LC3B-II (16 kDa) normalized with β-actin (42 kDa) (**a**) and p-mTOR (289 kDa) normalized with mTOR (289 kDa) (**b**). $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared with CT of respective age. Data represent means ± SE from three rats of three different litters

We further correlated the increased neuroinflammation with autophagy in the hippocampal neurons, where MZ appeared to enhance the co-immunolabeling of LC3B (Fig. 5c) and Beclin-1 (Fig. 5d) with β-tubulin III, which could be reduced by T4, as well as IL-1Ra (Fig. 5c, d). Thus, our results appear to indicate an IL-1-dependent autophagy in the hippocampal neurons during TH deficiency and T4- or IL-1Ra-mediated protection at postnatal stages.

Effect of T4, IL-1Ra, and 3-MA on Hippocampal Neuronal Apoptosis and Neuronal Density in Postnatal Hypothyroid Rats

Hypothyroidism induces hippocampal neuronal damage [9, 44–46], and using IL-1Ra and the autophagy inhibitor, 3-MA, we examined whether the inflammation-dependent autophagy pathway affected the hippocampal neurons of hypothyroid rats. We first verified the effect of 3-MA on hippocampal neurons of MZ-treated rats, showing a reduced LC3B expression **(Suppl. 2)**. We then examined the effect on apoptosis, where MZ appeared to enhance the c-caspase-3 co-immunolabeling with β-tubulin III, which could be reduced by T4, IL-1Ra, and 3-MA **(**Fig. 6a**)**. Corroborating these findings, we observed an MZ-mediated increased apoptotic TUNEL staining and % apoptotic index in the hippocampal neurons, and their T4, IL-1Ra, and 3-MA-mediated reduction (Fig. 6b).

Through Nissl staining, we further detected an MZ-mediated decrease in hippocampal neuronal count (%), which (like T4) could be inhibited by IL-1Ra and 3-MA (Fig. 7). Thus, our results indicate a neuroinflammation- and autophagy-dependent increased apoptosis and loss of hippocampal neurons in TH-deficient postnatal rats.

Table 3 Serum T3 and T4 levels

	MZ + T4	
	T3 (nmol/l)	T4 (nmol/l)
PND16	$1.40 \pm 0.16^{*}$	$42.22 \pm 7.42^{***}$
PND60	$1.53 \pm 0.32^{**}$	$57.41 \pm 4.64^{***}$

$^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared with MZ of respective age

Effect of T4 Supplementation, IL-1Ra, and 3-MA on Learning-Memory Functions in Postnatal Hypothyroid Rats

Hypothyroidism triggers impairment in learning-memory functions that are regulated by hippocampal neurons [9]. Hence, through Y-maze and passive avoidance tests, we assessed whether the MZ-induced hippocampal neuroinflammation and autophagy participated in the learning-memory dysfunctions. We found that like T4, IL-1Ra and 3-MA reduced the error (%) and enhanced the saving memory (%) in Y-maze test (Fig. 8a), and caused significant recovery of the transfer latency time in passive avoidance test (Fig. 8b) in the MZ-treated rats. Thus, our data indicate an increased hippocampal inflammation and autophagy-dependent learning-memory impairment in TH-deficient postnatal stages.

