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ORIGINAL ARTICLE

Thyroid Hormone Accelerates the Differentiation of Adult Hippocampal Progenitors

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Disrupted thyroid hormone function evokes severe physiological consequences in the immature brain. In adulthood, although clinical reports document an effect of thyroid hormone status on mood and cognition, the molecular and cellular changes underlying these behavioural effects are poorly understood. More recently, the subtle effects of thyroid hormone on structural plasticity in the mature brain, in particular on adult hippocampal neurogenesis, have come to be appreciated. However, the specific stages of adult hippocampal progenitor development that are sensitive to thyroid hormone are not defined. Using nestin-green fluorescent protein reporter mice, we demonstrate that thyroid hormone mediates its effects on hippocampal neurogenesis by influencing Type 2b and Type 3 progenitors, although it does not alter proliferation of either the Type 1 quiescent progenitor or the Type 2a amplifying neural progenitor. Thyroid hormone increases the number of doublecortin (DCX)-positive Type 3 progenitors, and accelerates neuronal differentiation into both DCX-positive immature neurones and neuronal nuclei-positive granule cell neurones. Furthermore, we show that this increase in neuronal differentiation is accompanied by a significant induction of specific transcription factors involved in hippocampal progenitor differentiation. *In vitro* studies using the neurosphere assay support a direct effect of thyroid hormone on progenitor development because neurospheres treated with thyroid hormone are shifted to a more differentiated state. Taken together, our results indicate that thyroid hormone mediates its neurogenic effects via targeting Type 2b and Type 3 hippocampal progenitors, and suggests a role for proneural transcription factors in contributing to the effects of thyroid hormone on neuronal differentiation of adult hippocampal progenitors.

Key words: hyperthyroid, hypothyroid, neurospheres, proneural transcription factors.

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Thyroid hormone (T3) profoundly regulates the development of the mammalian nervous system. T3 deficiency during critical developmental time windows results in irreversible structural and functional changes within the brain. During neurodevelopment, T3 exerts a powerful influence on proliferation, survival and differentiation of neuronal and glial progenitors (1). By contrast, in the mature brain, although functional consequences on mood and cognition are observed after perturbations of T3 levels, structural correlates of such functional consequences are poorly understood. Within the past decade, several studies have demonstrated that altered T3 levels in adulthood influence ongoing hippocampal neurogenesis (2–4), a process strongly correlated with cognitive performance and mood. Although these studies have demonstrated the neurogenic effects of T3, they do not provide an insight into the specific stages of adult hippocampal progenitor development that are sensitive to T3.

Hippocampal neurogenesis involves several stages of development that a stem or progenitor cell transits through before forming a granule cell neurone within the dentate gyrus (DG) subfield (5,6). The putative quiescent stem cell or Type 1 quiescent neural progenitor (QNP), which expresses the intermediate filament marker nestin and glial fibrillary acidic protein (GFAP), divides to give rise to transit amplifying neural progenitors (ANPs or Type 2a progenitors) that lose GFAP but retain nestin expression. The ANPs mature to form Type 2b progenitors that express markers of neuronal fate, such as the bHLH transcription factor NeuroD and the microtubule associated protein doublecortin (DCX), and continue to express nestin. Once committed to a neuronal fate, these DCX-positive progenitors

now called Type 3 progenitors, start migrating into the granule cell layer (GCL) and no longer express nestin. Immature neurones within the GCL transiently express the calcium binding protein calretinin, followed by more mature neuronal markers such as neuronal nuclei (NeuN) and calbindin. Several of these progenitor stages within the hippocampus are often indistinguishable when examined using exogenous mitotic markers, such as the thymidine analogue 5-bromo-2-deoxyuridine (BrdU). The proliferative stages of hippocampal neurogenesis quantified using BrdU, would include Type 1 progenitors to a small extent, largely Type 2a cells, and some Type 2b and Type 3 progenitors. The survival of these progenitors encompasses the maturational stages of progenitors, including Type 3 progenitors, as well as immature and mature neurones. Although diverse environmental stimuli may result in the common overall outcome of increasing hippocampal neurogenesis, individual progenitor stages are often differentially sensitive to specific stimuli (7–10).

To date, studies examining the influence of T3 status on adult hippocampal neurogenesis have utilised exogenous markers such as BrdU that do not allow a deeper characterisation of the stage-specific effects of T3 (2–4). In the present study, we utilised nestin-green fluorescent protein (GFP) reporter mice to delineate the specific progenitor stages that are responsive to altered T3 status in the hippocampal neurogenic nice. We show that T3 influences Type 2b and Type 3 hippocampal progenitors in the adult neurogenic niche, increasing the total number of DCX-positive cells, as well as accelerating neuronal differentiation. Furthermore, our results suggest that T3 influences the expression of key proneural transcription factors, which may contribute to its effects with respect to enhancing neuronal differentiation.

Materials and methods

Animals and treatments

Transgenic mice from a mixed C57BL/6 and CD2 background, expressing GFP under the control of the nestin promoter (11) were used to address the stage-specific effects of T3 on hippocampal neurogenesis. For all other experiments, C57BL/6 mice were used. Two to 3-month-old-male mice were maintained under a 12 : 12 h light/dark cycle with access to food and water *ad lib.* All animal treatments and procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals, and were approved by the TIFR Institutional Animal Ethics committee.

To examine the effects of hyperthyroidism, nestin-GFP mice were administered T3 (3,3′,5-triiodo-thyronine; 0.5 μg/ml; Sigma, St Louis, MO, USA) in drinking water for 10 days (n = 5 per group) (Fig. 1A). Serum samples from T3-treated mice were assayed in duplicate for free thyroxine (T4) and thyrotrophin (TSH) using an enzyme-linked immunosorbent assay based

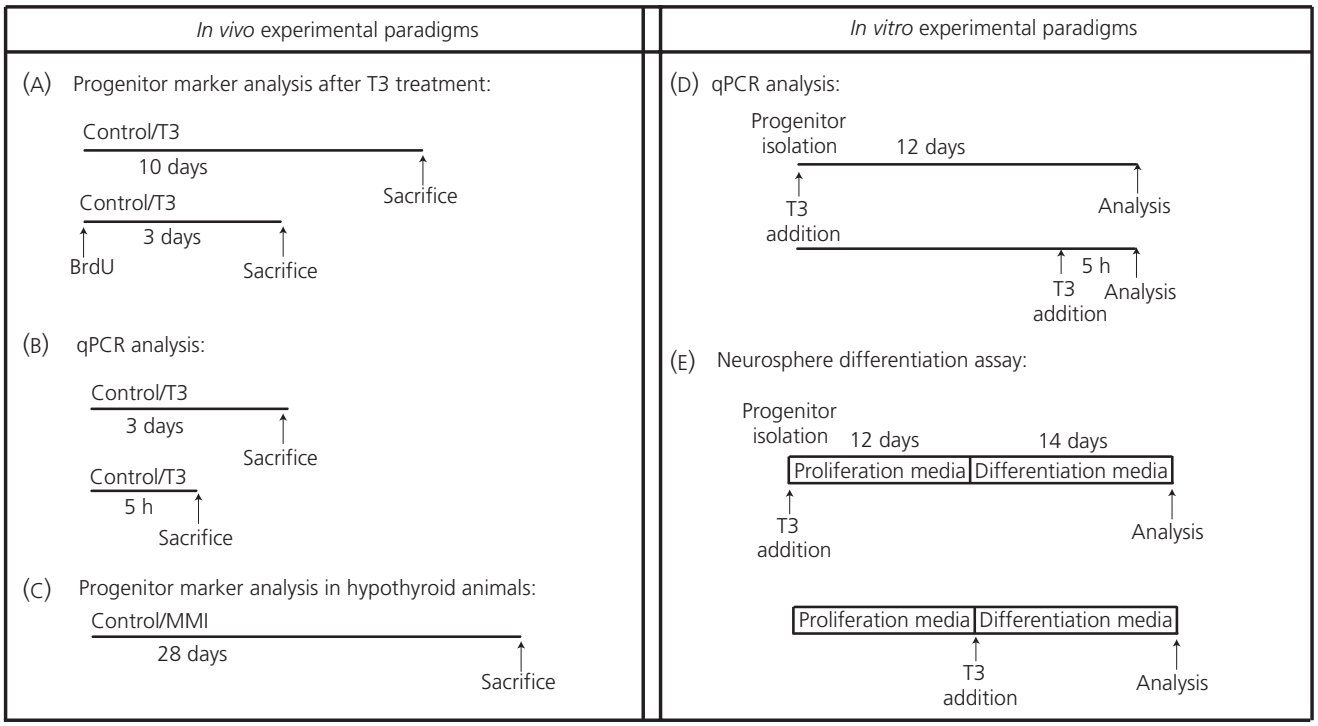


Fig. 1. Experimental design. (A) To examine the effects of hyperthyroidism, animals were administered thyroid hormone (T3) in drinking water for 10 days. To examine colocalisation of the mitotic marker 5-bromo-2-deoxyuridine (BrdU) with markers for specific progenitor cell stages, animals received BrdU, followed by T3 in drinking water for 3 days. (B) For quantitative polymerase chain reaction (qPCR) studies, animals were divided into Control and T3 groups, and the T3 group received either a single s.c. injection of T3 for 5 h or T3 in drinking water for 3 days before sacrifice. (C) To examine the effects of hypothyroidism, animals received 2-mercapto-1-methylimidazole (MMI) in drinking water for 28 days. (D) For experiments examining the direct effect of T3 on neurospheres, T3 was added to the media at the time of plating for 12 days *in vitro* (DIV) or for 5 h on 12 DIV. (E) To examine the effects of T3 on neurosphere differentiation, T3 was added to the media at the time of plating or at the time of transfer to the differentiation media, 12 days after plating.

