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Effect of Combined Levothyroxine (L-T₄) and 3-Iodothyronamine (T₁AM) Supplementation on Memory and Adult Hippocampal Neurogenesis in a Mouse Model of Hypothyroidism

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Citation: Rutigliano, G.; Bertolini, A.; Grittani, N.; Frascarelli, S.; Carnicelli, V.; Ippolito, C.; Moscato, S.; Mattii, L.; Kusmic, C.; Saba, A.; et al. Effect of Combined Levothyroxine (L-T₄) and 3-Iodothyronamine (T₁AM) Supplementation on Memory and Adult Hippocampal Neurogenesis in a Mouse Model of Hypothyroidism. *Int. J. Mol. Sci.* **2023**, *24*, 13845. <https://doi.org/10.3390/ijms241813845>

Academic Editor: Aleksey Zaitsev

Received: 25 July 2023

Revised: 30 August 2023

Accepted: 6 September 2023

Published: 8 September 2023



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Abstract: Mood alterations, anxiety, and cognitive impairments associated with adult-onset hypothyroidism often persist despite replacement treatment. In rodent models of hypothyroidism, replacement does not bring 3-iodothyronamine (T₁AM) brain levels back to normal. T₁AM is a thyroid hormone derivative with cognitive effects. Using a pharmacological hypothyroid mouse model, we investigated whether augmenting levothyroxine (L-T₄) with T₁AM improves behavioural correlates of depression, anxiety, and memory and has an effect on hippocampal neurogenesis. Hypothyroid mice showed impaired performance in the novel object recognition test as compared to euthyroid mice (discrimination index (DI): 0.02 ± 0.09 vs. 0.29 ± 0.06; $t = 2.515$, $p = 0.02$). L-T₄ and L-T₄+T₁AM rescued memory (DI: 0.27 ± 0.08 and 0.34 ± 0.08, respectively), while T₁AM had no effect (DI: −0.01 ± 0.10). Hypothyroidism reduced the number of neuroprogenitors in hippocampal neurogenic niches by 20%. L-T₄ rescued the number of neuroprogenitors (mean diff = 106.9 ± 21.40, $t = 4.99$, $p_{\text{corr}} = 0.003$), while L-T₄+T₁AM produced a 30.61% rebound relative to euthyroid state (mean diff = 141.6 ± 31.91, $t = 4.44$, $p_{\text{corr}} = 0.004$). We performed qPCR analysis of 88 genes involved in neurotrophic signalling pathways and found an effect of treatment on the expression of *Ngf*, *Kdr*, *Kit*, *L1cam*, *Ntf3*, *Mapk3*, and *Neurog2*. Our data confirm that L-T₄ is necessary and sufficient for recovering memory and hippocampal neurogenesis deficits associated with hypothyroidism, while we found no evidence to support the role of non-canonical TH signalling.

Keywords: methimazole; novel object recognition; thyroperoxidase; mass spectrometry; doublecortin; neuroendocrinology

1. Introduction

Thyroid hormones (TH) play an essential role in brain development during gestational and perinatal periods and contribute to maintaining brain health in adulthood [1]. Adult-onset hypothyroidism is associated with depression, anxiety [2], and memory deficits [3,4] both in humans and in animal models [5–9]. Poor memory performance is accompanied by altered synaptic plasticity in hippocampal circuits [10–12] and impaired neurogenesis in the neurogenic niche of the sub-granular zone (SGZ) of the dentate gyrus [13–16]. Adult neurogenesis is highly sensitive to the hormonal, neurotransmitter, and growth

factor milieu within the neurogenic niche, including TH signalling [7,12,17]. Levothyroxine (L-T₄) monotherapy is the treatment of choice for hypothyroidism [18]. However, about 10–15% of patients complain of persisting symptoms of TH deficiency, such as cognitive disturbances, fatigue, mood swings, and anxiety [19], despite thyroid-stimulating hormone (TSH) levels being within the normal range. Serum TSH may not reflect intracellular TH levels [19,20]. Additionally, generation of triiodothyronine (T₃) from thyroxine (T₄) by deiodinases may not be equivalent to thyroidal secretion of T₃ [21]. Finally, we hypothesise that persistent symptom of hypothyroidism may be due to decreased brain levels of downstream TH derivatives, in particular, 3-iodothyronamine (T₁AM). In fact, in mice treated with methimazole—a thyroperoxidase inhibitor—and L-T₄-replacement, brain T₁AM levels remained undetectable despite an almost full recovery of free T₄ and T₃ levels (fT₄ and fT₃) [22]. T₁AM is an endogenous trace amine involved in the regulation of metabolic homeostasis and central nervous system (CNS) function [23]. T₁AM showed pro-cognitive and anti-amnesic effects [24], and neuroprotective actions in several models, including seizure-related excitotoxic damage, altered autophagy, amyloidosis, and ischemia-reperfusion injury [25–28]. It is unknown if T₁AM participates in the regulation of adult hippocampal neurogenesis. Here, we investigated whether augmenting L-T₄ treatment with T₁AM improves neurocognitive and neurobiological alterations associated with adult-onset hypothyroidism and has an effect on hippocampal neurogenesis. In a rodent pharmacological model of hypothyroidism, we compared performance in memory, locomotion, and anxiety- and depression-related tasks in response to different replacement strategies combining L-T₄ and T₁AM. We evaluated the effect of these replacement strategies on neurogenetic capacity in the SGZ using histological markers and the expression of genes involved in neurogenetic pathways.

2. Results

2.1. Generation of Rodent Model of Hypothyroidism

The experimental protocol is depicted in Figure 1A. Control euthyroid mice ($n = 17$) had an initial average body weight (BW) of 21.38 ± 0.45 g at day 0, which increased over the 3 weeks to 25.88 ± 0.46 g. BW changed less in hypothyroid mice ($n = 15$), from 22.48 ± 0.18 g to 23.89 ± 0.18 g. Two-way ANOVA analysis found a statistically significant interaction between treatment and time on BW ($F(3, 279) = 35.21, p < 0.0001$). Hypothyroid mice treated with L-T₄ ($n = 18$, final BW: 26.46 ± 0.42 g) and L-T₄+T₁AM ($n = 17$, final BW: 26.79 ± 0.45 g) partially recovered BW, while BW of T₁AM-treated ($n = 15$, final BW: 25.58 ± 0.45 g) and untreated hypothyroid mice ($n = 15$, final BW: 26.0 ± 0.49 g) remained significantly lower than that of euthyroid mice ($n = 17$, final BW: 27.79 ± 0.46 g). The two-way ANOVA showed a significant effect of replacement treatment ($F(4, 542) = 11.66, p < 0.0001$), time ($F(7, 542) = 54.37, p < 0.0001$), and their interaction ($F(28, 542) = 1.63, p = 0.022$) on BW (Figure 1B).

