

Unliganded thyroid hormone receptor $\alpha 1$ impairs adult hippocampal neurogenesis

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ABSTRACT Thyroid hormone regulates adult hippocampal neurogenesis, a process involved in key functions, such as learning, memory, and mood regulation. We addressed the role of thyroid hormone receptor TR $\alpha 1$ in adult hippocampal neurogenesis, using mice harboring a TR $\alpha 1$ null allele (TR $\alpha 1^{-/-}$), overexpressing TR $\alpha 1$ 6-fold (TR $\alpha 2^{-/-}$), and a mutant TR $\alpha 1$ (TR $\alpha 1^{+/m}$) with a 10-fold lower affinity to the ligand. While hippocampal progenitor proliferation was unaltered, TR $\alpha 1^{-/-}$ mice exhibited a significant increase in doublecortin-positive immature neurons and increased survival of bromodeoxyuridine-positive (BrdU⁺) progenitors as compared to wild-type controls. In contrast, the TR $\alpha 1^{+/m}$ and the TR $\alpha 2^{-/-}$ mice, where the overexpressed TR $\alpha 1$ acts as an aporeceptor, showed a significant decline in surviving BrdU⁺ progenitors. TR $\alpha 1^{-/-}$ and TR $\alpha 2^{-/-}$ mice showed opposing effects on neurogenic markers like polysialylated neural cell adhesion molecule and stathmin. The decreased progenitor survival in the TR $\alpha 2^{-/-}$ and TR $\alpha 1^{+/m}$ mice could be rescued by thyroid hormone treatment, as was the decline in neuronal differentiation seen in the TR $\alpha 1^{+/m}$ mice. These mice also exhibited a decrease in NeuroD⁺ cell numbers in the dentate gyrus, suggesting an effect on early postmitotic progenitors. Our results provide the first evidence of a role for unliganded TR $\alpha 1$ in modulating the deleterious effects of hypothyroidism on adult hippocampal neurogenesis.—Kapoor, R., van Hogerlinden, M., Wallis, K., Ghosh, H., Nordstrom, K., Vennstrom, B., Vaidya, V. A. Unliganded thyroid hormone receptor $\alpha 1$ impairs adult hippocampal neurogenesis. *FASEB J.* 24, 4793–4805 (2010). www.fasebj.org

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THYROID HORMONE PERTURBATIONS in development cause major neuroanatomical and neurological deficits (1). Though adult-onset hypothyroidism does not manifest as severely as developmental hypothyroidism (2), it can precipitate depressive behavior and deteriorate cognitive function (3, 4). In particular, adult hypothyroidism impairs hippocampus-dependent behaviors, resulting in learning, memory, and mood-related deficits

(5, 6). Adult hippocampal neurogenesis plays an important role in these hippocampus-dependent tasks (7, 8), and is regulated by thyroid hormone (9–11). Decreased hippocampal neurogenesis has been postulated to contribute to the deficits in hippocampal functions observed in adult-onset hypothyroidism.

Adult neurogenesis encompasses progenitor proliferation, survival and differentiation, and the maturation and functional integration of newborn neurons (12). The developmental stages of adult neurogenesis are characterized by stage-specific markers, such as nestin, NeuroD, doublecortin (DCX), polysialylated neural cell adhesion molecule (PSA-NCAM), stathmin, and calretinin (13, 14). Adult hypothyroidism decreases progenitor survival, DCX-positive (DCX⁺) immature neuron number, and neuronal differentiation, with no effect on progenitor proliferation (9, 10). *In vitro* evidence suggests a direct effect of thyroid hormone on adult hippocampal progenitors (10). However, the role of thyroid hormone receptors (TRs) and their contribution to the damaging effects of hypothyroidism on adult hippocampal neurogenesis is unknown.