kit (Krishgen Biosystems, Whittier, CA, USA) in accordance with the manufacturer's instructions. TSH and T4 levels were suppressed in animals that were administered T3 in drinking water for 10 days. TSH levels – Control: 2.7 ± 0.49; T3-treated: 0.74 ± 0.28; T4 levels – Control: 16.35 ± 2.66; T3-treated: 1.93 ± 0.52 (n = 5 per group, *P < 0.05; Student's t-test). Results are expressed as the mean ± SEM nanomols (nM). To examine colocalisation of the mitotic marker BrdU (Sigma) with markers for specific progenitor cell stages, animals received BrdU (200 mg/kg body weight) via i.p. injections (n = 4 per group), and the T3 group received T3 in drinking water for 3 days, at the end of which all animals were sacrificed (Fig. 1A). T3 treatment for 3 days did not alter either T4 or TSH levels. TSH levels – Control: 2.85 ± 0.39; T3-treated: 2.6 ± 0.58; T4 levels – Control: 17.0 ± 2.3; T3-treated: 22.23 ± 6.6 (n = 4 per group, P > 0.05; Students t-test). Results are expressed as the mean ± SEM (nM). For gene expression studies, animals were divided into Control and T3 groups (n = 6–10 per group), and the T3 group received either a single s.c. injection of T3 (0.2 mg/kg body weight) for 5 h or T3 in drinking water (0.5 μg/ml) for 3 days before sacrifice (Fig. 1B). To examine the stage-specific effects of hypothyroidism on adult hippocampal progenitors, we utilised a paradigm that successfully induces hypothyroid status in rats and mice (2,12,13). Nestin-GFP mice received the goitrogen 2-mercapto-1-methylimidazole (MMI; Sigma), in drinking water at the final concentration of 0.025% for 28 days (n = 5 per group) (Fig. 1C).

Neurosphere assay

Bilateral hippocampi from postnatal day 3–4 C57BL/6 male mice were minced using a scalpel blade. Tissue was digested in trypsin-ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA, USA) for 7 min at 37 °C, followed by treatment with trypsin-inhibitor (Invitrogen). The tissue was washed, resuspended with complete neurosphere medium and triturated to obtain a single cell suspension (14). The medium consisted of mouse NeuroCult NSC basal medium containing mouse NeuroCult proliferation supplements (StemCell Technologies, Vancouver, Canada), 0.02% bovine serum albumin (Invitrogen), 2 mg/ml heparin (Sigma), 20 ng/ml epidermal growth factor (EGF; Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) and 10 ng/ml basic fibroblast growth factor (bFGF; Roche Diagnostics, Indianapolis, IN, USA). Cells from each pup (n = 5–6 animals) were plated in a 96-well plate, with 48 wells treated with T3 (20 nM; Sigma). The number of differentially-sized neurospheres from 12 random wells/condition was counted on day 12 *in vitro* (12 DIV) and expressed as a percentage of the total neurospheres for each condition (Fig. 1E). For gene expression analysis, cells from each pup were plated in a 96-well plate. Twenty-four wells/pup were treated with T3 (20 nM; Sigma) at the time of plating and neurospheres were harvested after 12 DIV. To examine the acute effects of T3, 12 DIV control neurospheres were treated with T3 (20 nM; Sigma) for 5 h (Fig. 1E). To examine the effects of thyroid hormone on the differentiation of neurospheres, neurospheres that were generated in proliferation media for 12 days were plated on poly-D-lysine and laminin-coated dishes in media lacking EGF and bFGF for 14 days to promote differentiation. T3 (20 nM) was added to media at the time of progenitor isolation or after transfer to differentiation media (Fig. 1F).

Immunohistochemistry and immunofluorescence

Mice were sacrificed by transcardial perfusion with 4% paraformaldehyde (PFA; Sigma). Serial coronal sections (50 μm) through the rostro-caudal extent of the hippocampus were generated using a Vibratome (Leica Microsystems, Wetzlar, Germany). To address the effect of T3 on the nestin-positive pool of adult hippocampal progenitors, sections were subjected to immunofluorescence for GFP. Sections were incubated overnight with the rabbit anti-GFP (dilution 1 : 1000; Invitrogen) and, after washes, sections were incubated with Alexa 488-conjugated donkey anti-rabbit (dilution 1 : 250; Invitrogen) for 3 h at room temperature. To examine the expression of proliferating cell nuclear antugen (PCNA), sections were incubated with mouse anti-PCNA (dilution 1 : 250; Accurate Biochemicals, Westbury, NY, USA) followed by Alexa 488-conjugated donkey anti-mouse (dilution 1 : 250; Invitrogen) antibody. Sections were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) and viewed using a Nikon Eclipse 90i fluorescence microscope (Nikon, Tokyo, Japan). To examine the influence of altered T3 status on the number of DCX positive cells, free-floating tissue sections were incubated with goat anti-DCX (dilution 1 : 250; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were then incubated with the biotinylated horse anti-goat (dilution 1 : 250; Vector Laboratories) antibody. After signal amplification with an avidin-biotin complex (Vector Laboratories), the signal was detected using diaminobenzidine (Sigma). Sections were DPX mounted and observed using a Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

For triple immunofluorescence experiments, sections were incubated overnight with a cocktail of antibodies: (i) rabbit anti-GFP (dilution 1 : 1000; Invitrogen); (ii) mouse anti-GFAP (dilution 1 : 500; Chemicon, Temecula, CA, USA); and (iii) goat anti-DCX (dilution 1 : 250; Santa Cruz Biotechnology). After washes, sections were incubated with secondary antibodies: (i) Alexa 488-conjugated donkey anti-rabbit (dilution 1 : 500; Invitrogen); (ii) Alexa 555-conjugated donkey anti-mouse (dilution 1 : 250; Invitrogen); and (iii) biotinylated horse anti-goat (dilution 1 : 250; Vector Laboratories). Signal amplification was performed using Alexa 647-conjugated streptavidin (dilution 1 : 500; Invitrogen) and sections were mounted on slides using Vectashield (Vector Laboratories). Colocalisation was determined using confocal Z-plane sectioning with a Zeiss LSM5 Exciter laser scanning microscope.

For colocalisation experiments with BrdU, sections were subjected to DNA denaturation and acid hydrolysis, and then incubated overnight with a cocktail of primary antibodies: (i) mouse anti-BrdU (dilution 1 : 250; Roche) and goat anti-DCX (dilution 1 : 250; Santa Cruz Biotechnology), or (ii) rat anti-BrdU (dilution 1 : 500; Accurate Biochemicals) and mouse anti-NeuN (dilution 1 : 1000; Chemicon). After washes, sections were incubated with the secondary antibody cocktail: (i) biotinylated horse anti-mouse (dilution 1 : 250; Vector Laboratories) and Alexa 488-conjugated donkey anti-rabbit (dilution 1 : 250; Invitrogen), and (ii) biotinylated goat anti-rat IgG (dilution 1 : 500; Chemicon) and Alexa 555-conjugated donkey anti-mouse (dilution 1 : 250; Invitrogen). Signal amplification of biotinlyated secondary antibody was achieved using: (i) Alexa 555-conjugated streptavidin (dilution 1 : 500; Invitrogen) and (ii) Alexa 488-conjugated streptavidin (dilution 1 : 500; Invitrogen) and the sections were mounted using Vectashield (Vector Laboratories).