2.2. Serum Levels of TT₄ but Not TT₃ Were Different between the Experimental Groups

Hypothyroidism almost completely suppressed total T₄ levels (TT₄) as compared to euthyroid mice (2.53 ± 0.12 ng/mL and 32.77 ± 1.48 ng/mL, $p < 0.01$). Serum TT₄ levels were restored by L-T₄ and L-T₄+T₁AM (38.92 ± 10.49 ng/mL and 38.88 ± 6.13 ng/mL) but not by T₁AM (3.40 ± 1.01 ng/mL) (Figure 1C). We observed no significant differences in total T₃ levels (TT₃) between treatments (Figure 1D).

2.3. Behavioural Correlates of Hypothyroidism and Replacement Treatments

Data relative to behavioural measures are reported in Table 1. In the elevated plus maze (EPM), hypothyroid mice showed a significantly lower number of stretch-attend postures (SAP), indicating reduced risk-assessment behaviours compared to euthyroid mice ($t = 3.03, p_{\text{corr}} = 0.006$, surviving Bonferroni correction for multiple comparison). None of the other EPM measures were significantly different between hypothyroid and euthyroid mice. One-way ANOVA did not detect any significant differences in primary and

secondary anxiety-related behaviours or decision-making and risk-assessment measures between treatments (Figure S1B–F).

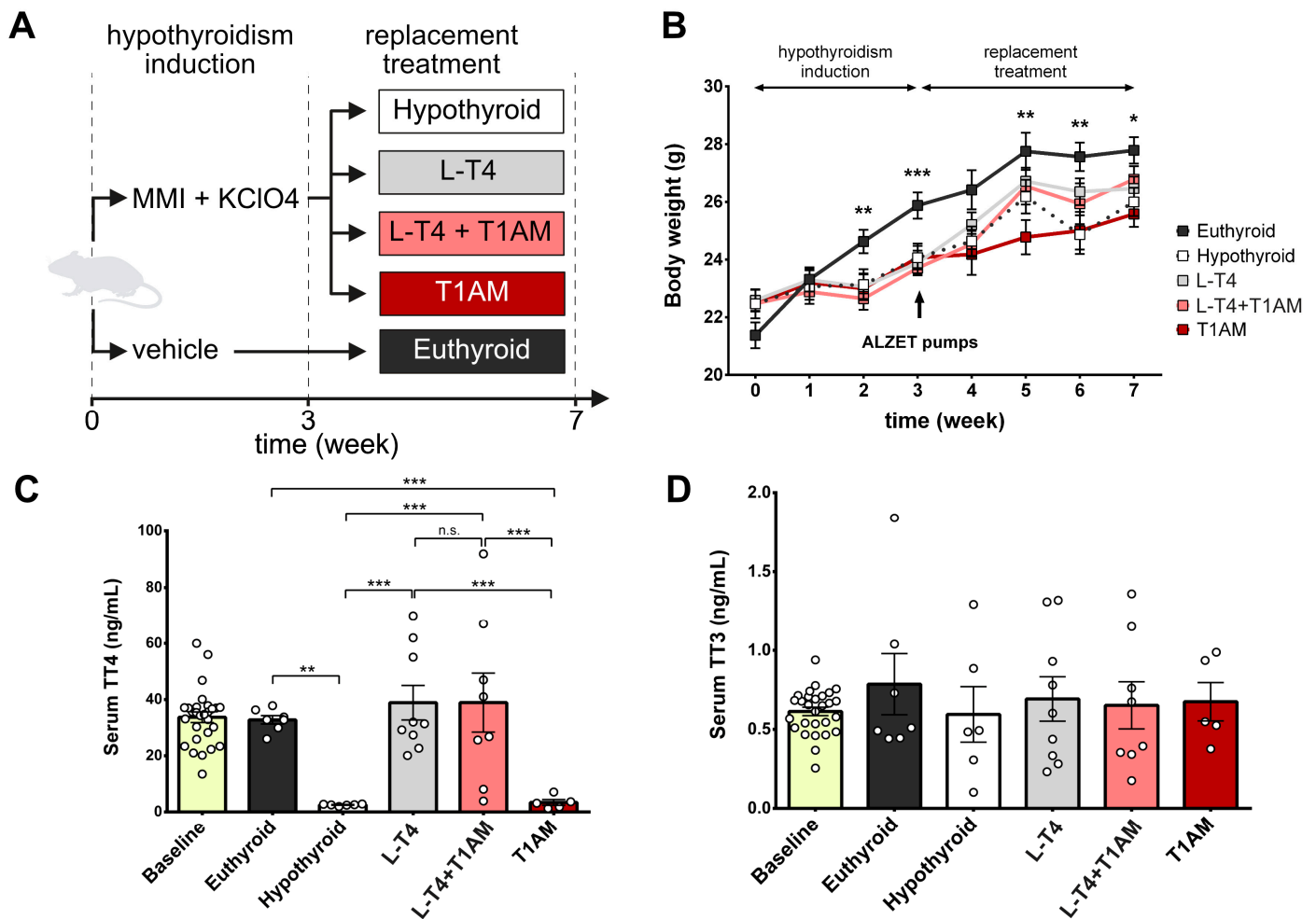


Figure 1. Murine model of pharmacological hypothyroidism. (A) Experimental protocol. Animals were divided into 5 groups: euthyroid ($n = 17$), hypothyroid ($n = 15$), hypothyroid treated with L-T₄ ($n = 18$), hypothyroid treated with L-T₄+T₁AM ($n = 17$), and hypothyroid treated with T₁AM ($n = 15$). MMI, methimazole; KClO₄, potassium perchlorate. (B) Body weight (BW) changes throughout the induction of hypothyroidism and replacement treatment. BW of hypothyroid mice started to diverge from euthyroid mice at week 2. Hypothyroid mice treated with L-T₄ and L-T₄+T₁AM partially recovered BW, while T₁AM-treated and untreated hypothyroid mice remained significantly lighter than euthyroid mice. Two-way ANOVA testing the effect of treatment, time and their interaction and Tukey’s post-hoc comparison were used to test differences between the groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (C) Serum TT₄ levels were almost completely suppressed in the hypothyroid group and restored by L-T₄ and L-T₄+T₁AM. (D) We observed no significant changes in serum TT₃ levels between the groups. ANOVA and Tukey’s multiple comparison were used to test differences between the groups (** $p < 0.01$, *** $p < 0.001$, n.s. not significant). Results are plotted as mean \pm SEM using GraphPad Prism 6 software. Baseline $n = 28$, hypothyroid $n = 6$, euthyroid $n = 7$, L-T₄ $n = 9$, L-T₄+T₁AM $n = 8$, T₁AM $n = 6$. The remaining samples were used to analyse serum fT₄ and fT₃ on an AIA 2000LA immunoassay platform.