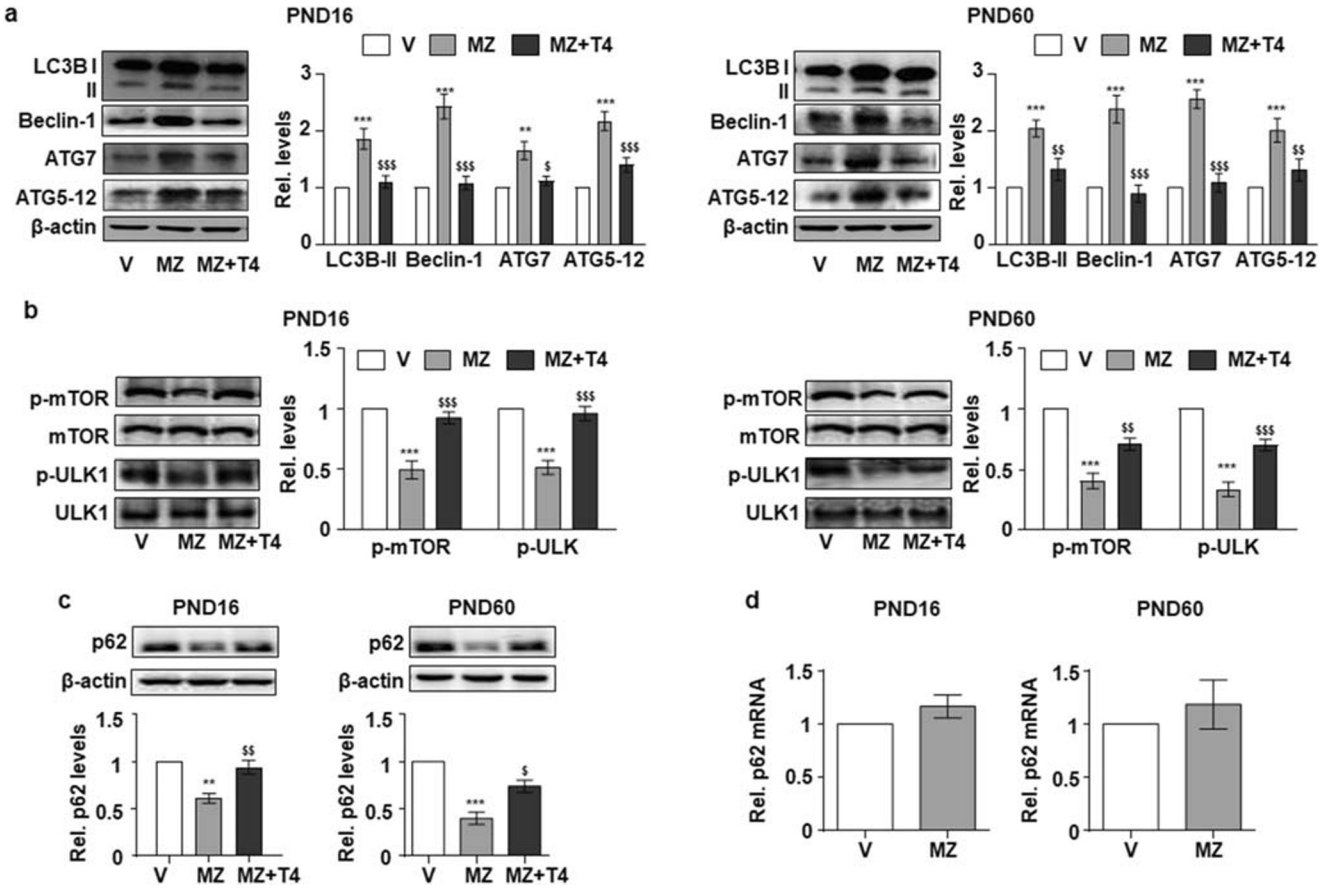

Fig. 2 Hypothyroidism-induced hippocampal autophagy is reduced by T4. Hippocampal tissues of PND16 and PND60 rats from vehicle control (V), MZ or MZ+T4 sets were isolated. **a**–**c** Representative western blots and densitometry relative to V of LC3B-II, Beclin-1 (60 kDa), ATG7 (78 kDa), and ATG5–12 (55 kDa) normalized with β-actin (**a**), p-mTOR normalized with mTOR and p-ULK1 (150 kDa) normalized with ULK1 (150 kDa) (**b**), and p62 (62 kDa) normalized with β-actin (**c**) for PND16 (left panel) and PND60 (Right Panel) sets. **d** mRNA levels relative to V of p62 normalized with GAPDH. $^{***}p < 0.001$ and $^{**}p < 0.01$ compared with V and $^{\$\$\$}p < 0.001$, $^{\$\$}p < 0.01$, and $^{\$}p < 0.05$ compared with MZ. Data represent means ± SE from three rats of three different litters

Discussion

The present study reveals a novel mechanism of hypothyroidism-induced cognitive decline during brain development and maturation. We distinctively identified that TH deficiency stimulated hippocampal neuronal autophagy via an inflammation-dependent mechanism, which ultimately emerged as the key reason for neuronal apoptosis and learning-memory loss. TH replenishment or a reduction of the hypothyroidism-induced hippocampal neuroinflammation and autophagy suppressed hippocampal neuronal damage and restored cognitive functions (Fig. 9).

Thyroid hormones have a vital role in influencing neurological and cognitive functions [47, 48], and the current study for the first time reveals the need for a regulated autophagy mechanism in TH-mediated neuroprotection. The findings appeared relevant for both early stages and young adulthood. Our study specifically indicated that TH deficiency stimulates the autophagy process, marked by upregulated Beclin-1 and LC3B-II that are central proteins in the autophagy pathway. Delving into the regulatory components demonstrated an inhibited mTOR1 and ULK1 as important nodes through which the TH deficiency-induced stress responses transmit to the autophagy machinery. Our findings showing increased ATG5–12 conjugate and ATG7 levels (suggestive of upregulated ubiquitin-proteasome system and the extension of phagophoric membrane in autophagic vesicles [17, 49]) corroborated that TH deficiency truly deregulates autophagy via increased cell degradation and recycling within the hippocampus. Additionally, hypothyroidism-mediated reduction in protein and not mRNA levels of the cargo adaptor protein, P62, hints at its own degradation in the process of autophagic flux. It also suggests the delivery of autophagy substrates to autophagosomes for degradation. In the concept of TH-dependent functions, a link between subclinical hypothyroidism and abnormal skeletal development has been reported. The changes associated with a reduced expression of Beclin-1 and LC3-II, upregulated mTOR and decreased autophagosome and p62 accumulation in the chondrocytes [50]. Likewise, in the hypothyroid state, muscle nerve