To examine the differentiation of neurospheres, cells were fixed in 4% PFA. After washes, cells were blocked and incubated overnight with a cocktail of antibodies: (i) rabbit anti-βIII tubulin (dilution 1 : 1000; Covance, Princeton, NJ, USA) and (ii) mouse anti-GFAP (dilution 1 : 1000; Sigma). After washes, sections were incubated with secondary antibodies: (i) Alexa 555-conjugated donkey anti-rabbit (dilution 1 : 500; Invitrogen) and (ii) Alexa 488-conjugated donkey anti-mouse (dilution 1 : 500; Invitrogen). Cells were washed and counterstained with Hoechst 33342 (Invitrogen) before coverslipping with Vectashield. The percentage of neurones generated after differentiation in control or T3-treated conditions was determined by counting the number of βIII tubulin immunopositive neurones in the total number of Hoechst positive cells in four random fields/well and in four wells per condition.

Cell counting

The number of GFP- or DCX-positive cells within the GCL of the DG hippocampal subfield was quantified (four sections per animal, n = 5 per group) using a Zeiss Axioskop at a magnification of × 400, by an experimenter blind to the treatment conditions. The morphological status of DCX-positive

cells was quantified by categorising them as: (i) DCX-positive cells without tertiary dendrites or (ii) DCX-positive cells with complex tertiary arbours (15). For triple immunofluorescence experiments, 100 GFP-positive cells per animal (n = 5 per group) were analysed for colocalisation with GFAP or DCX. For double immunofluorescence experiments, 50–70 BrdU-positive cells per animal (n = 4 per group) were analysed for colocalisation with DCX and NeuN. Z-plane sectioning with 1-μm steps on a Zeiss LSM5 Exciter laser scanning microscope was used to confirm colocalisation.

Quantitative polymerase chain reaction (qPCR)

RNA purification, cDNA synthesis and qPCR were performed as described previously (16). In brief, total RNA was isolated using Tri Reagent (Sigma). The RNA was quantified using Nanodrop (Eppendorf, Hamburg, Germany) and 2 μg of RNA per sample was used to prepare cDNA using a reverse transcription kit (Applied Biosystems, Foster City, CA, USA). cDNA was amplified in a Realplex mastercycler (Eppendorf) and visualised using a SYBR Green kit (Applied Biosystems). Hypoxanthine phosphoribosyl transferase (Hprt) was used as an endogenous housekeeping gene control. Hprt mRNA levels were confirmed to be unchanged by thyroid hormone treatment. To compare the expression of Hprt and target genes, the comparative C_T method was used, as described previously (12). Primer sequences are provided in the Supporting information (Table S1).

Chromatin immunoprecipitation (ChIP) assay

ChIP was carried out as described previously (12). Briefly, bilateral hippocampi were dissected and fixed to cross-link the DNA with the bound proteins. The tissue was dounce homogenised, sonicated and immunoprecipitated using a pan-acetylation histone 3 (H3) or pan-acetylation histone 4 (H4) antibody (1 μg; Cell Signaling Technologies, Beverly, MA, USA). After reverse cross-linking and chromatin precipitation, qPCR analysis was performed within upstream regions of the Tis21, Dlx2, Tlx, Klf9 and Hes5 genes. The 5′ upstream sequences of the mouse Tis21, Dlx2 and Tlx gene were analysed for putative T3 receptor binding sites (TRE) using ALIBABA, version 2.1 (http://www.gene-regulation.com/pub/programs.html). Although Tis21 has a putative TRE 152 bp upstream of the transcriptional start site, Dlx2 and Tlx do not contain putative TRE sequences and hence a 200-bp region immediately upstream of the transcriptional start site was analysed. For the Klf9 and Hes5 genes, we performed qPCR analysis to examine the possible enrichment of acetylated histone H3 and H4 within upstream regions that contain characterised T3 receptor binding sites (17,18). In each sample, the results were normalised to a region amplified from the GAPDH promoter. Primer sequences used in the ChIP experiments are provided in the Supporting information (Table S2).

Statistical analysis

Results were subjected to statistical analysis using Student's unpaired t-test (GRAPHPAD PRISM; GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

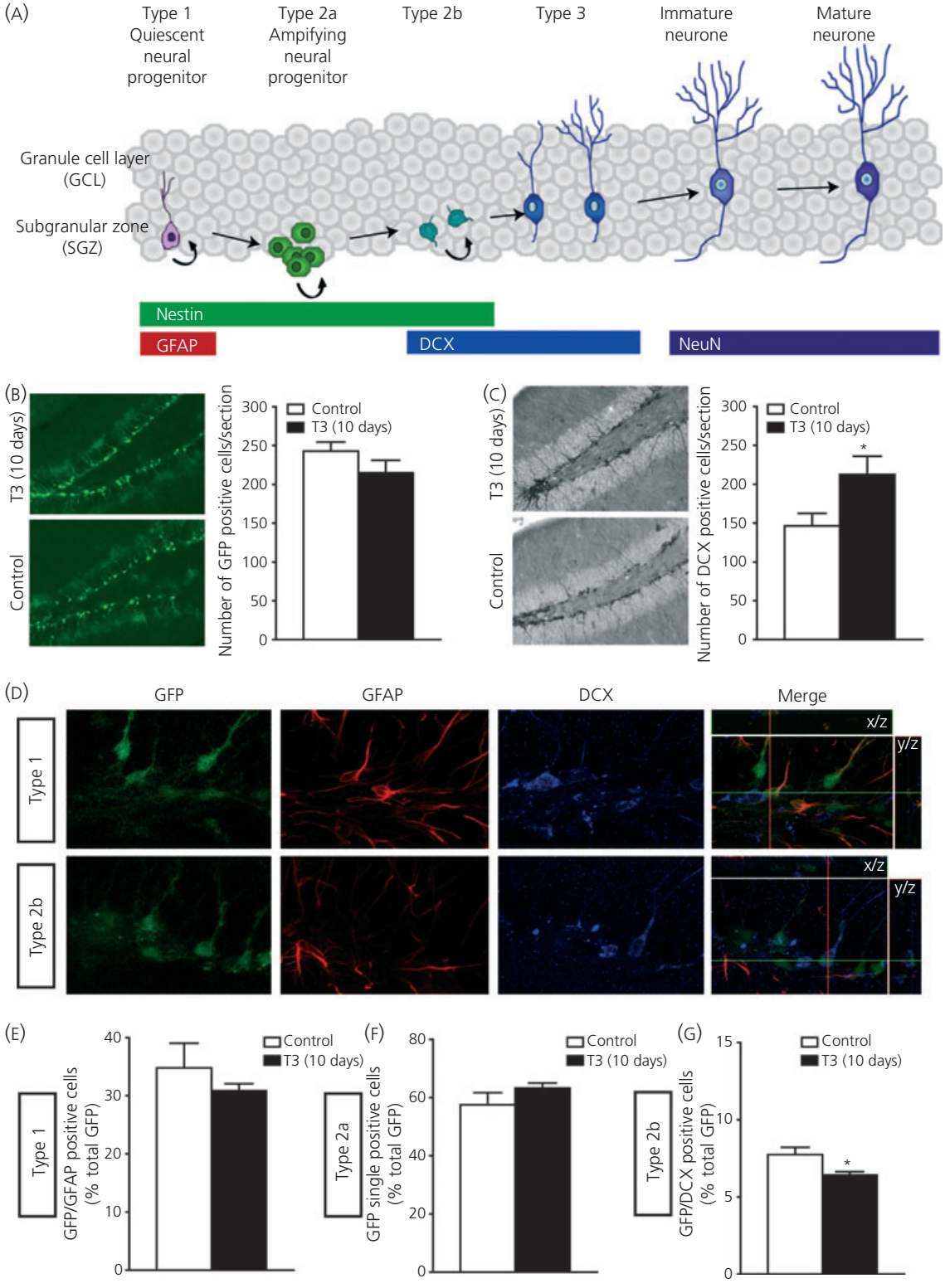
Adult-onset hyperthyroidism increases the number of immature neurones in the DG hippocampal subfield

Adult hippocampal progenitors express various endogenous markers that allow identification of the distinct stages of progenitor cell development (Fig. 2A). Within the adult hippocampal neurogenic niche, the putative stem cell (Type 1/QNP) expresses GFAP and nestin. QNPs are slowly dividing cells that undergo asymmetric divisions to give rise to the nestin-positive but GFAP-negative, ANP/Type 2a progenitor cell. The ANPs divide quite rapidly to give rise to Type 2b cells that express nestin, as well as DCX. As the Type 2b cells mature, they start migrating into the GCL and become Type 3 cells that retain DCX expression but lose nestin expression. Once in the GCL, these cells integrate functionally into hippocampal networks and express mature neuronal markers such as NeuN and calbindin.