TRs are transcription factors that bind thyroid hormone response elements and activate or repress target genes as ligand-receptor complexes or aporeceptors (15). TR α and β genes generate several TR isoforms, of which TR $\alpha 1$, TR $\alpha 2$, TR $\beta 1$, and TR $\beta 2$ are predominant in the adult mammalian brain (2). TR $\alpha 1$ contributes 70–80% of total TR expression in the brain (16). TR $\alpha 2$ does not bind thyroid hormone, though some reports implicate TR $\alpha 2$ in the transcriptional repression of thyroid hormone-responsive genes (17). It is of interest to note that the phenotype in TR $\alpha 2^{-/-}$ mice, which as a consequence of ablation of TR $\alpha 2$ inevitably overex-

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press TR α 1 severalfold, has been ascribed to TR α 1 aporeceptor effects in many tissues (18).

It remains unclear whether the deleterious effects of hypothyroidism are due to insufficient target gene activation or a consequence of the aporeceptor acting as a transcriptional regulator (15). The focus of the present study was to investigate the role of TR α 1 in adult hippocampal neurogenesis, using TR α 1^{-/-}, TR α 2^{-/-}, and TR α 1^{+/-} heterozygous mice carrying a point mutation (TR α 1R384C) that lowers thyroid hormone affinity 10-fold (18–20). Further, TR α 1-GFP-expressing mice were used to address the stage-specific expression of TR α 1 in adult hippocampal progenitors. Our results demonstrate a key role for TR α 1 in the regulation of the postmitotic survival of adult hippocampal progenitors, and indicate that an unliganded TR α 1, acting as an aporeceptor, is responsible for the deleterious effects of hypothyroidism on adult hippocampal neurogenesis.

MATERIALS AND METHODS

Thyroid hormone receptor mutant mice

TR α 1^{-/-} and TR α 2^{-/-} mice were generated as described previously (18, 19). The mouse strain carrying the dominant-negative R384C mutation in TR α 1 (TR α 1^{+/-}) was generated as described previously (20). TR α 1-GFP-knock-in mice were constructed by inserting the coding sequence of eGFP in frame 3' to exon 9 of the TR α 1 gene (21). Heterozygote offspring were bred against C57BL/6 for 3 generations and then intercrossed to generate TR α 1-GFP mice homozygous for the chimeric gene. TR α 1-GFP-knock-in mice have normal body and organ weights and T3, T4, and TSH levels, and show no overt morphological or physiological phenotype. The littermate mutant and wild-type mice were kept at 21°C on a 12:12-h light-dark cycle, and 2-mo-old male mice were used in the study. Animal care procedures were in accordance with the guidelines set by the European Community Council Directives (86/609/EEC) and were approved by the Karolinska Institutet and Tata Institute of Fundamental Research (TIFR) institutional animal ethics committees.

BrdU-labeling paradigms and drug treatments

To determine whether TR α 1 is expressed by proliferating adult hippocampal progenitors, we injected heterozygote TR α 1-GFP mice with a single intraperitoneal (i.p.) injection of the mitotic marker 5-bromo-2'-deoxyuridine [BrdU, 150 mg/kg body weight (bw); Sigma, St. Louis, MO, USA], 2 h prior to sacrifice ($n=3$). Male wild-type mice were used as a negative control to ensure specificity of the GFP signal. To address the effects on adult hippocampal progenitor proliferation, TR α 1^{-/-} and TR α 2^{-/-} mice, as well as littermate wild-type controls, received a single i.p. injection of BrdU (100 mg/kg bw; Sigma) and were sacrificed 2 h later ($n=4$ or 5/group). To address the role of an unliganded TR α 1 receptor on adult hippocampal progenitor proliferation, TR α 1^{+/-} and wild-type littermate controls received a single BrdU (100 mg/kg bw) injection and were sacrificed 2 h later ($n=4$ –5/group).