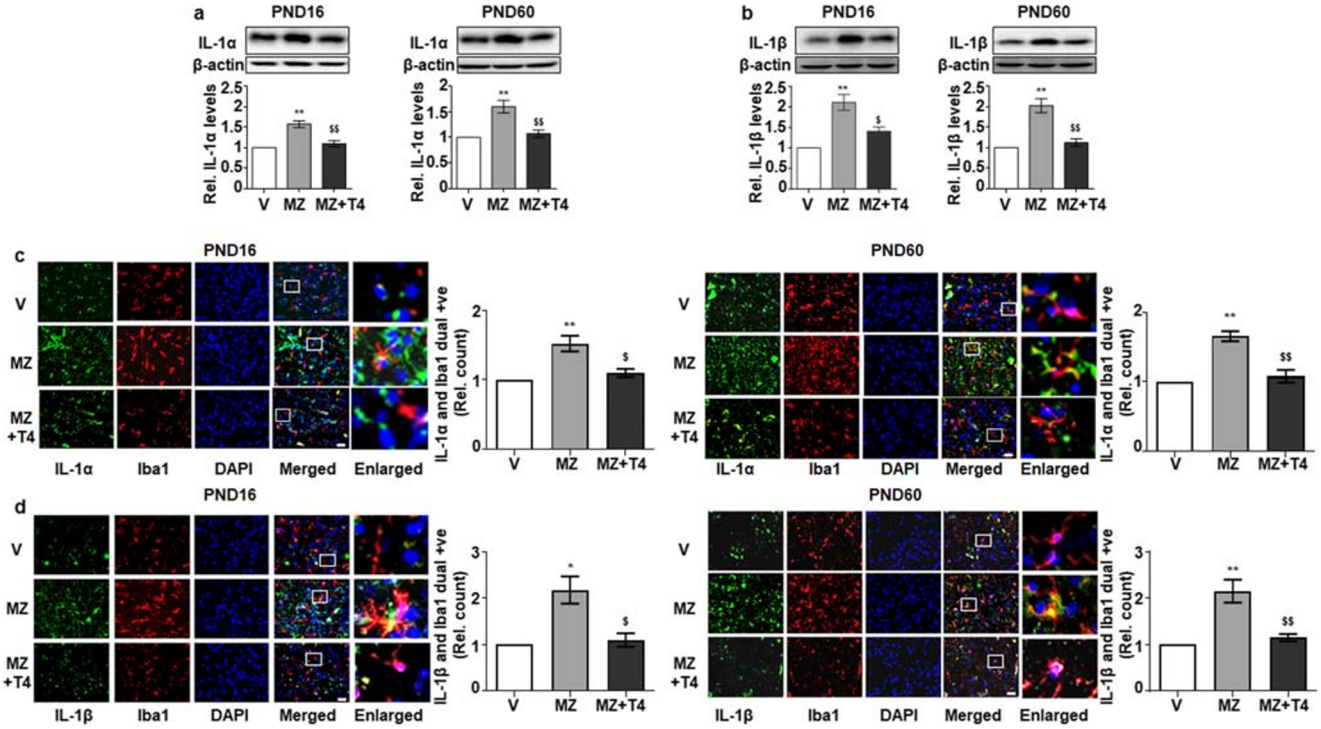

Fig. 3 Hypothyroidism increases hippocampal IL-1α, IL-1β and microglial activation. Hippocampal tissues of PND16 and PND60 rats from V, MZ, and MZ + T4 sets were isolated. **a**, **b** Representative western blots and densitometry relative to V of IL-1α (17 kDa) (**a**) and IL-1β (17 kDa) (**b**) normalized with β-actin. $^{**}p < 0.01$ compared with V and $^{\$\$}p < 0.01$ and $^{\$}p < 0.05$ compared with MZ. Data represent means ± SE from three rats of three different litters. Immunofluorescence was performed on brain sections from PND16 and PND60 rats of V, MZ, and MZ + T4 sets. **c**, **d** Representative fluorescence photomicrographs (× 40) of the dentate gyrus region of hippocampus in sections co-immunostained for IL-1α (**c**) or IL-1β (**d**) with Iba1 and co-stained with DAPI. Scale bar: 20 μm. Enlarged: inset area of the merged image. Bar diagrams represent the dual IL-1α (**c**) or IL-1β (d) and Iba1 + ve counts in the merged photomicrographs, relative to V. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with V and $^{\$\$}p < 0.01$ and $^{\$}p < 0.05$ compared with MZ. Data represent means ± SE from three rats of three different litters

stimulation modulated the autophagy process, conferring TH as a critical predictor of exercise-related muscle conditions [51]. Within the brain, although the involvement of TH-targeted genes in autophagy-related cellular events has been shown for neurological and psychiatric disorders [52], to the best of knowledge, the current study appeared first in identifying the effect of TH deficiency on autophagy in the brain, or more specifically hippocampal neurons. Notably, although we detected that T4 replenishment reduced hypothyroidism-induced hippocampal autophagy, we found that T4 treatment in control rats promoted LC3B-II and beclin-1 levels and decreased mTOR phosphorylation in the hippocampus **(Suppl. 3)**. The latter corresponds with earlier findings demonstrating a hyperthyroidism-induced autophagy in the skeletal muscle [53] and liver [54]. Nonetheless, the differential impact of T4 on the hippocampal autophagy process in control compared with hypothyroidism warrants further investigation, which may be carried out as a separate study. Irrespective of thyroid hormones, there also have been contradictory observations on the role of autophagy in hippocampal functions. On the one hand, hippocampal injections of genetic and pharmacological modulators of autophagy showed that autophagy induction promoted short-term synaptic functions and plasticity, and reduced the age-dependent memory deficits [55]. Contrarily, hippocampal injury in transient hypoxia, long-term ischemia [56, 57], chronic cerebral hypoperfusion [58], and early stages of aging [18] involved an increased autophagy. Matching the current findings, our recent study also demonstrated an augmented autophagy-dependent cognitive impairment in estrogen-deficient females [16], and another report claimed impaired insulin signaling as an inducer of hippocampal autophagy and spatial working memory deficits [59]. Thus, the above studies point to the non-specific or rather situation and condition-dependent pattern in which autophagy participates in hippocampal functions, of which few also strongly express the vital link between hippocampal neuronal autophagy and hormone physiology [16, 59].