Using nestin-GFP transgenic mice, we performed experiments aiming to characterise the specific stages of hippocampal progenitor cell development that are sensitive to T3. The GFP-expressing cells would encompass the early stages or the proliferative pool of progenitors, namely the Type 1, Type 2a and Type 2b cells. The total number of GFP-positive progenitors remained unchanged after T3 treatment for 10 days (Fig. 2B), suggesting that the total number of nestin-positive cells or basal proliferation of these progenitors remained unaltered by increased T3 levels. However, the total number of GFP-positive cells is controlled by both the basal proliferation rate and also by differentiation from Type 2b to Type 3 cells. To examine the potential effects of thyroid hormone on proliferation, we examined the expression of PCNA in control and thyroid hormone-treated animals. The number of PCNA positive cells per section did not significantly differ between the hyperthyroid and control animals (PCNA positive cells/section: Control: 59.06 $\pm$ 6.7; T3-treated: 67.4 $\pm$ 6.1; n = 4 per group, $P > 0.05$; Student's t-test). These results are consistent with earlier reports in rats where

Fig. 2. Stage-specific effects of adult-onset hyperthyroidism on hippocampal progenitors. (A) A schematic representation is shown of the various markers expressed by hippocampal progenitors at different stages of progenitor development. The quiescent neural progenitor (QNP) expresses nestin and glial fibrillary acidic protein (GFAP) and divides slowly to give rise to the amplifying neural progenitor (ANP) cells that lose their GFAP expression, but retain nestin expression. The Type 2a progenitors rapidly divide and give rise to Type 2b progenitors that begin to express doublecortin (DCX) in addition to nestin. Type 2b cells mature to form Type 3 progenitors that stop expressing nestin, but continue to express DCX. These progenitors eventually form neuronal nuclei (NeuN)-expressing mature neurones within the granule cell layer (GCL) of the dentate gyrus. (B) Representative confocal images are shown from the dentate gyrus subfield of nestin-green fluorescent protein (GFP) transgenic mice, treated with vehicle or thyroid hormone (T3) for 10 days. The total number of GFP-positive cells/section was unaltered in T3-treated and control animals. (C) Representative brightfield images are shown of DCX-immunopositive cells from control and T3-treated mice. The total number of DCX-positive cells/section was significantly increased in T3-treated animals compared to controls. (D) Confocal images are shown after triple immunofluorescence for GFP (green), GFAP (red) and DCX (blue) on hippocampal sections to distinguish Type 1 ($GFP^+GFAP^+DCX^-$), Type 2a ($GFP^+GFAP^-DCX^-$) and Type 2b ($GFP^+GFAP^-DCX^+$) cells within the dentate gyrus. (E) The number of GFP and GFAP double-positive Type 1 cells, expressed as a percentage of the total GFP-positive cells, was unchanged in control and T3-treated animals. (F) The percentage of the total pool of GFP-positive cells that were positive for GFP alone (Type 2a cells) remained unchanged between control and T3-treated animals. (G) The percentage of GFP and DCX double-positive Type 2b cells was decreased in T3-treated animals compared to controls. All results are expressed as the mean $\pm$ SEM (n = 5 per group). *$P < 0.05$ compared to controls (Student's *t*-test).

adult-onset hyperthyroidism did not affect the total pool of proliferating hippocampal progenitors (2,3). We next examined the influence of T3 treatment on the predominantly post-mitotic pool of progenitor cells that express DCX. DCX positive cells would include both Type 2b and Type 3 progenitors (19, 20). We show that adult-onset hyperthyrodism significantly increases the number of DCX-positive cells within the DG subfield of the murine hippocampus (Fig. 2c). During the process of hippocampal progenitor maturation, the dendritic branching becomes more elaborate with the formation of tertiary dendritic arbours (15). Although the total number of



DCX-positive cells was significantly increased within the hippocampus of hyperthyroid animals, the percentage of immature, DCX-positive cells with complex tertiary dendrites remained unaltered in T3-treated animals compared to controls (data not shown).

Stage-specific effects of adult-onset hyperthyroidism on Type2b hippocampal progenitors

Although the total pool of GFP-expressing cells was unaffected by T3 treatment, it remained possible that sub-categories of cells may be sensitive to changes in circulating levels of T3. We performed triple immunofluoresence experiments for progenitor markers on nestin-GFP mice to identify the effects of T3 on various stages of progenitor development, namely the Type 1 (nestin-GFP and GFAP double positive), the Type 2a (nestin-GFP positive but GFAP negative) and Type 2b (nestin-GFP and DCX double positive) progenitors. Type 1 and Type 2a neural progenitors remained unaffected by increased T3 levels (Fig. 2D–F). Interestingly, the percentage of cells that were double positive for nestin-GFP and DCX (Type 2b cells) showed a small but significant decline within the hippocampi of hyperthyroid animals compared to controls (Fig. 2G). In view of the enhanced total DCX-positive cell number, such a decline may be suggestive of a possible accelerated transition of these cells from Type 2b to Type 3 after T3 treatment.

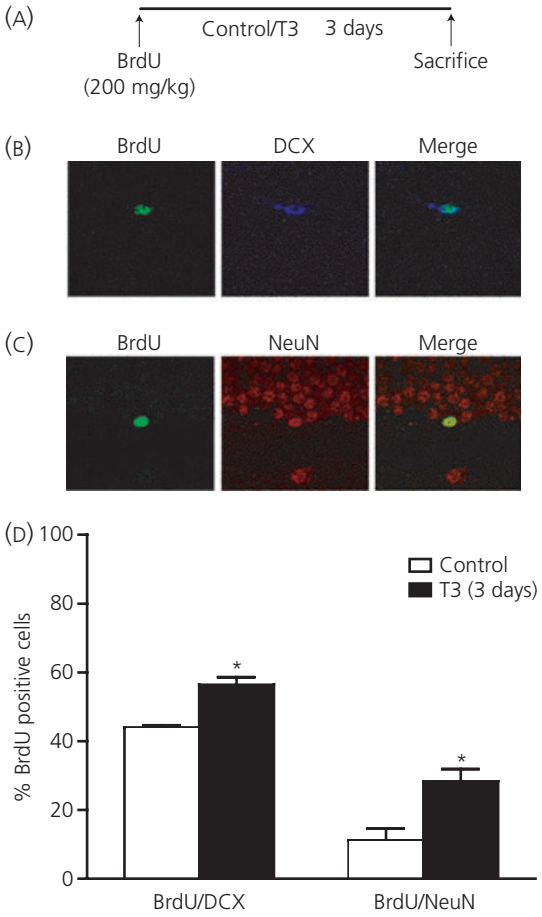


Fig. 3. Thyroid hormone (T3) treatment results in the accelerated maturation of 5-bromo-2-deoxyuridine (BrdU)-positive progenitors into neurones. (A) Adult male mice received a single BrdU injection (200 mg/kg) and were then administered T3 (0.5 μg/ml) in drinking water for 3 days. (B–C) Representative confocal images are shown after double immunohistochemistry for BrdU (green) and doublecortin (DCX) (blue) or neuronal nuclei (NeuN) (red) within the dentate gyrus subfield of the hippocampus. (D) T3 administration for 3 days resulted in an increase in the percentage of BrdU-positive cells that colocalised with DCX and NeuN compared to control animals. The results are expressed as the mean ± SEM (n = 5 per group). *P < 0.05 compared to controls (Student's *t*-test).

T3 treatment accelerates the maturation of BrdU-positive progenitors into immature neurones

To directly examine the hypothesis that T3 accelerates the differentiation of hippocampal progenitors into immature neurones, we pulse-labelled adult hippocampal progenitors using the mitotic marker BrdU followed by short-term T3 treatment for 3 days (Fig. 3A). At the 3-day timepoint, although the BrdU-labelled progenitors are likely to have acquired DCX-immunopositivity, very few of these progenitors are expected to express markers of mature neurones such as NeuN. We chose this intermediate timepoint to assess whether T3-treated animals exhibit an accelerated neuronal differentiation of adult hippocampal progentiors. Double immunofluorescence was carried out for BrdU and DCX, as well as for BrdU and the mature neuronal marker, NeuN (Fig. 3B,C). T3 treatment for 3 days significantly increased the

Table 1. Quantitative Polymerase Chain Reaction (qPCR) Analysis of Neurogenesis-Associated Genes After Acute and Short-Term Thyroid Hormone (T3) Treatment *In Vivo*.