Table 1. Measures of anxiety-related behaviours, spontaneous locomotor activity, memory, and depression-related behaviours in the mouse model of hypothyroidism and replacement treatments.

Experiment	Hypothyroid	Euthyroid	L-T ₄	L-T ₄ +T ₁ AM	T ₁ AM	F (DFn, DFd), <i>p</i>
EPM:						
% of time spent in open arms	7.51 ± 0.99	9.81 ± 2.85	11.83 ± 2.65	9.34 ± 1.67	7.06 ± 1.77	0.79 (4, 57), 0.79
<i>n</i> of entries into open arms	3.17 ± 0.49	Mdn = 3 IQR = 2–4	4.08 ± 0.73	2.92 ± 0.43	2.33 ± 0.51	3.69, 0.45 ^a
<i>n</i> of entries into closed arms	7.00 ± 0.73	9.67 ± 0.64	8.83 ± 0.76	7.69 ± 0.87	8.58 ± 0.72	1.87 (4, 56), 0.13
<i>n</i> of total head dips	11.67 ± 2.55	Mdn = 15 IQR = 10–20	13.25 ± 2.23	11.23 ± 2.09	12.25 ± 2.10	2.39, 0.66 ^a
<i>n</i> of unprotected head dips	4.30 ± 0.89	5.38 ± 2.40	5.50 ± 1.39	4.10 ± 1.00	3.50 ± 0.98	0.29 (4, 48), 0.89
<i>n</i> of protected head dips	9.70 ± 1.71	11.23 ± 1.21	10.40 ± 1.17	10.50 ± 1.21	11.2 ± 0.87	0.25 (4, 48), 0.25
<i>n</i> of end arm explorations	1.10 ± 0.35	1.31 ± 0.41	1.10 ± 0.50	0.45 ± 0.21	0.90 ± 0.35	0.77 (4, 49), 0.55
<i>n</i> of stretch attend postures	2.60 ± 0.65	6.69 ± 1.07	6.40 ± 1.51	6.50 ± 1.81	4.60 ± 0.87	1.96 (4, 48), 0.12
OF:						
total distance travelled (m)	789.1 ± 79.61	996.8 ± 65.35	846.7 ± 90.59	943.6 ± 84.08	Mdn = 901.9 IQR = 694.8–1025	3.77, 0.44 ^a
time spent in open vs. closed arms (s)	Mdn = 0.81 IQR = 0.70–0.86	0.79 ± 0.02	0.85 ± 0.02	0.79 ± 0.04	0.83 ± 0.04	2.97, 0.56 ^a
ORT:						
discrimination index	0.02 ± 0.09	0.29 ± 0.06	0.27 ± 0.08	0.34 ± 0.08	−0.01 ± 0.10	3.15 (3, 42), 0.03
TST:						
% of immobility time	61.86 ± 1.73	59.03 ± 2.52	62.13 ± 2.83	62.1 ± 2.88	60.72 ± 1.84	0.32 (4, 43), 0.86

^a Differences between groups were assessed with the non-parametric Kruskal–Wallis test.

We did not find any significant differences in spontaneous locomotor activity and thigmotaxis in the open field test (OF) or percentage immobility time in the tail suspension test (TST) between treatments (Figure 2 and Figure S2B). Hypothyroid mice showed significantly lower discrimination index (DI) in the ORT (DI: hypothyroid: 0.02 ± 0.09; euthyroid: 0.29 ± 0.06; $t = 2.515$, $p = 0.02$, Figure 3). One-way ANOVA revealed a significant effect between treatments ($F(3, 42) = 3.15$, $p = 0.03$). Subsequent post-hoc tests only detected a significant difference between hypothyroid and L-T₄+T₁AM-treated mice (mean diff. = −0.31, $p < 0.05$). The visual inspection of the DI does, however, suggest an almost complete recovery of memory in L-T₄-treated relative to hypothyroid mice, while T₁AM had no effect (DI: −0.01 ± 0.10). DIs diverged significantly from a hypothetical value of 0 (no discrimination) in euthyroid ($t = 4.82$, $p < 0.001$), L-T₄-treated ($t = 3.53$, $p < 0.01$), and L-T₄+T₁AM-treated ($t = 4.04$, $p < 0.01$) mice, confirming the ability to discriminate the novel object and therefore intact memory in these groups (Figure 3).

2.4. L-T₄ and L-T₄+T₁AM Restored the Number of DCX+ Cells

Hypothyroidism induced a 20% reduction in the number of doublecortin-positive (DCX+) cells (mean diff = −53.50 ± 23.81, $t = 2.25$, $p_{\text{uncorr}} = 0.07$). One-way ANOVA revealed a trend toward a significant effect of treatment ($F(4, 15) = 2.79$, $p = 0.06$). We performed pair-wise comparisons between the five groups using a *t*-test, using Bonferroni correction for multiple comparisons ($0.05/10 = 0.005$). Compared to hypothyroidism, L-T₄ increased the number of cells by 45.58% (mean diff = 106.9 ± 21.40, $t = 4.99$, $p_{\text{corr}} = 0.003$) while L-T₄+T₁AM produced a 60.44% increase (mean diff = 141.6 ± 31.91, $t = 4.44$, $p_{\text{corr}} = 0.004$), the latter corresponding to a 30.61% rebound relative to euthyroidism (mean diff = 88.13 ± 36.99, $t = 2.38$, $p_{\text{uncorr}} = 0.05$). T₁AM had no effect (mean diff = 72.13 ± 58.84, $t = 1.23$, $p_{\text{uncorr}} = 0.27$) (Figure 4). These results confirm that L-T₄ is a crucial endocrine signal controlling progenitor development within neurogenic niches, while there are no significant effects of T₁AM in the modulation of adult hippocampal neurogenesis.