In continuation with the concept of endocrine-related effects, studies from a group showed both altered apoptosis and autophagy in relation to neuronal damage [60, 61]. Few studies demonstrated an apoptosis-independent autophagic flux or a switch between apoptotic and autophagic cell death within the hippocampus in situations like withdrawal of insulin hormones, etc. [62–65]. However, supporting our earlier study, demonstrating estrogen deficiency–induced autophagy and subsequent neuronal apoptosis [16], the current study

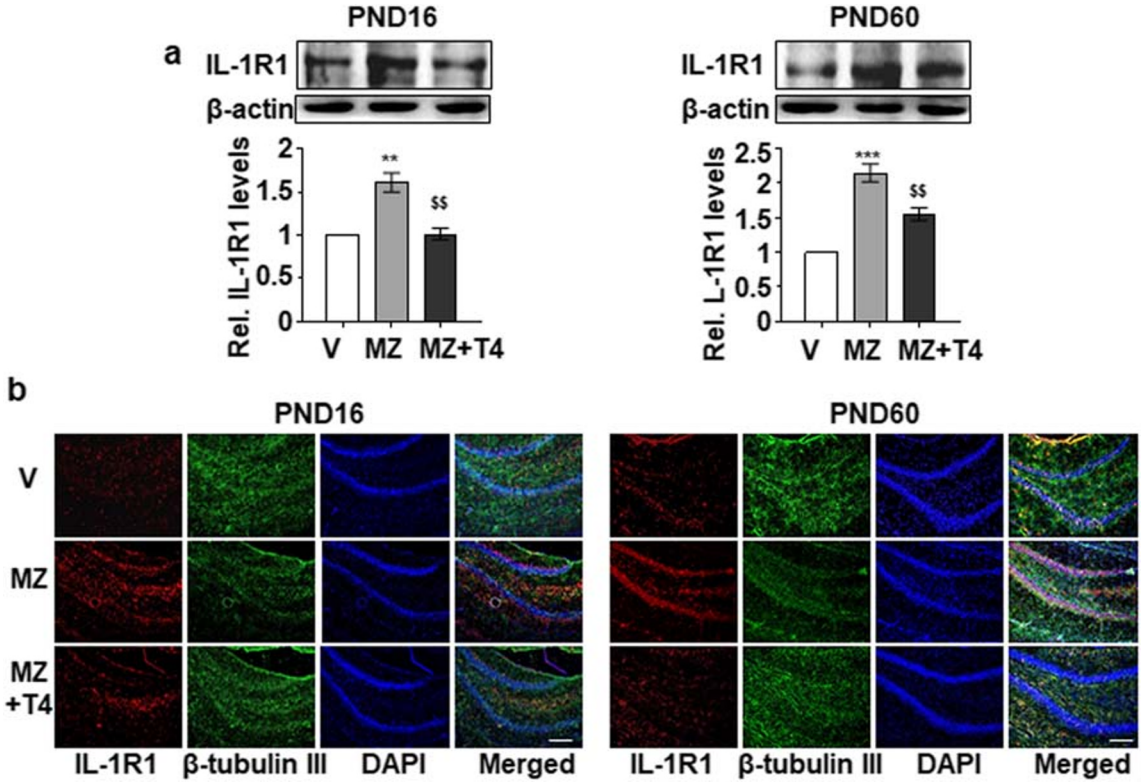

Fig. 4 Hypothyroidism increases hippocampal neuronal IL-1R1 levels. Hippocampal tissues of PND16 and PND60 rats from V, MZ and MZ + T4 sets were isolated. **a** Representative western blots and densitometry relative to V of IL-1R1 (80 kDa) normalized with β-actin. $^{***}p < 0.001$ and $^{**}p < 0.01$ compared with V and $^{\$\$}p < 0.01$ compared with MZ. Data represent means ± SE from three rats of three different litters. Immunofluorescence was performed on brain sections from PND16 and PND60 rats of V, MZ, and MZ + T4 sets. **b** Representative fluorescence photomicrographs (× 10) of the dentate gyrus region of hippocampus in sections immuno-stained for IL-1R1 and β-tubulin III, and co-stained with DAPI. Scale bar: 200 μm. Photomicrographs represent brain sections from three rats of three different litters

delineated a strong causal relationship between autophagy and TH deficiency–induced apoptotic hippocampal neuronal cell death. More specifically, it showed enhanced autophagy induction as a requisite for caspase-3-dependent apoptosis, which then critically governed cognitive dysfunction. Moreover, our data showing a decrease in surviving hippocampal neurons using autophagy inhibitor, 3MA, also sustained the concept of an interplay between autophagy and apoptotic pathways in the pathogenesis of neurodegeneration, or the need for an intermediate apoptosis in autophagy-induced neuronal cell death [66, 67].

Corroborating findings from earlier reports [23–25], we spotted a hypothyroidism-induced hippocampal interleukin-1 activation and microglial activation, which could be reduced by TH supplementation. Subsequently, delving deeper into the hypothyroidism-induced mechanism revealed neuroinflammation as the pivotal regulator of the autophagy mechanism and its downstream hippocampal neuronal damage. Thyroid hormones have been shown to suppress hepatocellular inflammation and ultimate carcinogenesis involving selective autophagy process [68], while another study, without linking the inflammation and autophagy mechanisms, demonstrated the protective role of T3 in combination with docosahexaenoic acid against inflammasome activation and via enhanced hepatic bioenergetics and lipid homeostasis in ischemia-reperfusion [69]. Thus, an inverse relation between inflammation and autophagy had been generally shown in terms of thyroid hormone functioning, and opposed to that, the current study appears first in linking the thyroid hormone levels with both hippocampal inflammation and autophagy by showing a positive association between the two mechanisms in hypothyroidism or TH-mediated hippocampal recovery. Putting aside thyroid hormone functioning and considering hippocampal inflammation and autophagy as such, studies showed that mutations within the lysosomal-associated membrane protein type-2 (LAMP2) gene, which participates in autophagy and lysosomal degradation of accumulated proteins in neurodegeneration [70], led to inflammation alteration and memory loss [71]. Similarly, drugs activating the autophagy process inhibited the PI3K/AKT/mTOR/ULK1 signaling pathway with a concomitant clearance of hippocampal amyloid beta (Aβ) and reduction in inflammation [72]. Moreover, inhibition of the inflammatory response in the transgenic Alzheimer's disease (AD) mice models not only reinstated a normal LC3-II and p62 level but also inhibited Aβ accumulation [73]. On the other hand, a decreased cell death and