	T3 (3 days)		T3 (5 h)	
Gene	Fold change (mean ± SEM)	*P*	Fold change (mean ± SEM)	*P*
Dlx2	**1.50 ± 0.11***	0.02	1.13 ± 0.15	0.43
Emx2	1.23 ± 0.30	0.52	0.83 ± 0.08	0.14
Hes5	0.62 ± 0.13	0.09	1.00 ± 0.10	1.00
Klf9	0.89 ± 0.12	0.57	1.19 ± 0.08	0.12
Math-1	0.77 ± 0.22	0.48	**2.04 ± 0.42***	0.02
NeuroD	1.44 ± 0.27	0.13	1.15 ± 0.14	0.35
Ngnl	1.29 ± 0.32	0.45	**2.04 ± 0.52***	0.04
Ngn2	0.85 ± 0.15	0.54	1.21 ± 0.21	0.30
Tis21	**2.65 ± 0.13***	< 0.001	1.25 ± 0.25	0.31
Tlx	**2.09 ± 0.29***	< 0.001	**1.32 ± 0.08***	0.01
TRα1	**1.63 ± 0.22***	0.036	1.15 ± 0.04	0.11
TRα2	0.97 ± 0.09	0.755	1.18 ± 0.08	0.07
TRβ1	0.96 ± 0.09	0.797	1.33 ± 0.22	0.20
TRβ2	1.01 ± 0.11	0.971	1.46 ± 0.31	0.13

qPCR was performed on hippocampal tissue obtained from adult male mice treated with T3 (0.5 μg/ml) in drinking water for 3 days or a single injection of T3 (0.2 mg/kg body-weight), and sacrificed 5 h later, and were then compared with their respective vehicle-treated controls. The expression of the thyroid hormone receptor isoforms (TRα1, TRα2, TRβ1 and TRβ2) was also determined at these time-points. The results are expressed as the mean ± SEM fold change (n = 5–10 per group). *P < 0.05 compared to controls (Student's t-test). Significant values indicated in bold.

percentage of BrdU-positive cells that were also immunopositive for DCX and NeuN (Fig. 3D) compared to control animals. This suggests that, in the presence of T3, BrdU-positive progenitors show a faster acquisition of immunopositivity for DCX and NeuN, which are markers of immature and mature neurones, respectively.

Short-term treatment with T3 increases the expression of genes involved in neuronal differentiation within the adult hippocampus

We next examined whether T3 treatment influences the expression of transcription factors involved in neuronal differentiation. There are several genes that have been considered to be involved in promoting neuronal cell fate choice, and early or terminal neuronal differentiation. T3 treatment for 3 days resulted in the significant up-regulation of two genes, Tis21 and Dlx2 (Table 1), that have been suggested to play an important role in neuronal differentiation (21, 22). To determine whether the increase in Tis21 is associated with epigenetic changes in the Tis21 promoter, we performed ChIP experiments for acetylated histone H3 and H4 within the putative TRE-containing promoter region of Tis21. T3 treatment resulted in a significant up-regulation of both acetylated H3 and H4 within the Tis21 promoter compared to control animals (Fig. 4B). Histone acetylation (AcH3 and AcH4) within the promoter regions of two other genes, Klf9 (Fig. 4D) and Hes5 (data not shown), which contain known TREs, remained unchanged after T3 treatment for 3 days. Although Dlx2 contained no putative TRE sequences upstream of its transcriptional start site, AcH3 was significantly increased within the Dlx2 promoter after T3 treatment (Fig. 4A), which is consistent with increased Dlx2 mRNA expression.

We next sought to determine whether T3 evokes transcriptional changes in neurogenic transcription factors after a single treatment, before the onset of increased neuronal differentiation observed after 3 days of T3 treatment. Acute T3 treatment resulted in a distinct pattern of changes in the expression of proneural genes within the hippocampus. Although the transcription factors Dlx2 and Tis21 were unaffected by acute T3 exposure, Math-1 and Ngn1, which are involved in neural fate determination, were significantly up-regulated. Although 5 h and 3 days of T3 treatment resulted in largely differing patterns of gene expression, a common feature of

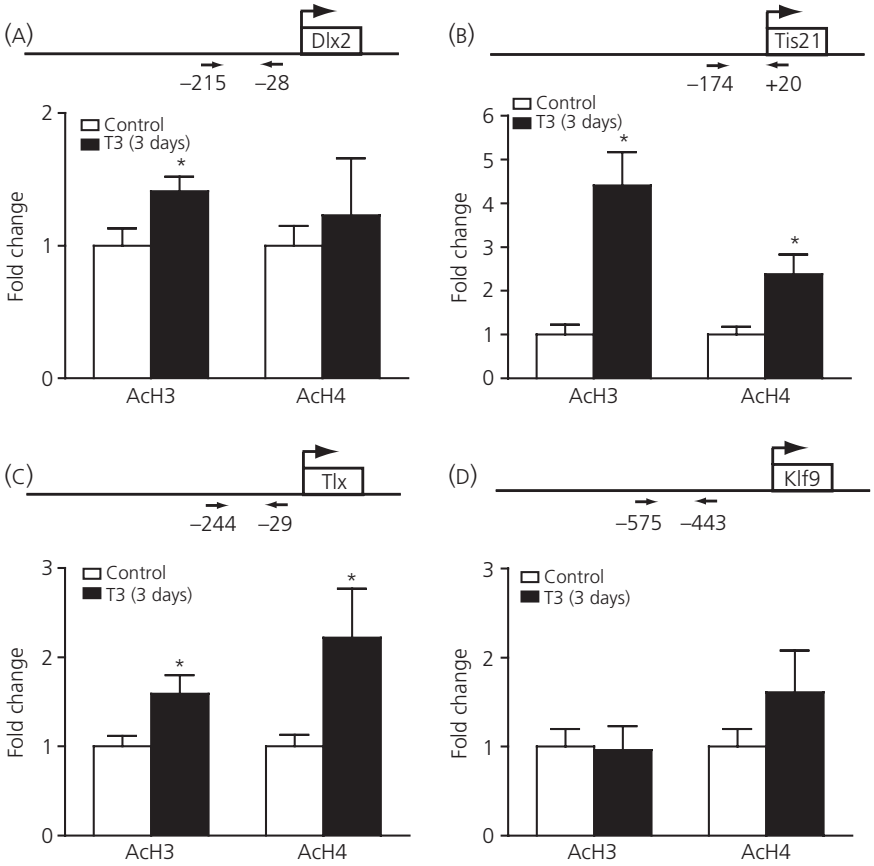


Fig. 4. Chromatin immunoprecipitation (ChIP) analysis of histone acetylation within the promoter regions of Dlx2, Tis21, Tlx and Klf9 after short-term thyroid hormone (T3) treatment. Hippocampal tissue derived from mice that received 3 days of T3 administration (0.5 μg/ml in drinking water) were subjected to ChIP analysis to examine acetylation of histone H3 (AcH3) and H4 (AcH4) within the promoter regions of the neurogenesis-associated genes, Dlx2, Tis21, Tlx and Klf9. T3 treatment increased AcH3 within the promoter region of the *Dlx2* gene (A) and enhanced AcH3 and AcH4 within upstream regulatory sequences of the Tis21 (B) and Tlx (C) genes. T3 treatment for 3 days did not influence AcH3 and AcH4 within the Klf9 gene promoter (D). Arrows indicate the location of the quantitative polymerase chain reaction primer binding within upstream regulatory sequences of the analysed genes. The results are expressed as the mean ± SEM fold change (n = 5–10/group). *P < 0.05 compared to controls (Student's *t*-test).

T3 exposure was the induction of the orphan nuclear receptor, Tlx (Table 1). In addition, AcH3, as well as AcH4, was significantly induced at the Tlx promoter by 3 days of T3 treatment (Fig. 4C). Besides its role in the maintenance of stem cell fate, Tlx has also been shown to influence neural fate commitment in cultured adult hippocampal progenitors (23). Interestingly the expression of the T3 receptor (TR) α1 was significantly induced by T3 treatment for 3 days. This is particularly interesting because this TR isoform has been shown to modulate adult hippocampal neurogenesis (24).

Survival of DCX-positive immature neurones and Type 2b hippocampal progenitors is compromised after adult-onset hypothyroidism

To examine the influence of adult-onset hypothyroidism, nestin-GFP transgenic animals were made hypothyroid using the goitrogen MMI for 28 days. Similar to results from hyperthyroid animals, adult-onset hypothyroidism did not affect the total number of GFP-positive progenitors within the adult hippocampus (Fig. 5A). Although hyperthyroid animals displayed an increase in the number of immature hippocampal neurones, decreased levels of circulating T3 significantly decreased the number of DCX-positive immature neurones within the DG (Fig. 5B). We next examined the specific stages of hippocampal progenitor maturation that were sensitive to adult-onset hypothyroidism using triple immunofluorescence for various stage-specific markers. Although the Type 1 and Type 2a progenitors remained unaffected by decreased T3 levels (Fig. 5C,D), the percentage of Type 2b cells was significantly lower within the hippocampi of hypothyroid animals compared to controls (Fig. 5F). These results suggest a reduced survival of DCX-positive progenitor cells within the hippocampal neurogenic niche of adult-onset hypothyroid animals. Our findings agree with studies that utilised the exogenous marker, BrdU, in rats to demonstrate that decreased circulating T3 levels significantly decreased the survival and neuronal differentiation of adult hippocampal progenitors (2,3).