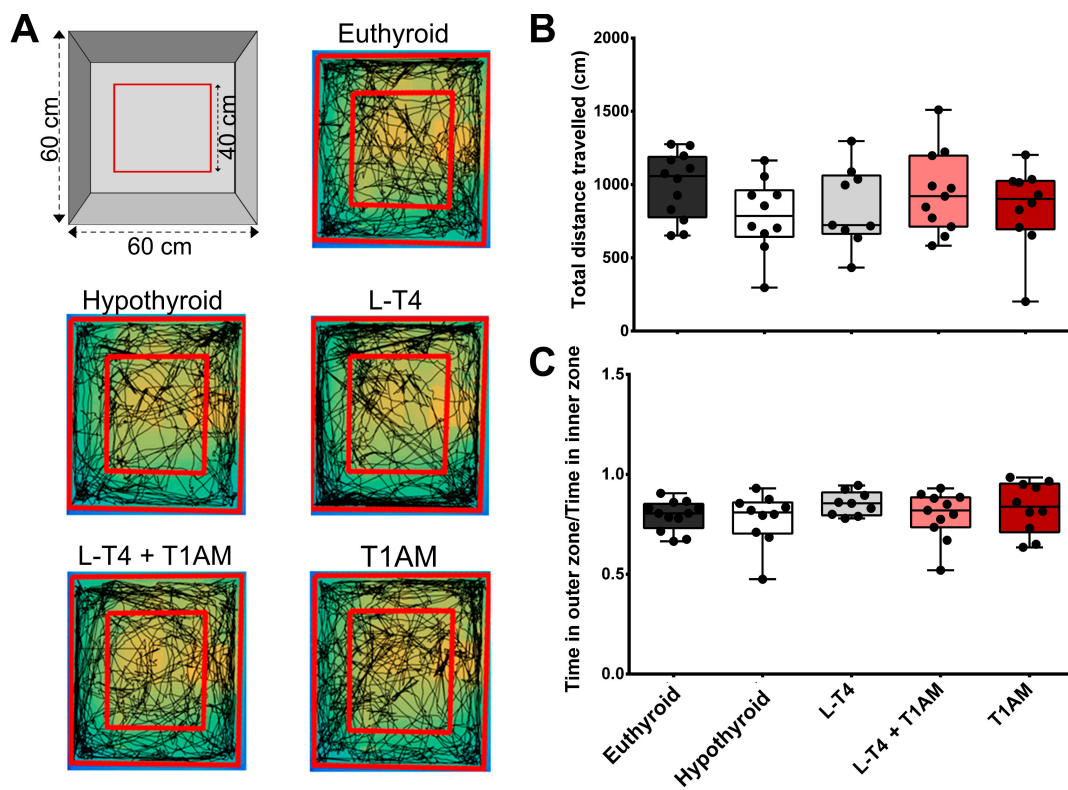


Figure 2. Effect of hypothyroidism and different replacement treatments on locomotion. (A) Open field apparatus and example trajectories in the open field test (10 min duration) for experimental mice in the five experimental groups. We found no between-group differences in total distance travelled (B) or thigmotaxis (C) in the open field test. Hypothyroid $n = 10$, euthyroid $n = 12$, L-T₄ $n = 9$, L-T₄+T₁AM $n = 11$, T₁AM $n = 10$. Results are plotted as mean \pm SEM using GraphPad Prism 6 software.

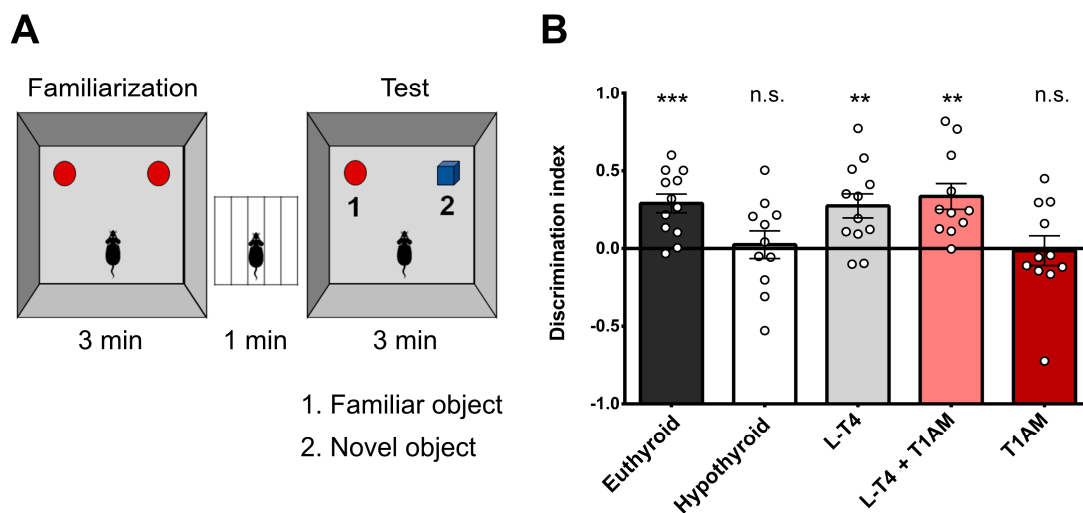


Figure 3. Effect of hypothyroidism and different replacement treatments on memory. (A) Graphical representation of the apparatus used for the novel object recognition test (ORT). (B) One sample t -test comparing DIs of the five experimental groups to a hypothetical value of 0 (no discrimination of the novel vs. familiar object) showed that DIs diverged significantly from 0 in euthyroid, L-T₄-treated and L-T₄+T₁AM-treated mice, confirming intact memory in these groups (** $p < 0.01$, *** $p < 0.001$, n.s. not significant). Results are plotted as mean \pm SEM using GraphPad Prism 6 software. Hypothyroid $n = 11$, euthyroid $n = 12$, L-T₄ $n = 12$, L-T₄+T₁AM $n = 11$, T₁AM $n = 11$.