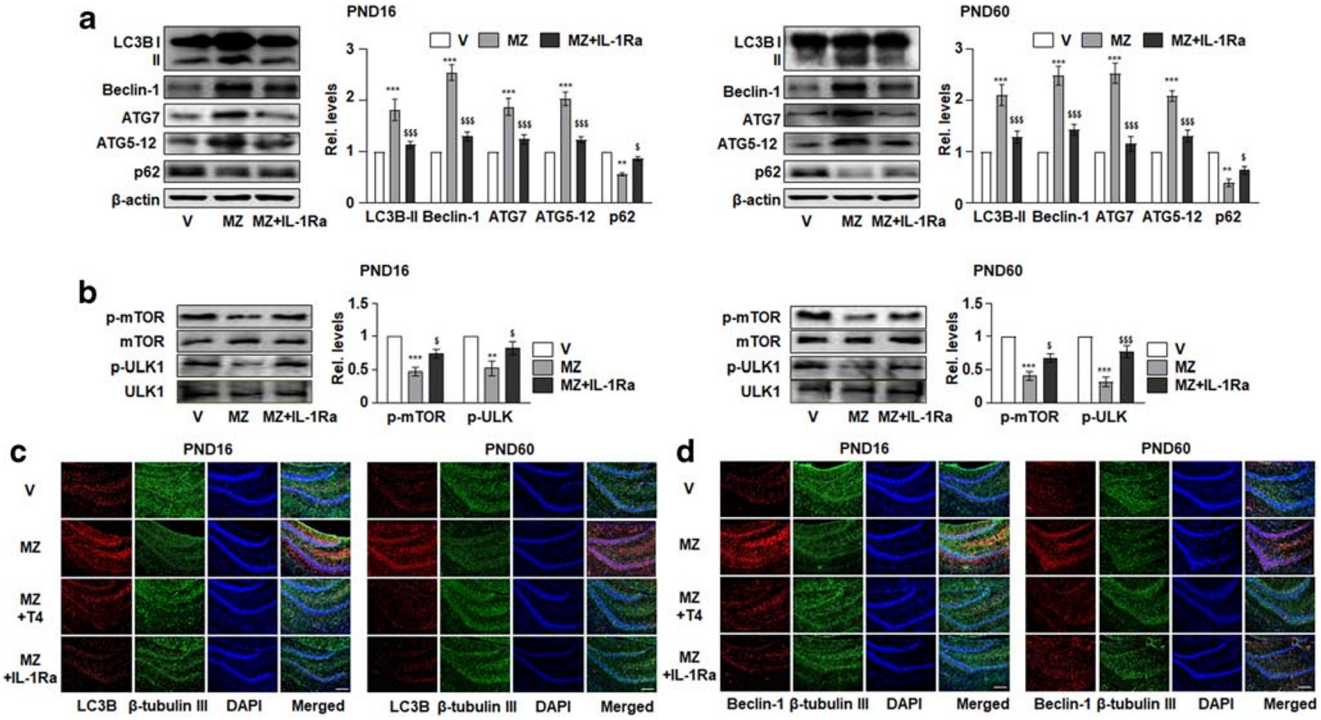

Fig. 5 IL-1Ra reduces hypothyroidism-induced hippocampal autophagy. Hippocampal tissues of PND16 and PND60 rats from V, MZ and MZ + IL-1Ra sets were isolated. **a**, **b** Representative western blots and densitometry relative to V of LC3B-II, Beclin-1, ATG7, ATG5–12, and p62 normalized with β-actin (**a**), and p-mTOR and p-ULK1 normalized with mTOR and ULK1 respectively (**b**). $^{***}p < 0.001$ and $^{**}p < 0.01$ compared with V and $^{\$\$\$}p < 0.001$ and $^{\$}p < 0.05$ compared with MZ. Data represent means ± SE from three rats of three different litters. Immunofluorescence was performed on brain sections from PND16 and PND60 rats of V, MZ, MZ + T4, and MZ + IL-1Ra sets. **c**, **d** Representative fluorescence photomicrographs (× 10) of the dentate gyrus region of hippocampus in sections co-immuno-stained for LC3B (**c**) or Beclin-1 (**d**) with β-tubulin III and co-stained with DAPI. Scale bar: 200 μm. Photomicrographs represent brain sections from three rats of three different litters