T3 treatment of hippocampal progenitors *in vitro* shifts the pattern of neurospheres generated in the neurosphere assay

Neurospheres were generated from postnatal mice for a period of 12 days in the presence or absence of T3 in the medium. T3 treatment did not significantly affect the total number of hippocampal neurospheres (Fig. 6D). T3-treated neurospheres were significantly smaller than the control neurospheres (Fig. 6A,C). By contrast to the large neurospheres observed after 12 DIV in control wells, T3-treated wells showed a marked reduction in larger neurospheres. Consistent with its role in increasing the number of immature neurones within the

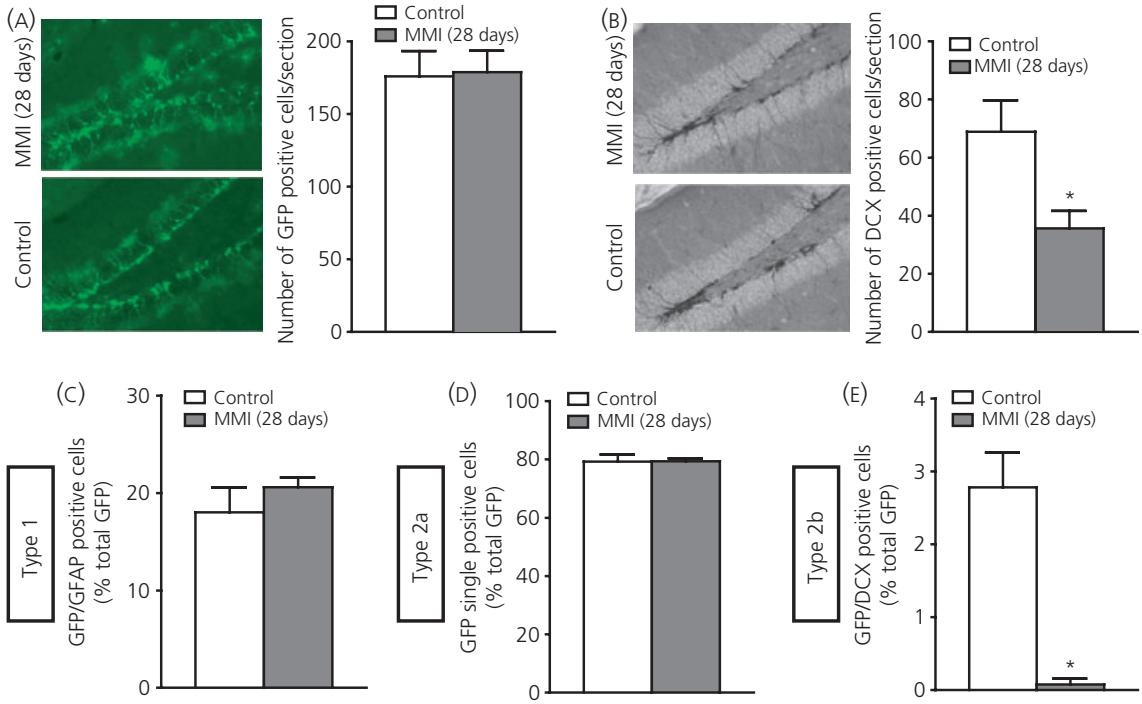


Fig. 5. Adult-onset hypothyroidism decreases the number of doublecortin (DCX)-positive progenitors and evokes a decline in Type 2b cells within the adult dentate gyrus subfield of the hippocampus. (A) Representative confocal images are shown of the dentate gyrus subfield from nestin-green fluorescent protein (GFP) transgenic mice, treated with vehicle or the goitrogen 2-mercapto-1-methylimidazole (MMI) for 28 days in drinking water. The number of GFP-positive cells/section in MMI-treated does not differ from controls. (B) Representative brightfield images are shown after DCX immunohistochemistry on hippocampal sections from control and MMI-treated mice. The number of DCX-positive cells/section was significantly reduced in MMI-treated animals compared to controls. The percentage of GFP and glial fibrillary acidic protein (GFAP) double-positive Type 1 cells (C) and GFP single positive (Type 2a) cells (D) was unaltered in MMI-treated animals compared to controls. The percentage of GFP and DCX double-positive (Type 2b) progenitors was significantly reduced in MMI-treated animals (E). All results are expressed as the mean ± SEM (n = 5/group). *P < 0.05 compared to controls (Student's *t*-test).

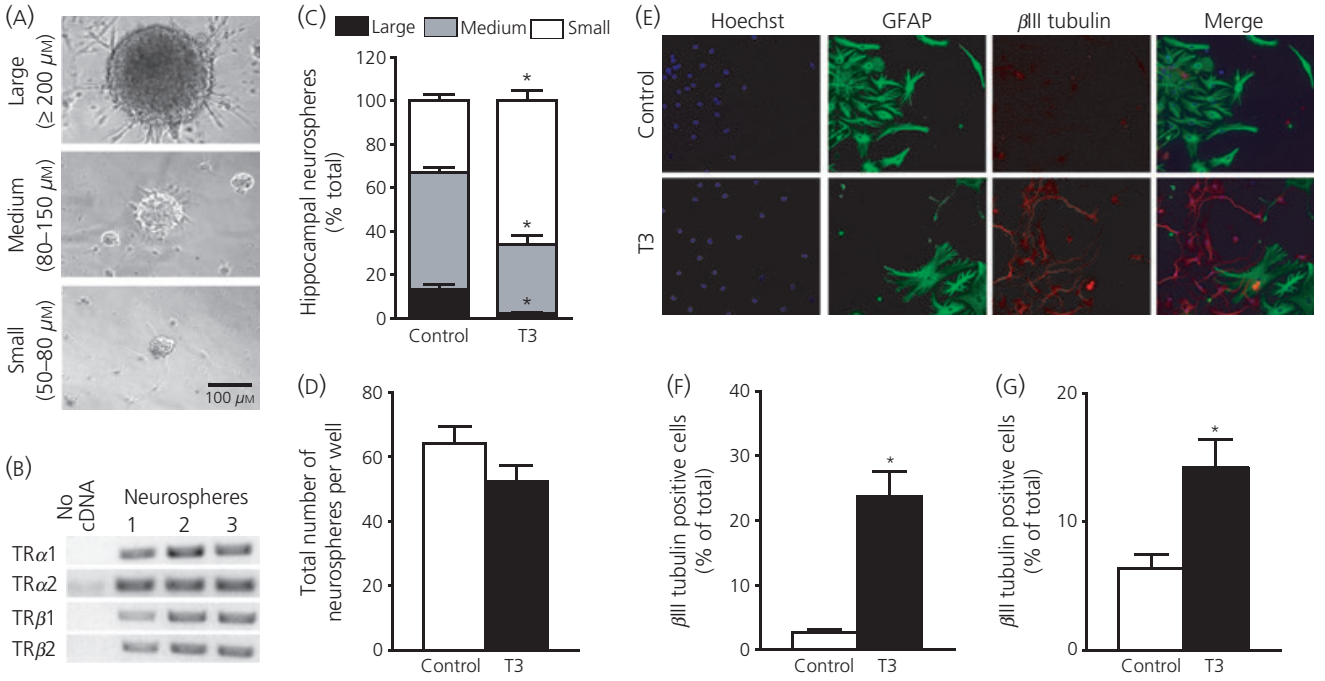


Fig. 6. Thyroid hormone (T3) treatment alters the size distribution of neurospheres generated in the neurosphere assay and enhances neuronal differentiation. Neurospheres were generated from postnatal male mice in the presence or absence of T3 (20 nM) for a period of 12 days *in vitro* (12 DIV). (A) Representative brightfield images are shown of neurospheres of varying diameters that were characteristic of the three categories of neurospheres, quantitated based on size distribution in the neurosphere assay. (B) Quantitative polymerase chain reaction analysis of neurospheres generated from control animals (n = 3) revealed the presence of all thyroid hormone receptor isoforms (TRα1, TRα2, TRβ1, TRβ2). (C) The graph depicts the percentage of large, medium or small neurospheres from control and T3-treated cultures compared to the total number of neurospheres obtained for each treatment. T3-treated wells showed a significant increase in smaller-sized (50–80 μM) neurospheres and a decline in both mid-sized (80–150 μM) and large-sized (≥ 200 μM) neurospheres. (D) No significant difference was observed in the total number of neurospheres generated in the presence or absence of T3 (20 nM) for 12 DIV. (E) Representative images are shown of differentiated neurospheres in the presence or absence of T3 (20 nM), immunostained for glial fibrillary acidic protein (GFAP) and βIII tubulin and counterstained with Hoechst 33342. (F) The graph depicts the percentage of βIII tubulin-positive cells within differentiated neurospheres when T3 was added to the media from the time of progenitor isolation compared to controls. (G) The graph depicts the percentage of βIII tubulin-positive cells within differentiated neurospheres when T3 was added to the differentiation media after neurosphere formation compared to controls. All results are expressed as the mean ± SEM (n = 5–6 animals/group). *P < 0.05 compared to controls (Student's *t*-test).

hippocampus *in vivo*, neurosphere expansion into a large size indicative of continued proliferative activity, does not appear to take place in the presence of T3. Such a shift in distribution of differently-sized neurospheres in the neurosphere assay is suggestive of a reduced proliferative potential and the possibility of a more 'differentiated' state of the progenitor in the presence of T3. To directly test whether T3 treatment of hippocampal progenitors evokes enhanced neuronal differentiation, we treated neurospheres with T3 (20 nM) right from the time of plating or after transfer to the differentiation medium at 12 DIV. Both these treatment regimes evoked a significant increase in the percentage of βIII tubulin immunopositive neurones (Fig. 6E-G). qPCR analysis also revealed the presence of TR isoforms within hippocampal neurospheres (Fig. 6B). These results suggest a direct effect of T3 on hippocampal progenitors.