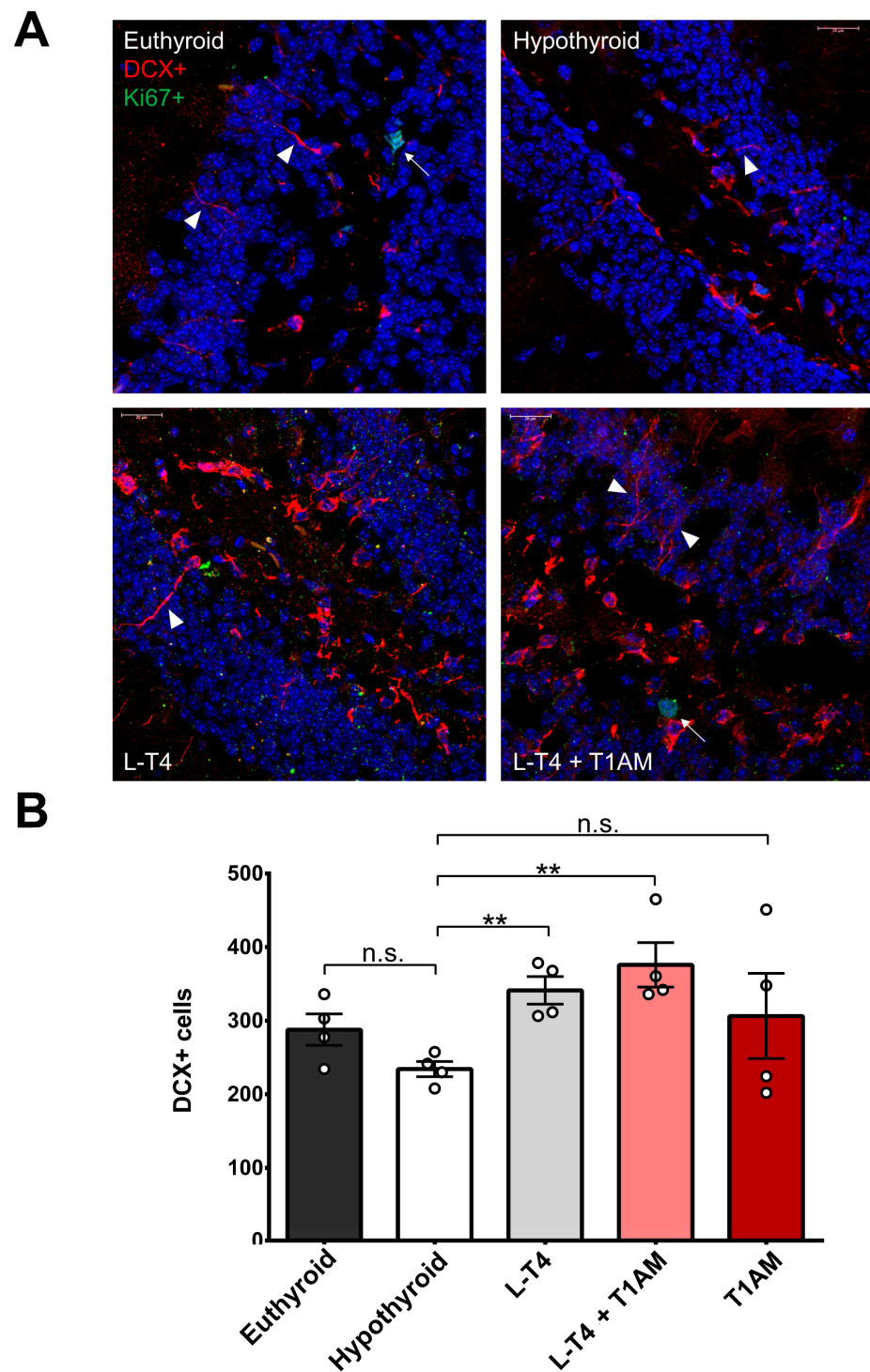


Figure 4. The effect of different replacement treatments on hippocampal neurogenesis. (A) Representative confocal microscopy images of sections illustrating immunofluorescence of DCX+ (red) and Ki67+ (green). DAPI was used to counterstain nuclei (blue). The arrows point at proliferating cells, and the arrowheads point at some neuroblast neurites. Scale bars 20 μ m. (B) DCX+ cell count. Each point represents the sum of DCX+ cells from four coronal sections taken across the hippocampus of a single mouse. Hypothyroidism caused a 20% reduction in the number of DCX+ cells in hippocampal neurogenic niches. L-T₄ rescued the number of DCX+ cells while L-T₄+T₁AM caused a 30.61% rebound relative to the euthyroid state. Pair-wise comparisons between the five groups using a *t*-test and Bonferroni-corrected for multiple comparisons (** $p < 0.01$, n.s. not significant). Hypothyroid $n = 4$, Euthyroid $n = 4$, L-T₄ $n = 4$, L-T₄+T₁AM $n = 4$, T₁AM $n = 4$.

2.5. L-T₄+T₁AM Enhanced the Expression of Genes Involved in Neurogenetic Pathways

The qPCR analysis revealed differentially expressed genes in neurogenetic pathways. One-way ANOVA revealed a significant effect of treatment on expression of *Kdr* ($F(4, 24) = 3.20, p = 0.03$), *L1cam* ($F(4, 24) = 2.84, p = 0.04$), *Mapk3* ($F(4, 23) = 2.96, p = 0.04$) and *Neurog2* ($F(4, 20) = 3.94, p = 0.02$). We observed a trend towards an effect of treatment on expression of *Ngf* ($F(4, 21) = 2.50, p = 0.07$), *Kit* ($F(4, 24) = 2.51, p = 0.07$) and *Ntf3* ($F(4, 23) = 2.51, p = 0.07$). We performed *t*-test pair-wise comparisons to test the differences between L-T₄+T₁AM and either L-T₄ or T₁AM, using Bonferroni correction for multiple comparisons ($0.05/14 = 0.004$). We found that *Mapk3* and *Neurog2* were significantly upregulated in L-T₄+T₁AM-treated relative to L-T₄-treated mice (*Mapk3*, $t = 4.51, p_{\text{corr}} = 0.002$; *Neurog2*, $t = 4.81, p_{\text{corr}} = 0.001$), with non-significant differences between L-T₄+T₁AM and T₁AM (*Mapk3*, $t = 0.16, p_{\text{uncorr}} = 0.87$; *Neurog2*, $t = 1.13, p_{\text{uncorr}} = 0.29$), suggesting that changes in gene expression could be induced by T₁AM on its own (Figure 5).