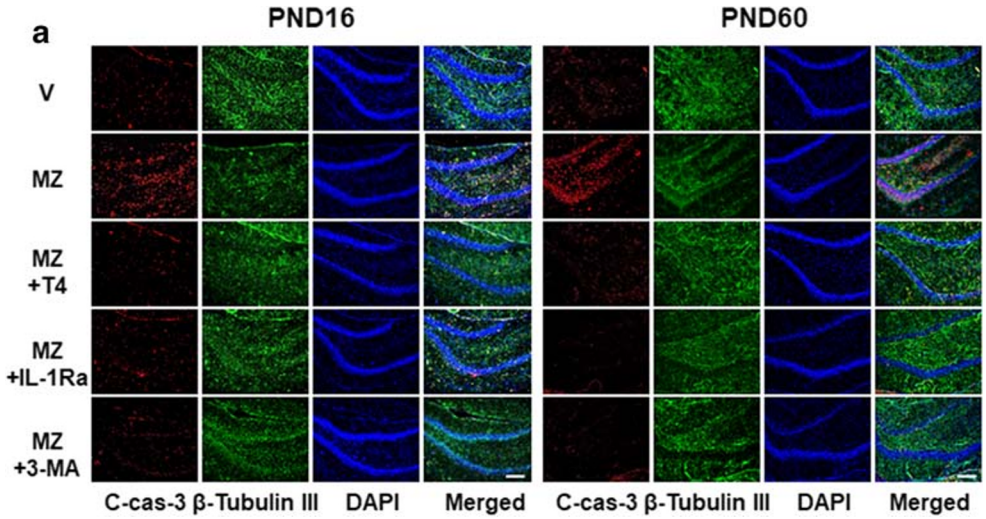

Fig. 6 Hypothyroidism-induced hippocampal neuronal apoptosis is reduced by T4, IL-1Ra, and 3-MA. Immunofluorescence and TUNEL staining were performed on sections from PND16 and PND60 rat brain of V, MZ, MZ + T4, MZ + IL-1Ra, and MZ + 3-MA sets. **a** Representative fluorescence photomicrographs (× 10) of the dentate gyrus region of hippocampus in sections co-immuno-stained for cleaved caspase-3 with β-tubulin III and co-stained with DAPI. Scale bar: 200 μm. Photomicrographs represent brain sections from three rats of three different litters. **b** Representative fluorescence photomicrographs (× 10) of the dentate gyrus region of hippocampus in sections stained for TUNEL and β-tubulin III antibody, and co-stained with DAPI. Scale bar: 200 μm. Enlarged: inset area of the merged image. Bar diagram represents Apoptotic index. $^{***}p < 0.001$ compared with V and $^{\$\$}p < 0.01$ and $^{\$}p < 0.05$ compared with MZ. Data represent means ± SE from three rats of three different litters

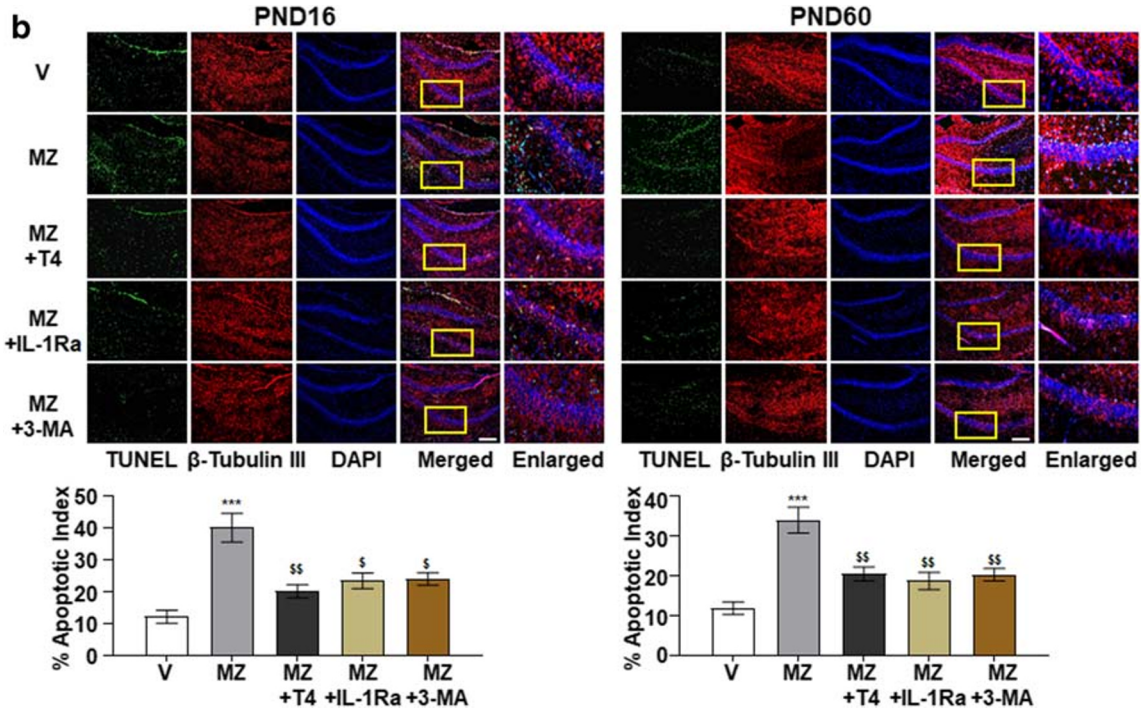

Fig. 6 (continued)

reduced cerebral expression of pro-inflammatory cytokines or autophagy markers in the ATG7 KO mice, as well as upon rosiglitazone or chloroquine treatments and toll-like receptor 4 knockdown following ischemia/reperfusion, traumatic brain injury or radiotherapy-induced neural stem cell and progenitor cell death closely matched our observations in hypothyroidism [74–79]. The expression of inflammatory factors at par with the increased Beclin-1, caspase-3 and LC3II/I in focal