To examine effects on the survival and differentiation of adult hippocampal progenitors, TR α 1^{-/-} and TR α 2^{-/-} mice, as well as littermate wild-type controls were adminis-

tered BrdU (150 mg/kg bw) once daily by i.p. injection for 3 d and were sacrificed 28 d after the last injection ($n=4$ or 5/group). To examine the role of an unliganded TR α 1 receptor on adult hippocampal progenitor survival and differentiation, TR α 1^{+/-} and wild-type littermate controls received a single daily BrdU (100 mg/kg bw) injection for 3 d and were sacrificed 30 d later ($n=4$ or 5/group).

Experiments to address the influence of thyroid hormone treatment on adult hippocampal progenitor survival in TR α 2^{-/-} mice had 4 experimental groups: wild type + vehicle, wild type + T3, TR α 2^{-/-} + vehicle, and TR α 2^{-/-} + T3 ($n=4$ or 5/group). The groups receiving thyroid hormone treatment were given T3 (0.5 μ g/ml, Sigma) in drinking water, as described previously (22) for 16 d prior to sacrifice.

In experiments to examine the effect of thyroid hormone treatment on adult hippocampal progenitor survival and differentiation in TR α 1^{+/-} mice, there were 4 experimental groups: wild type + vehicle, wild type + T3, TR α 1^{+/-} + vehicle, and TR α 1^{+/-} + T3 ($n=4$ –5/group). The treatment groups (wild type + T3, TR α 1^{+/-} + T3) received T3 (0.5 μ g/ml) in drinking water, as described previously (22) for 12 d. From d 10 to d 13, all animals were administered a single injection of BrdU (150 mg/kg bw), and were sacrificed 30 d after the last BrdU injection.

Immunohistochemistry

Mice were sacrificed by transcardial perfusion with 4% PFA, brains were removed, postfixed, and subsequently cryoprotected in 30% sucrose-PFA. Serial coronal sections (30 μ m) through the rostrocaudal extent of the hippocampus were generated using a freezing microtome (Leica, Wetzlar, Germany). Free-floating sections were processed for BrdU immunohistochemistry as described previously (10). In brief, after DNA denaturation and acid hydrolysis, sections were incubated overnight with mouse anti-BrdU antibody (1:500; Boehringer Mannheim, Indianapolis, IN, USA). Sections were then exposed to secondary antibody (biotinylated anti-mouse IgG, 1:500, Vector Laboratories, Burlingame, CA, USA). An avidin-biotin complex (Vector Laboratories) was used for signal amplification, which was detected using the substrate diaminobenzidine (Sigma).

For immunohistochemistry and immunofluorescence experiments to examine the expression of endogenous markers of either immature neurons (DCX, PSA-NCAM) or the neurogenic niche (NeuroD, stathmin) in the adult hippocampus, tissue sections were blocked using 10% horse serum in phosphate buffer prior to overnight exposure at room temperature to the following antibodies: goat anti-DCX (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-PSA-NCAM (1:500; kind gift from Prof. T. Seki, Juntendo University, Tokyo, Japan; ref. 23), rabbit anti-stathmin (1:250; Calbiochem, San Diego, CA, USA), and goat anti-NeuroD (1:200; Santa Cruz Biotechnology). Following washes in 0.1 M phosphate buffer, sections were incubated with secondary antibodies: biotinylated anti-goat IgG (1:250; Vector Laboratories), Alexa 488-conjugated donkey anti-mouse (1:250; Molecular Probes, Eugene, OR, USA), Alexa 488-conjugated donkey anti-rabbit (1:250; Jackson ImmunoResearch, Bar Harbor, ME, USA) at room temperature for 3 h. An avidin-biotin complex (Vector Laboratories) was used for signal amplification of biotinylated secondary antibodies, which was then detected with diaminobenzidine (Sigma). To visualize immunofluorescence, sections were mounted in Vectashield (Vector Laboratories) and viewed using a Nikon Eclipse 90i fluorescence microscope (Nikon, Tokyo, Japan).