T3 treatment increases the expression of genes involved in neuronal differentiation in hippocampal neurospheres

Neurospheres generated in the presence of T3 showed altered expression of several transcription factors involved in progenitor turnover, fate choice determination and neuronal differentiation (Table 2). Emx2 and Klf9 expression, although unchanged *in vivo*, were significantly induced by direct treatment of hippocampal progenitors with T3 for 12 days. Increased expression of Emx2 is assumed to increase the frequency of asymmetric adult stem cell divisions, thus promoting neuronal differentiation (25), and Klf9 is important for the terminal differentiation of adult hippocampal neurones (26). Similar to the effects noted within the hippocampus after exposure to T3 *in vivo*, Tlx expression was significantly induced by T3 within hippocampal progenitors *in vitro*. By contrast, T3 treatment significantly decreased the expression of Dlx2, as well as several genes involved in neuronal fate determination and differentiation, such as Math-1, NeuroD, Ngn1 and Ngn2. However, it is important to note that the pattern of neurospheres generated at this time-point was characteristically distinct in the presence and absence of T3 (Fig. 6C). This raises the caveat that the altered gene expression pattern noted at this time-point may be more reflective of the difference in the progenitors within the neurospheres, rather than an indication of a regulation of gene expression by T3 *per se*. This makes it difficult to determine whether the gene expression

Table 2. Quantitative Polymerase Chain Reaction (qPCR) Analysis of Neurogenesis-Associated Genes After the Generation of Neurospheres in the Presence of Thyroid Hormone (T3) (20 nM) for 12 Days *In Vitro* (DIV) and After an Acute T3 Exposure of 5 h to Neurospheres at the 12 DIV Time-Point.

	T3 (5 h)		T3 (12 days)	
Gene	Fold change (mean ± SEM)	*P*	Fold change (mean ± SEM)	*P*
Dlx2	0.96 ± 0.20	0.902	**0.41 ± 0.05***	0.004
Emx2	**1.40 ± 0.13***	0.038	**1.42 ± 0.07***	0.014
Hes5	0.82 ± 0.09	0.273	1.12 ± 0.15	0.478
Klf9	**1.90 ± 0.06***	< 0.001	**2.86 ± 0.32***	0.001
Math-1	1.16 ± 0.36	0.771	**0.31 ± 0.06***	0.004
NeuroD	1.15 ± 0.20	0.781	**0.34 ± 0.02***	0.001
Ngnl	0.90 ± 0.22	0.780	**0.23 ± 0.03***	0.001
Ngn2	1.09 ± 0.06	0.565	**0.35 ± 0.03***	0.002
Tis21	1.09 ± 0.06	0.501	0.74 ± 0.06	0.199
Tlx	0.84 ± 0.04	0.136	**3.32 ± 0.40***	< 0.001
TRα1	**1.60 ± 0.16***	0.045	**0.46 ± 0.10***	0.046
TRα2	**1.65 ± 0.19***	0.009	**1.57 ± 0.06***	0.008
TRβ1	1.59 ± 0.19	0.142	**0.43 ± 0.01***	0.002
TRβ2	1.22 ± 0.05	0.662	**0.36 ± 0.01***	0.002

The expression of the thyroid hormone receptor isoforms (TRα1, TRα2, TRβ1 and TRβ2) was also determined at these time-points. The results are expressed as the mean ± SEM fold change (n = 5–10 per group). *P < 0.05 compared to controls (Student's t-test). Significant values indicated in bold.

changes observed in the presence of T3, in progenitors treated for 12 DIV, arises as a result of observed differences in the kinds of neurospheres generated, or as a result of T3-induced gene expression changes. To rule out contributions from altered neurosphere composition, we first generated neurospheres for 12 DIV and then exposed them to T3 for 5 h before examining gene expression changes. Interestingly, acute T3 treatment of hippocampal neurospheres also significantly induced the expression of Emx2 and Klf9. Under these acute treatment conditions, when the hippocampal neurosphere composition is not altered, we did not observe the changes in gene expression of Dlx2, Math-1, NeuroD, Ngn1 and Ngn2 observed after T3 treatment for 12 DIV.

We also examined the expression of the TR isoforms both after 5 h of T3 treatment to neurospheres at the 12 DIV time-point, as well as in neurospheres generated with T3 in the medium for 12 DIV. Short-duration T3 exposure evokes an induction in TRα1 and TRα2 expression in neurospheres. By contrast when neurospheres were generated in the presence of T3 for 12 DIV, there was a decrease in the expression of all TR isoforms except TRα2, which showed an induction.

Discussion

In the past decade, altered T3 levels have been suggested to influence proliferation, survival and neuronal differentiation within the adult hippocampal neurogenic niche (2–4). However, the influence of T3 on adult hippocampal progenitors is poorly characterised, with no current understanding of the precise stages of hippocampal progenitor development that are particularly sensitive to T3. This is largely a consequence of the tools utilised to study hippocampal neurogenesis after perturbations of T3 status, which are based on administration of exogenous mitotic markers such as BrdU that do not allow a resolution of effects on individual stages of hippocampal progenitor development. In the present study, using nestin-GFP transgenic mice, we provide novel evidence that the neurogenic effects of T3 are mediated through effects on Type 2b and Type 3 hippocampal progenitor cells, with an increase in the total DCX-immunopositive pool of progenitors after T3 treatment, and an accelerated neuronal differentiation of these stages of progenitor development. However, the dendritic complexity of the DCX-positive pool of hippocampal progenitors is unaltered after T3 treatment, suggesting that the effects of T3 on increasing DCX-positive cell number and accelerating neuronal differentiation do not involve effects on morphological maturation. Interestingly, this is the same sub-category of hippocampal progenitors that is sensitive to decreased levels of T3. We, and others, have previously demonstrated that adult-onset hypothyroidism significantly decreases the survival and neuronal differentiation of hippocampal progenitors (2–4). Our results show that, along with an overall decrease in the number of DCX-positive immature neurones, the Type 2b cells are also significantly reduced in hypothyroid animals. Taken together, these results reveal that the proliferating pool of Type 1 and Type 2a hippocampal progenitors is insensitive to perturbations of T3 levels, and that the DCX-positive pool of Type 2b and Type 3 progenitors is selectively sensitive to altered T3 status (Fig. 7).

Our *in vitro* results suggest that T3 may exert direct effects on hippocampal progenitors. Consistent with our *in vivo* data, there was no significant difference in the number of neurospheres that were generated in the presence of T3, indicating no effect on proliferation. However, T3 resulted in a significant shift in the pattern of neurospheres generated with predominantly smaller neurospheres observed after T3 treatment. Larger neurospheres are considered to arise from a more latent stem cell pool in contrast to the smaller-sized neurospheres that possibly arise from a more restricted progenitor cell type. These results, along with evidence of expression of TR isoforms by hippocampal progenitors, support a role for a direct effect of T3 on progenitor cell development. In addition, T3 treatment resulted in significantly increased numbers of βIII tubulin positive neurones, providing further support for the neurogenic role of thyroid hormone on hippocampal progenitors. Previous studies reveal that T3 treatment of subventricular zone neurospheres results in an increased number of smaller-sized neurospheres (27). This suggests the possibility that progenitors derived from the subventricular zone and the subgranular zone may exhibit certain common responses to T3 treatment *in vitro*. By contrast, the *in vivo* literature suggests that T3 influences proliferation of subventricular zone progenitors, whereas the effect of T3 in the hippocampal neurogenic niche is predominantly on post-mitotic progenitors (24,28).