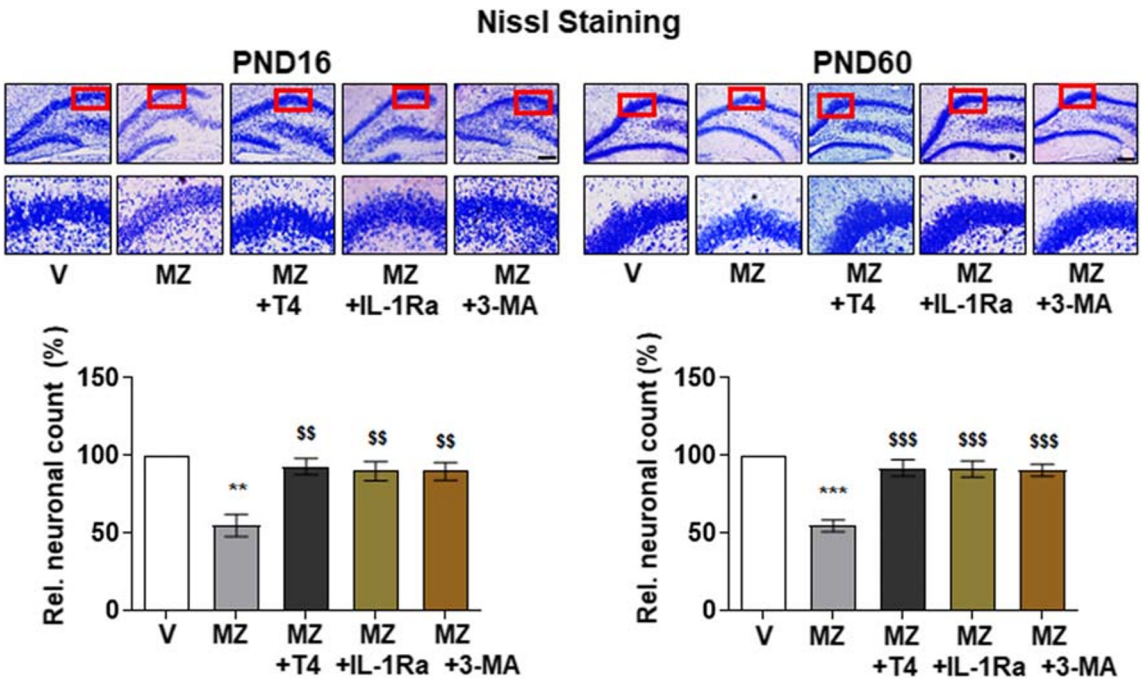

Fig. 7 Hypothyroidism-induced hippocampal neuronal loss is reduced by T4, IL-1Ra, and 3-MA. Nissl staining was performed on brain sections from PND16 and PND60 rats of V, MZ, MZ + T4, MZ + IL-1Ra, and MZ + 3-MA sets. Figure shows representative photomicrograph of Nissl-stained dentate gyrus region of hippocampus (× 10). Enlarged: inset area. Bar diagrams represent Neuronal count (%) relative to V. Scale bar: 200 μm. $^{***}p < 0.001$ and $^{**}p < 0.01$ compared with V and $^{\$\$\$}p < 0.001$ and $^{\$\$}p < 0.01$ compared with MZ. Data represent means ± SE from three rats of three different litters

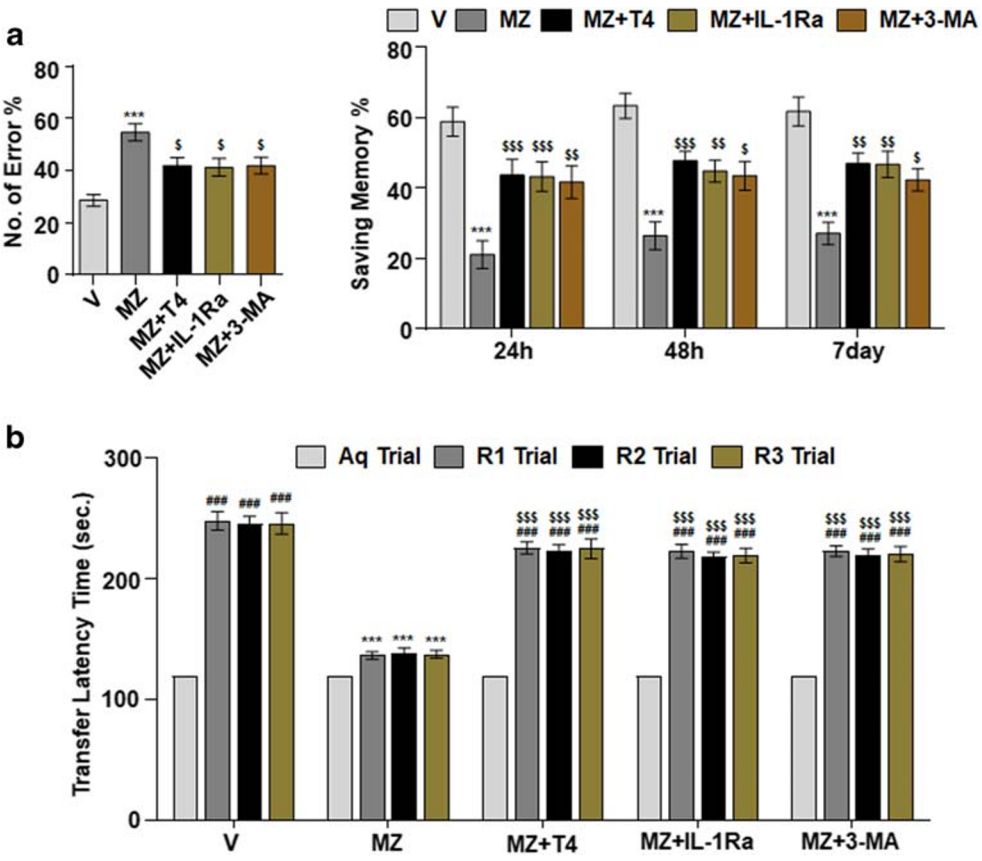

Fig. 8 Hypothyroidism-induced learning-memory impairment is reduced by T4, IL-1Ra and 3-MA. Y-maze and passive avoidance tests were performed on PND60 rats of V, MZ, MZ + T4, MZ + IL-1Ra, and MZ + 3-MA sets. **a** Y-maze data representing the number of errors (%) during learning and the memory retained (% saving memory) at 24 h, 48 h, and 7 days post learning trial. $^{***}p < 0.001$ compared with V and $^{\$\$\$}p < 0.001$, $^{\$\$}p < 0.01$, and $^{\$}p < 0.05$ compared with MZ. **b** Passive avoidance data representing transfer latency time in the first (R1), second (R2), and third (R3) retention trials (24 h, 48 h, and 72 h post acquisition (Aq) trial, respectively). $^{***}p < 0.001$ compared with V and $^{\$\$\$}p < 0.001$ compared with MZ for a particular trial, and $^{\#\#\#}p < 0.001$ compared with acquisition trial. Data represent means ± SE from ten rats of ten different litters