For experiments to address the neuronal differentiation of adult dentate granule cell progenitors, double-labeling experiments were carried out. Sections were incubated overnight

with the following primary antibody cocktails: rat anti-BrdU (1:500; Accurate Biochemicals, Westbury, NY, USA) with mouse anti-neuronal nuclei (NeuN) (1:1000; Chemicon, Temecula, CA, USA) to assess the neuronal differentiation of adult hippocampal progenitors; or rat anti-BrdU (1:500; Covance, Vienna, VA, USA) with rabbit anti-calretinin (1:250; Swant, Bellinzona, Switzerland) to examine the colocalization of BrdU with the transient immature neuron marker, calretinin. Sections were then incubated with the following cocktails of secondary antibodies: biotinylated anti-rat IgG (1:500; Chemicon) with Alexa 555-conjugated anti-mouse (1:250; Molecular Probes) or Cy3-conjugated donkey anti-rat and FITC-conjugated donkey anti-mouse (both 1:250; Jackson ImmunoResearch). Signal amplification was performed using FITC-conjugated streptavidin (1:250; Molecular Probes), following which sections were mounted using Vectashield (Vector Laboratories). Sections were observed with a Zeiss Axiovert confocal laser scanning microscope (510LSM; Carl Zeiss, Oberkochen, Germany) to determine immunofluorescence colocalization using confocal z -plane sectioning.

For experiments to determine the neuronal or glial differentiation of adult dentate granule cell progenitors, triple-labeling studies were carried out. Sections were incubated overnight with a cocktail of primary antibodies: rat anti-BrdU (1:500; Accurate Biochemicals) with mouse anti-NeuN (1:1000; Chemicon) and rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; Chemicon). Sections were incubated with a cocktail of secondary antibodies: biotinylated anti-rat IgG (1:500; Chemicon) with Alexa 555-conjugated anti-mouse (1:250; Molecular Probes) and Cy5-conjugated anti-rabbit (1:500; Chemicon), followed by incubation with Alexa 488-conjugated streptavidin (1:500, Molecular Probes). Sections were mounted using Vectashield (Vector Laboratories), and immunofluorescence colocalization was determined using confocal z -plane sectioning with an Olympus Fluoview FV 1000 laser scanning microscope (Olympus, Tokyo, Japan).

For double labeling in TR α 1-GFP mice, sections were incubated overnight with rabbit anti-GFP (1:5000; Abcam, Cambridge, UK) along with either goat anti-NeuroD (1:200) or goat anti-DCX (1:250; both Santa Cruz Biotechnology), followed by a cocktail of secondary antibodies: anti-goat Alexa 594 and anti-rabbit Alexa 488 (both 1:1000; Invitrogen, Stockholm, Sweden). For BrdU double-labeling, the sections were first incubated with anti-GFP primary and secondary antibodies before BrdU pretreatment and immunohistochemistry, as described above. The GFP signal was specific as demonstrated by the lack of any immunofluorescence with an anti-GFP antibody in wild-type mice. Colocalization of GFP signal in TR α 1-GFP mice with the markers (BrdU, NeuroD, and DCX) was examined using confocal z -stack images on a Zeiss Axiovert confocal laser-scanning microscope (510LSM). For all immunohistochemistry and immunofluorescence experiments, the specificity of antibody signal was confirmed using the following controls: absence of primary antibody, or isotype-matched IgG controls.

Cell counting

Quantitation of BrdU⁺ cell numbers in hippocampal sections was carried out using a previously described modified, unbiased stereology protocol (24) on a Zeiss Axioskop microscope. Sections spanned the rostrocaudal extent of the hippocampus (bregma -1.34 to -3.80) (25), and every 6th hippocampal section was processed for quantitation (12 sections/animal). Sections were coded, and the quantitation was conducted by an experimenter masked to the code. BrdU⁺ cells within dentate gyrus (DG) were counted as being in the subgranular zone (SGZ)/granule cell layer (GCL) when they were directly touching the SGZ or within it. The

total number of BrdU⁺ cells in the SGZ/GCL was estimated by multiplying the total number of BrdU cells counted from every 6th section by the section periodicity, 6.