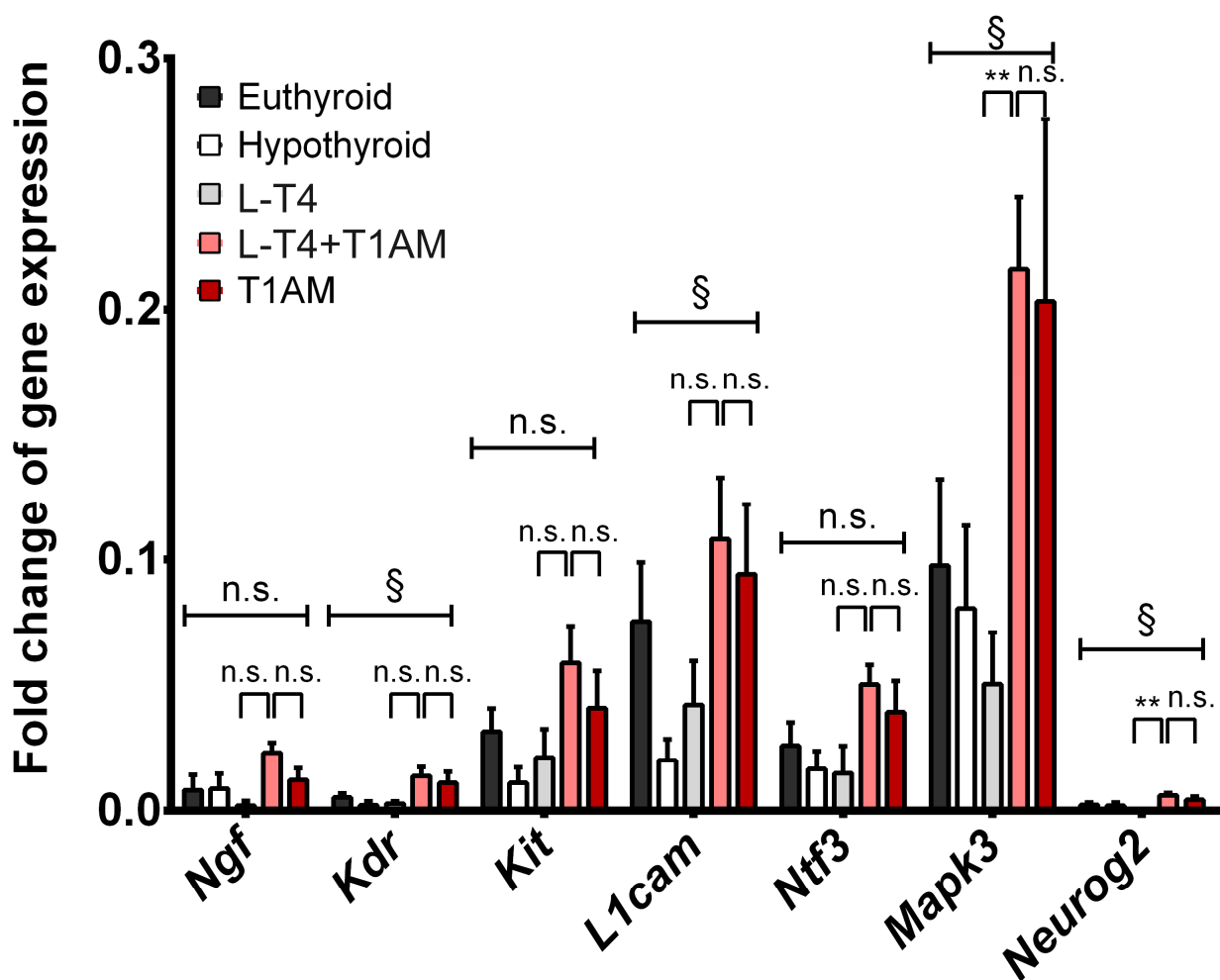


Figure 5. Analysis of expression of genes involved in neurogenetic pathways. One-way ANOVA showed significant between-treatment differences in gene expression (§ $p < 0.05$). *t*-test pair-wise comparisons were performed to test the differences between L-T₄+T₁AM and either L-T₄ or T₁AM (** $p < 0.01$, n.s. not significant). Hypothyroid $n = 5$, euthyroid $n = 4$, L-T₄ $n = 4$, L-T₄+T₁AM $n = 7$, T₁AM $n = 6$.

3. Discussion

In this study, we confirmed that L-T₄ monotherapy is necessary and sufficient to recover deficits in memory and adult hippocampal neurogenesis associated with adult-onset

hypothyroidism. We observed no further improvement in neurocognitive and neurobiological alterations from augmenting L-T₄ with T₁AM.

In our model of adult-onset hypothyroidism, we found no alterations in behavioural measures of locomotion, anxiety, or depression, in contrast with empirical clinical observation and previous reports [7,29–31]. Our hypothyroidism induction protocol almost completely suppressed serum TT₄ levels, while we observed no changes in serum TT₃ levels, suggesting that our protocol induced moderate hypothyroidism. Residual thyroperoxidase activity may be able to ensure a sufficient production of T₃ to prevent alteration in measures of depression or anxiety.

On the other hand, our model of moderate hypothyroidism showed alterations in memory, suggesting that cognitive functions are more susceptible to TH deficiency. The effect of TH deficit on cognitive functions and synaptic plasticity has been extensively demonstrated in rodents [7–10,32] and corresponds to clinical evidence of deficits in spatial, associative and verbal memory in hypothyroid patients [3,4]. In our hypothyroid mice, we found a 20% decrease in newborn neuroblasts in the SGZ of the dentate gyrus. A 4-week treatment with L-T₄ was necessary and sufficient to recover both memory and number of neuroblasts, consistent with previous evidence of the rescuing effects of L-T₄ on hypothyroidism-induced behavioural, electrophysiological, morphological, and molecular alterations [7,9,17,32]. TH play a crucial role toward the postmitotic survival of adult dentate granule cell progenitors [17]. It remains unclear if the decline in survival is due to a delay/block in neuronal differentiation or whether these cells may be targeted for cell death. The susceptibility of cognitive functions and neurogenesis to moderate TH changes may explain the relationship between TH and neurodegenerative diseases, such as Alzheimer's disease, observed in clinical populations [33–35], including those with subclinical hypothyroidism [2].

We found no significant differences between L-T₄ monotherapy and L-T₄+T₁AM in memory and number of DCX+ cells. T₁AM did not show any effect per se. We focused on T₁AM because we hypothesise that some of the neurocognitive symptoms of hypothyroidism traditionally attributed to lack of TH are partly caused by reduced availability of downstream metabolites. It has been demonstrated that T₁AM remains undetectable in the tissues of animals pharmacologically depleted of TH, even following full recovery of fT₄ and fT₃ [22]. Additionally, evidence has been accumulating about the effects of T₁AM in the CNS. T₁AM is a modulator of noradrenergic, dopaminergic, and histaminergic transmission [23]. It has been observed to modulate sleep [36], exploratory activity, memory acquisition and retention [24]. T₁AM showed neuroprotective actions in models of seizure-related excitotoxic damage, altered autophagy, amyloidosis, ischemia–reperfusion injury, and neuroinflammation [25–28,37]. In these studies, T₁AM was administered either through perfusion on ex vivo brain acute/organotypic slices or through intracerebral microinjection, while we used a systemic administration. Studies employing radioactively labelled T₁AM showed that T₁AM crosses the blood–brain barrier [38].