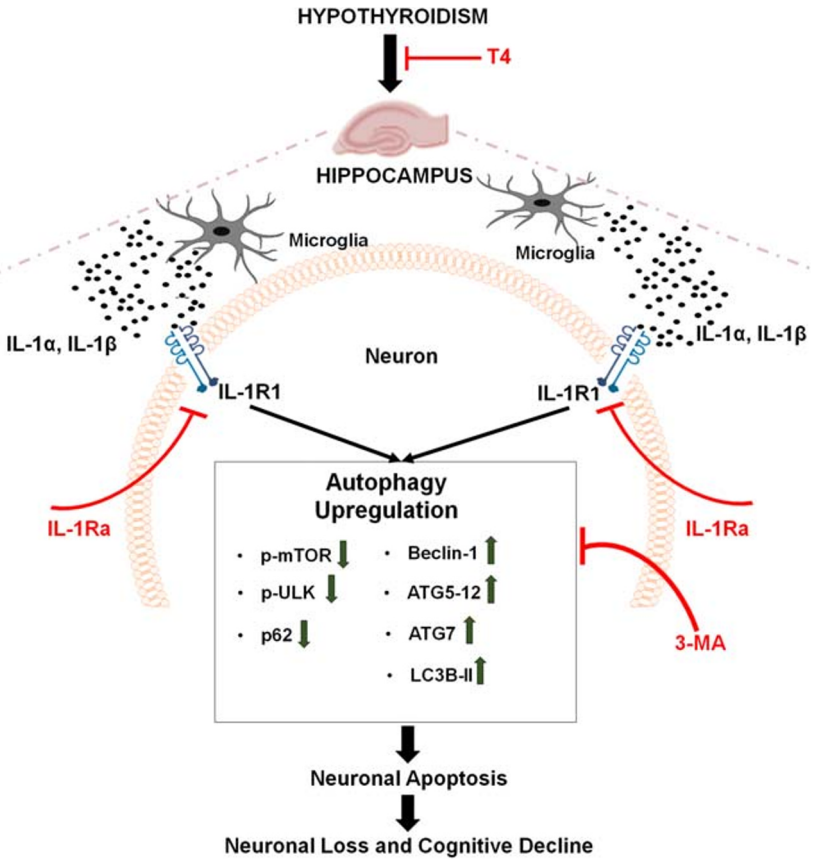

Fig. 9 Proposed schematic depicting hypothyroidism-induced hippocampal neuroinflammation and autophagy leading to increased neuronal apoptosis, neuronal loss, and learning-memory dysfunction. Hypothyroidism induces microglial activation, which generates IL-1α and IL-1β and induces neuronal IL-1R1 expression in the hippocampus. This in turn stimulates autophagy and subsequent apoptosis and loss in hippocampal neurons, ultimately resulting in cognitive decline. Inhibition of IL-1 and autophagy pathway, like T4 treatment, reduced the hypothyroidism-induced hippocampal neuronal damage. (The schematic diagram has been created with BioRender.com)

cerebral ischemia-reperfusion injury [80] also showed a direct link between inflammation and autophagy. Thus, it may be postulated that hippocampal autophagy and inflammation bear an inverse relationship in some situations of neurodegeneration, where the former is essential for clearance of protein oligomers. On the other hand, autophagy and hippocampal inflammation bear a direct relationship in situations, such as hypothyroidism (as observed by us here), etc., culminating in hippocampal neuronal death and damage. Increased hippocampal autophagy downstream of neuroinflammation in the hypothyroid conditions could also be indicative of a defense mechanism for impending neurodegeneration. This may be more relevant for situations like hypothyroidism-induced AD-like pathology [25, 81], marked by hippocampal neurodegeneration. This draws support from earlier studies that indicate an activated autophagy or inhibited mTOR-mediated alleviation in neuronal cell death and memory impairment [82, 83]. Nonetheless, the condition or situation-dependent relationship between hippocampal autophagy, inflammation, and apoptosis, and how thyroid hormones interact in the recovery against neurodegeneration await investigation as an extension to this study.

In conclusion, the present study showed that thyroid hormones play a critical contributory role in sustaining a normal hippocampal autophagy mechanism, which undergoes disruption via interleukin activation, culminating in hippocampal neuronal loss and learning-memory impairment. Moreover, our study proposes microglia and autophagy as important partners in hypothyroidism-induced hippocampal neuronal damage. Accordingly, as suggested earlier [84], it is possible that the aberrant microglial activation has functional consequences in microglial phagocytosis, leading to abnormal engulfment of hippocampal neurons, together with autophagy-induced flux dysregulation and the subsequent apoptotic neuronal cell death. Thus, the crossroads of inflammation, autophagy, apoptosis, and neuronal survival are critical points, which may serve as appropriate targets for reducing cognitive dysfunction in juveniles and adults hypothyroids. Exploring the hypothyroidism-induced dysregulation of glia-neuron communication involving microglial phagocytosis and neuronal autophagy may be a future direction of research to understand the pathophysiological mechanisms associated with hypothyroidism-induced decline in cognitive function.

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Compliance with Ethical Standards

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

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