Quantitation of DCX⁺ cells in hippocampal sections was carried out by an experimenter masked to the study code. The number of DCX⁺ cells in the SGZ of the DG were quantitated (4 sections/animal, $n=5$ /group). The results were expressed as the number of DCX⁺ cells per section. We also addressed the morphological status of DCX⁺ cells by categorizing them as DCX⁺ cells without tertiary dendrites or DCX⁺ cells with complex tertiary arbors (26). Quantitation of both DCX⁺ cell numbers and morphological category was performed under masked conditions using a Zeiss Axioskop at $\times 400$.

Quantitation of proliferating cell nuclear antigen (PCNA), PSA-NCAM, stathmin, and NeuroD⁺ cells was performed using the same approach used for quantitation of DCX⁺ cell numbers.

To examine the differentiation of BrdU⁺ progenitors into neurons or glia in the TR α 1^{-/-} and TR α 2^{-/-} mice, the percentage of BrdU⁺ cells that colocalized with the neuronal marker NeuN or the glial marker GFAP was determined using confocal microscopy. In each animal ($n=5$ /group), 20 BrdU⁺ cells were analyzed using z -plane sectioning with 0.41- μ m steps on an Olympus Fluoview FV100 laser-scanning confocal microscope to confirm colocalization with either NeuN or GFAP. In the TR α 1^{+/-m} mice, 50 BrdU⁺ cells in each animal per marker were analyzed to confirm colocalization with either the transient neuronal marker calretinin or the mature neuronal marker NeuN, using z -plane sectioning on a Zeiss Axiovert 510LSM confocal laser-scanning microscope. Percentage colocalization of BrdU with either NeuN or calretinin was determined for BrdU⁺ cells within the SGZ/GCL or directly in contact with the SGZ and was confirmed using z -plane stack analysis of confocal images.

In TR α 1-GFP mice, 20 BrdU, NeuroD, or DCX-immunopositive cells per animal ($n=3$) were analyzed for colocalization with GFP using z -plane sectioning on a Zeiss Axiovert 510LSM confocal laser-scanning microscope.

Statistical analysis

Results were subjected to statistical analysis using the program Prism (GraphPad, San Diego, CA, USA). Experiments with two groups were analyzed for differences using the unpaired Student's t test, with significance determined at $P < 0.05$. Experiments with 4 groups were subjected to statistical analyses using analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test, with significance determined at values of $P < 0.05$.

RESULTS

TR α 1 is expressed in newborn neurons of the adult hippocampus

Given recent evidence that thyroid hormone regulates adult hippocampal neurogenesis (9–11) and that the most abundant thyroid hormone receptor isoform in the brain is TR α 1 (16), we sought to further elucidate the function of this receptor in hippocampal neurogenesis. To examine the expression of the TR α 1 receptor in adult hippocampal progenitors, we used a novel knock-in TR α 1-GFP mouse strain generated by inserting the coding sequence of eGFP in frame with the

TR α 1 gene. In the hippocampus, GFP expression was observed within nuclei of cells in the SGZ and the GCL of the DG subfield. Double immunohistochemistry for the mitotic marker BrdU and GFP was performed to determine whether TR α 1 is expressed by the proliferating pool of adult hippocampal progenitors. The lack of colocalization of TR α 1-GFP with BrdU in hippocampal progenitors (Fig. 1A) indicated that this receptor was not expressed by proliferating progenitor cells within the SGZ in the DG. To address whether TR α 1 is expressed later in neuronal development, *i.e.*, in the largely postmitotic pool of adult hippocampal progenitors, we performed double immunohistochemistry for GFP and 2 markers for immature neurons; NeuroD and DCX. Confocal analysis revealed that TR α 1 was expressed in both NeuroD and DCX⁺ progenitors in the SGZ and GCL within the DG (Fig. 1B, C). These results indicate that within the neurogenic niche of the adult hippocampus, TR α 1 is predominantly expressed by postmitotic progenitors destined to acquire a neuronal fate, and does not appear to be present in proliferating progenitors. On the basis of these findings along with our previous results (10), we hypothesized that TR α 1 plays a role in the survival of adult hippocampal progenitors, and used mice lacking or harboring a mutation in the TR α isoforms to further investigate this hypothesis.