We found an effect of replacement treatment on expression of several genes, including *Ngf*, *Kdr*, *Kit*, *L1cam*, *Ntf3*, *Mapk3*, and *Neurog2*. In particular, L-T₄+T₁AM-treated mice displayed an upregulation of *Mapk3* and *Neurog2* as compared to L-T₄-treated mice. However, we did not observe significant differences between L-T₄+T₁AM and T₁AM in *Mapk3* and *Neurog2* expression, suggesting that changes in gene expression could be induced by T₁AM on its own. These genes are involved in neurogenetic mechanisms, such as increased proliferation of DCX+ cells in the hippocampus [39], initiation, and progression through the G1 phase of the cell cycle [40]; neuronal cell fate determination [40]; and suppression of glial fate promoting neurogenesis [41]. TH regulate gene transcription through thyroid receptors (TR), widely expressed in different stages of granule cells progenitors [17]. T₁AM does not bind to TR but to several molecular targets, including the trace amine-associated receptor 1 (TAAR1), alpha2 adrenergic receptors, transient receptor potential channels, and ApoB-100 [42]. How its distinct target profile links to gene expression will need to be clarified in future studies. However, we hypothesise a role for TAAR1 due to growing evidence of its involvement in neuropsychiatric disorders [43,44]. Furthermore, we have

previously demonstrated that T1AM neuroprotective actions in models of amyloidosis, ischemia–reperfusion injury, and neuroinflammation are dependent on TAAR1 [27,28,37]. TAAR1 was reported to activate distinct signalling pathways depending on its localization in different cellular compartments. When on the plasma membrane, TAAR1 activation leads to increased intracellular cAMP levels and activation of the protein kinase A through coupling to G α s proteins and adenylyl cyclase activation [45–47]. Intracellular TAAR1 activation induces its coupling with G α 13 subunits to activate the GTPase RhoA [48]. TAAR1 activation is also associated with G protein-coupled inwardly rectifying potassium channels [49,50] and G-protein-independent inhibition of glycogen synthase kinase 3 β [51].

The present study has some limitations. First, our findings do not clarify if the effects of replacement treatments on memory are causally mediated by changes in hippocampal neurogenesis. Future studies should investigate if blocking neurogenesis during L-T₄ and/or L-T₄+T₁AM treatment prevents memory rescue. Second, for some comparisons, the sample size was not sufficient to have enough statistical power. This is probably due to our protocol inducing moderate hypothyroidism (compensated TT₃ levels), which may have caused behavioural changes of small-to-moderate effect size, while our statistical analysis was powered to detect larger effect sizes, based on previous reports in thyroidectomized or transgenic rodents. Finally, to test our hypothesis that some of hypothyroidism-induced cognitive impairments are explained by T₁AM deficiency, we planned to measure brain T₁AM in different treatments. However, similarly to others [52], we found no clear endogenous signal of T₁AM (below detection limit) in most of our pilot samples. Due to these technical reasons, we therefore decided to abort this objective.

Our data show that moderate TH changes are sufficient to induce significant impairments in memory and neurogenesis. L-T₄ was necessary and sufficient to recover both memory and number of neuroprogenitors to the euthyroid state, while we found no evidence to support the role of non-canonical TH signalling. This was despite an effect of T₁AM on expression of genes involved in neurogenetic pathways, which should be further explored in future studies.

4. Materials and Methods

4.1. Mouse Model of Pharmacological Hypothyroidism

Six-week-old C57BL/6J male mice (maintained in a 12 h light/dark cycle with ad libitum access to diet and water) were given methimazole (0.20 mg/g/day) and potassium perchlorate (0.30 mg/g/day) [22] in drinking water for 49 days, while control littermates received water. At day 21, mice were anesthetised with 1.5% isoflurane and implanted with ALZET[®] subcutaneous osmotic pumps (Charles River Laboratories, Milan, Italy) delivering replacement treatments for 28 days (reservoir volume of 100 μ L, delivery rate of 0.11 μ L/h). Mice were divided into 5 groups: euthyroid, hypothyroid, L-T₄ (0.04 μ g L-T₄/g BW/day [53]), L-T₄+T₁AM (0.04 μ g L-T₄ + 0.004 μ g T₁AM/g BW/day) and T₁AM (0.004 μ g T₁AM/g BW/day) [27] (Figure 1A). All drugs were purchased from Sigma Aldrich-Merck (Milan, Italy). BW was monitored weekly throughout treatment. All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals of the Italian Ministry of Health (Legislative Decree n. 116/92) and the European Community (European Directive 86/609/EEC). The Italian Ministry of Health approved the use of animals in this protocol (65E5B.10, n.734/2017-PR, 10 October 2017).

4.2. HPLC-MS/MS Analysis of Thyroid Hormones

Serum samples were processed to perform a HPLC-MS/MS quantification of TT₃ and TT₄ based on the previously reported methods [54] described in the Supplementary Materials and Table S1.

4.3. Behavioural Tests

Before testing, mice were habituated to experimental handling daily for 10 min. Over the last 3 days before the experiment, mice were habituated to the testing room

for 1 h, i.e., the experimenter moved them from their usual holding room to the testing room, a soundproof environment with 100 lux illumination level. Afterwards, mice underwent behavioural tests over three consecutive days, in the following order: day 1, EPM; day 2, OF and ORT; day 3, TST. Mouse behaviour was videotaped and scored manually by investigators blind to the experimental condition. For the automatic scoring of the OF, we used the toolbox developed by Patel [55].

4.3.1. Elevated Plus Maze Test

The EPM apparatus consists of 4 arms (25 cm length \times 5 cm width) forming a plus sign, elevated 50 cm above the floor. Two opposite arms have walls (16 cm height, closed arms), while the other two do not (open arms) (Figure S1A). Mice were placed in the centre and allowed to freely explore the maze for 5 min. Recorded variables and their interpretation [56–59] are detailed in Table 2.

Table 2. Behavioural interpretation of the variables recorded in the elevated plus maze test.

Variable	Behavioural Interpretation
Time in open arms (%)	Anxiety-related
<i>n</i> of entries into open arms	Anxiety-related
<i>n</i> of entries into closed arms	Spontaneous locomotor activity
Total <i>n</i> of head dips	Anxiety-related
<i>n</i> of unprotected head dips	Anxiety-related
<i>n</i> of protected head dips	Decision-making; assessment of height and openness
<i>n</i> of end-arm explorations	Anxiety-related
<i>n</i> of SAPs	Risk-assessment

4.3.2. Open Field Test

Mice were allowed to freely explore the OF arena for 10 min (Figure 2A). We scored total travelled distance, and thigmotaxis, i.e., time spent in outer zones versus inner zones.