Mechanistically, T3 has been considered to act as a ligand cue to determine the timing of cell cycle exit or to promote fate commitment to a specific lineage. This role for T3 appears to be conserved

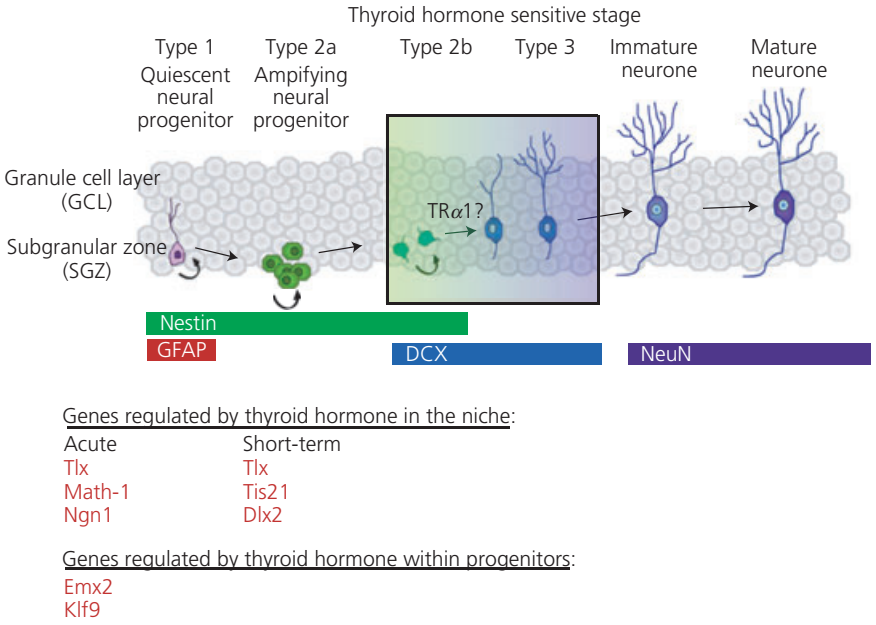


Fig. 7. Shown is a schematic indicating the stage-specific effects of thyroid hormone (T3) on adult hippocampal progenitor cell development. T3 influences the Type 2b ($GFP^+GFAP^-DCX^+$) and Type 3 ($GFP^-GFAP^-DCX^+$) progenitors within the dentate gyrus subfield of the hippocampus. Adult-onset hyperthyroidism enhances the total pool of doublecortin (DCX)-positive progenitors. Despite this enhancement in DCX-positive cell number, the percentage of Type 2b progenitor cells is reduced, likely as a result of the effects of T3 in accelerating neuronal maturation. Adult-onset hypothyroidism results in a robust decline in the total pool of DCX-positive cells, including a decrease in Type 2b progenitors. The expression of several neurogenic genes is altered after acute as well as short-term treatment with T3 within hippocampal progenitors themselves, as well as within the hippocampal neurogenic niche. A common feature of T3 treatment is the induction of the thyroid hormone receptor alpha 1 (TRα1), both within hippocampal progenitors *in vitro*, as well as the hippocampus *in vivo*, implicating a potential role for this TR isoform in mediating the neurogenic effects of thyroid hormone.

in diverse systems, such as muscle, bone and neural precursors (29,30). For example, in muscle cells, T3 induces the expression of the transcription factor MyoD that is critical for myogenic cell fate commitment and promotes skeletal muscle differentiation (31,32). In oligodendrocytic precursors, T3 is considered to be critical in determining the timing of oligodendrocytic differentiation (33–35). For most of these progenitor cell types, there appears to be a distinct stage of T3 sensitivity. However, it remains to be determined whether this critical window of sensitivity arises as a result of a dynamic change in the expression of TR isoforms or through changes in the local availability of T3 that may regulate the expression of key fate determining genes. The transient period of T3 sensitivity may serve as a 'gate' through which progenitors pass before the acquisition of a particular fate (Fig. 7). Our results indicate that TRs are expressed within the adult hippocampal niche, as well as by hippocampal progenitors. Interestingly, our previous work revealed that the TRα1 isoform appears to be important to mediate the neurogenic effects of T3 within the hippocampus, and is enriched in expression within DCX-positive and NeuroD-positive progenitors within the hippocampal neurogenic niche (24). We have previously shown that unliganded TRα1, similar to hypothyroidism, results in the decreased survival of hippocampal progenitors with a decline in the NeuroD-positive pool of progenitors probably representing a decrease in the Type 2b and Type 3 progenitors, an effect completely rescued by T3 treatment. By contrast, in TRα1 knockout animals, where the complete absence of TRα1 may serve to remove the sensitivity of these progenitor cell stages to T3, we find robust increases in the number of DCX-positive cells. Taken together, our current results and previous work with TRα1 mutant mice indicate that the Type 2b and Type 3 cells show a significant decrease in survival under conditions where TRα1 is likely to be unliganded, such as in dominant negative TRα1 mice or under hypothyroid conditions. However, when TRα1 is likely to be saturated by the ligand (e.g. after T3 treatment or when the dependence on the liganded TRα1 isoform is removed such as in loss of function TRα1 mutants), DCX-positive cells encompassing these particular stages of progenitor development show robust increases in number. A detailed analysis using both pharmacological tools and TR isoform specific mouse mutants is required to gain a mechanistic insight of the manner in which postmitotic hippocampal progenitors, cell cycle exit and cell fate acquisition are regulated by T3 and TRs in the adult hippocampal neurogenic niche.

Although our results reveal that Type 2b and Type 3 progenitors are sensitive to perturbations of T3 status, the molecular mechanisms underlying the effects of T3 are unclear. Our results examining the temporal expression of various genes involved in neuronal differentiation both *in vivo* and *in vitro* provide some insights into the potential candidates for mediating the effects of T3 within the hippocampal neurogenic niche. Short-term T3 exposure for 3 days *in vivo* resulted in the robust induction and enhanced promoter histone acetylation of the neurogenic transcription factors Dlx2 and Tis21. Tis21 is expressed in Type 2 and Type 3 progenitors, and

activation of Tis21 results in the accelerated differentiation of hippocampal progenitors (36,37), an effect strikingly similar to that observed with T3 treatment. Acute treatment with T3 enhanced the expression of two proneural genes, Math-1 and Ngn1, suggesting a potential activation of a neurogenic programme early after exposure to T3. Although there are distinct genes induced by T3 within the hippocampus after acute versus short-term T3 treatment, a common feature of T3 exposure is the regulation of Tlx. Tlx is classically considered to be required for the maintenance of an undifferentiated state within adult stem cells; however, Tlx also promotes neural commitment in adult hippocampal progenitors (23,38). At present, it is unclear whether the effects of T3 on hippocampal progenitors are cell autonomous or involve niche-mediated effects. Previous results suggest that long distance morphogen cues may contribute to the neurogenic effects of thyroid hormone. We have recently shown that expression of Shh, a developmental morphogen required for the maintenance of the adult hippocampal stem cell niche, is also regulated by T3 in adulthood (12). Our results also provide support for direct effects at the level of modulation of gene expression by T3 within progenitors themselves. Acute treatment of neurospheres with T3 evoked a robust induction of two proneural genes Emx2 and Klf9, suggesting that they may serve as T3 target genes within hippocampal progenitors. Previous reports suggest a role for Emx2 in neuronal differentiation, and for the thyroid hormone responsive gene Klf9 in regulating the neuronal maturation of adult hippocampal progenitors (26,39). Our results suggest that these proneural genes may contribute to the effects of T3 treatment on enhanced neuronal differentiation of hippocampal progentiors observed *in vitro*. It is interesting that we noted different patterns of gene expression evoked by short-term T3 treatment *in vitro* compared to *in vivo*. Although this might reflect different effects of T3 directly on progenitors versus those on the neurogenic niche, we cannot preclude the possibility that the difference may also arise because the *in vitro* progenitors were derived from young postnatal brains versus the studies *in vivo* that were performed on adults. Taken together, the *in vitro* and *in vivo* gene expression and ChIP studies suggest an influence of T3 treatment on several genes strongly implicated in the modulation of a neurogenic fate.

In conclusion, we provide novel evidence of T3 action on specific stages of hippocampal progenitor development, in particular implicating the Type 2b and Type 3 progenitor cells as being sensitive to perturbations of T3. We demonstrate an increase in the total number of DCX-positive cells and an accelerated neuronal maturation of hippocampal progenitors after exposure to T3, likely through influencing the expression of proneural genes. Our results motivate future studies for dissecting out the mechanistic contributions of specific proneural genes to the effects of T3 within the hippocampal neurogenic niche.

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Supporting information

The following supplementary material is available:

Table S1. Sequence information for reverse transcriptase-polymerase chain reaction primers.

Table S2. Sequence information for chromatin immunoprecipitation primers.

This supplementary material can be found in the online article.

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