Proliferation of adult hippocampal progenitors is unaffected in the DG of TR α 1^{-/-} and TR α 2^{-/-} mice

Male TR α 1^{-/-} mice and wild-type littermate controls were injected with BrdU 2 h prior to sacrifice in order to assess the influence of TR α 1 receptor loss on the proliferation of adult hippocampal progenitors (Fig. 2). Stereological analysis indicated no change in the number of BrdU⁺ progenitors in the SGZ/GCL of TR α 1^{-/-} mice as compared to wild-type animals (Fig. 2A, left graph). We also examined the expression of an endogenous marker of cell division, PCNA, and the number

of PCNA⁺ cells in the SGZ/GCL was unaltered in TR α 1^{-/-} mice (Fig. 2A, right graph). We next examined the numbers of BrdU- and PCNA⁺ cells in the SGZ/GCL of TR α 2^{-/-} mice that overexpress TR α 1 as a direct consequence of the gene-targeting strategy. TR α 2^{-/-} mice do not exhibit any change in the number of BrdU- or PCNA⁺ cells within the SGZ/GCL, indicating no effect in these mice on hippocampal progenitor proliferation (Fig. 2B, graphs). In both wild-type and TR α 1- and TR α 2-deficient mice, BrdU- and PCNA⁺ cells were observed in clusters within the SGZ at the border of the GCL and the hilus.

TR α 1^{-/-} and TR α 2^{-/-} mice show opposing survival of adult hippocampal progenitors

To examine the postmitotic survival of adult hippocampal progenitors, TR α 1^{-/-} and TR α 2^{-/-} knockout mice and their respective wild-type controls received daily BrdU injections for 3 d and were sacrificed 28 d later. TR α 1^{-/-} mice showed a significant increase in the number of BrdU⁺ cells in the SGZ/GCL as compared to wild-type controls, which indicated an increased survival of adult hippocampal progenitors in the mutants (Fig. 3A, right panel). In striking contrast, TR α 2^{-/-} mice demonstrated a significant decline in the number of persisting BrdU⁺ cells in the SGZ/GCL (Fig. 3B, right panel). The surviving BrdU⁺ cells exhibit a characteristic ovoid shape and are not observed in clusters but are seen dispersed through the GCL (Fig. 3, image panels).

TR α 1^{-/-} and TR α 2^{-/-} mice exhibit contrasting effects on the numbers of DCX⁺ immature neurons in the adult DG

DCX is a microtubule-associated protein expressed by postmitotic and migratory adult hippocampal progenitors (12) and is a useful endogenous marker for adult hippocampal neurogenesis). TR α 1^{-/-} mice exhibited