4.3.3. Novel Object Recognition Test

After a 10 min habituation, mice were allowed to freely explore two identical objects for 3 min (familiarization). Then, one of the two objects was replaced with a novel object. In the test phase, mice were left to freely explore the objects for 3 min (Figure 3A). Exploration time was counted when the mice were within 2 cm from the object with the nose directed towards it, sniffing, or touching the object with the nose but not when the nose was pointing away from the object even if the mice were beside the object, running around it, sitting, or climbing on it. DI were calculated according to the formula:

$$DI = (\text{time at novel} - \text{time at familiar}) / (\text{time at novel} + \text{time at familiar}) \quad (1)$$

4.3.4. Tail Suspension Test

Mice were suspended from a shelf, with climb-stoppers around the tail (Figure S2A). Escape-related behaviours were counted when mice: (a) tried to reach the apparatus walls and suspension bar; (b) shook the body; (c) showed movements akin to running. Escape was not counted when mice showed small movements confined to the front legs without involvement of the hind legs or in cases of oscillations due to the momentum gained during mobility bouts. Lack of escape-related behaviour was considered as immobility and was taken as a measure of depression.

4.4. Immunofluorescence

We performed immunofluorescence for Ki67 for cell proliferation and DCX for newborn Type 2b and Type 3 neuroblasts. After transcardial perfusion with 4% paraformaldehyde, brains were removed, cryoconserved in 30% sucrose and stored at -80°C . We cut 10 μm thick coronal sections using a cryostat (Leica CM3050 S, Leica Biosystems,

Milan, Italy) at 180 μm intervals across the hippocampus (-1.82 mm to -2.36 mm from bregma). For antigen retrieval, we immersed the slices in sodium citrate pH 6.0 at $85\text{ }^\circ\text{C}$ three times for 1 min each time. Slices were hydrated in phosphate buffer saline (PBS, Sigma Aldrich-Merck) for 20 min at room temperature (RT) and washed twice with 0.3% TritonTM X-100 (Sigma Aldrich-Merck) in PBS. Following a one-hour blocking step in 5% bovine serum albumin (BSA) and 0.5% TritonTM X-100 solution in PBS at RT, hippocampal slices were incubated with anti-Ki67 (Rat monoclonal, #14569880, Life Technologies, Monza, Italy) (1:500) and anti-DCX (Rabbit, #Ab18723, Abcam, Cambridge, UK) (1:800) primary antibodies (dissolved in 0.1% BSA, 0.1% TritonTM X-100 in PBS, Sigma Aldrich-Merck) at $4\text{ }^\circ\text{C}$ overnight. After washing, slides were incubated for 2 h at RT, in the dark, with goat anti-rat Alexa-488-conjugated (1:1000, Invitrogen, Waltham, MA, USA, #A11006) and donkey anti-rabbit Alexa-647-conjugated (1:1000, Abcam, #Ab150075) secondary antibodies (dissolved in 1% BSA and 0.1% TritonTM X-100 in PBS). Slices were mounted with the FluoroshieldTM medium containing DAPI (Abcam, #Ab104139) for staining nuclei. Images were captured using a Leica TCS SP8 Laser Scanning Confocal Microscope (Leica Biosystems) and analysed using Fiji 2.9.0.

4.5. Gene Expression Analysis

RNA was isolated from hippocampi dissected and homogenized in TRIzolTM (ThermoFisher Scientific, Milan, Italy). RNA yield was determined with the Qubit Fluorometer (ThermoFisher Scientific). After DNase digestion, RNA was retrotranscribed using the iScript Clear cDNA Synthesis kit (Bio-Rad, Coralville, IA, USA). qPCR analysis was performed in PrimePCRTM "Neurogenesis Tier 1 M96" collection panel (Bio-Rad), a pre-designed 96-well PCR plate containing primer sets for 88 gene targets involved in neurogenesis pathway for use with SYBR[®] Green (Table S2) on a CFX Connect Real Time System (BioRad). Ct values > 35 were considered to be no expression. Fold change calculation by $\Delta\Delta\text{Ct}$ method [60] was performed with the CFX MaestroTM Software version 2.2 (Bio-Rad, Hercules, CA, USA) using three reference genes for normalization: TATA-binding protein (*Tbp*), glyceraldehyde-3-phosphate dehydrogenase (*Gadph*), and Hypoxanthine-guanine phosphoribosyltransferase (*Hprt1*).

4.6. Statistical Analysis

Outliers were removed with the ROUT method ($Q = 1\%$). Normal distribution was assessed with the D'Agostino and Pearson test. Data were reported as mean \pm standard error of the mean (SEM) or as median and interquartile ranges as appropriate. BW were analysed using two-way ANOVA for repeated measures, followed by Sidak's post hoc tests. Differences between groups in hormonal level, behavioural performance, immunofluorescence, and gene expression were assessed with one-way ANOVA, followed by Tukey's post-hoc tests or with a Kruskal–Wallis test as appropriate. For the ORT, one-sample *t*-test was used to determine whether the average DI for each group was different from chance (hypothetical value = 0). Statistical significance was set at $p < 0.05$. Statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Supplementary Materials: The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms241813845/s1>.

Author Contributions: Conception of the work, G.R., L.M., C.K., N.O. and R.Z.; data collection, G.R., N.G., A.B., S.F., V.C., C.I., S.M. and A.S.; data analysis and interpretation, G.R., A.B., N.G., V.C., A.S., N.O. and R.Z.; drafting and critical revision of the article, G.R., A.B., A.S., N.O. and R.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the University of Pisa (PRA 2018 to R.Z.) and the NextGenerationEU-MIUR-CNR project "THE—Tuscany Health Ecosystem" (ECS 00000017, CUP B83C22003930001 to N.O.).

Institutional Review Board Statement: All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals of the Italian Ministry of Health (Legislative Decree n. 116/92) and the European Community (European Directive 86/609/EEC). The Italian Ministry of Health approved the use of animals in this protocol (65E5B.10, n. 734/2017-PR, 10 October 2017).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: The Centre for Instrumentation Sharing of University of Pisa (CISUP) is kindly acknowledged for providing the Sciex QTrap 6500+ mass spectrometer used for the mass spectrometric assays. The authors gratefully acknowledge Silvia Burchielli, Cecilia Ciampi, and Francesca Biondi for the excellent animal care.

Conflicts of Interest: The authors declare no conflict of interest.

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