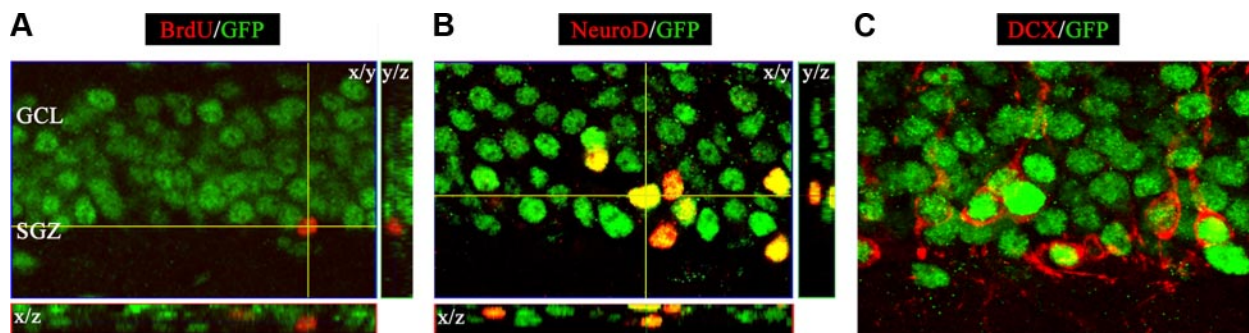


Figure 1. TR α 1 is expressed in Neuro-D- and DCX⁺ progenitors within the DG. TR α 1-GFP-knock-in mice generated by insertion of the coding sequence of eGFP in frame with the TR α 1 gene were utilized to study the expression of TR α 1-GFP chimeric protein using double immunofluorescence studies. TR α 1-GFP-knock-in mice received a single injection of BrdU (150 mg/kg, *n*=3) and were sacrificed 2 h later. A) Confocal z-stack image at $\times 630$, indicating the lack of colocalization of TR α 1-GFP with BrdU within the SGZ of the DG. B) NeuroD⁺ progenitors within the SGZ and GCL of the DG colocalized with GFP, as shown in a representative confocal z-stack image ($\times 630$). C) DCX-immunopositive immature neurons were also observed to exhibit colocalization with GFP at $\times 630$, indicating the presence of TR α 1 in DCX⁺ immature neurons.

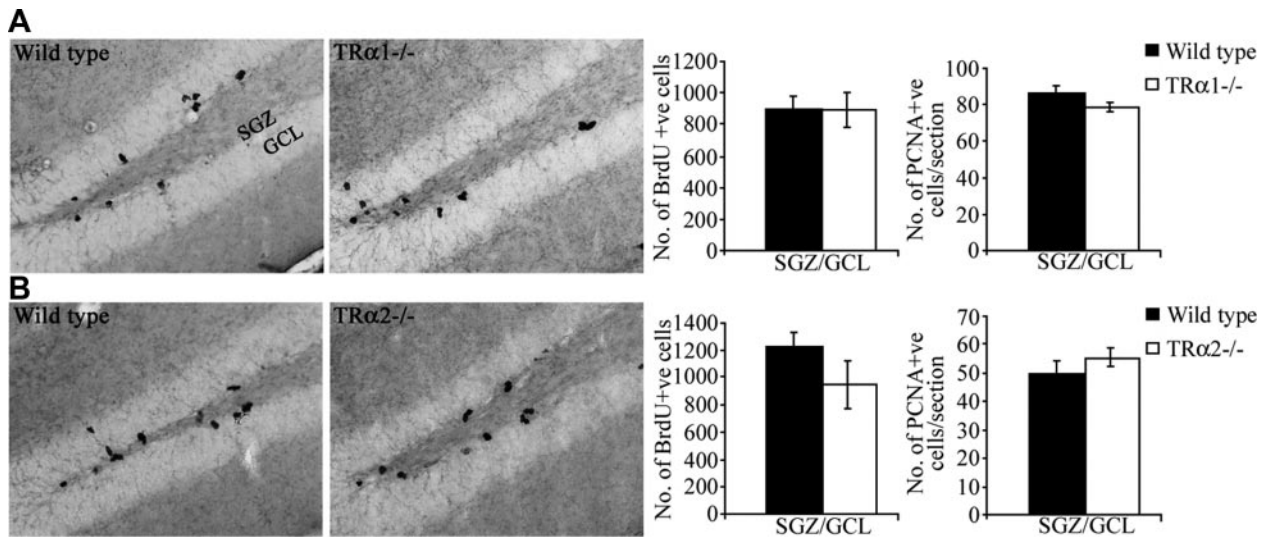


Figure 2. TRα1^{-/-} and TRα2^{-/-} mice do not exhibit any change in the proliferation of adult hippocampal progenitors. TRα1^{-/-} and TRα2^{-/-} mice and respective wild-type littermate controls received a single injection of BrdU (100 mg/kg, *n*=3–5/group) and were sacrificed 2 h later to assess the numbers of proliferating adult hippocampal progenitors in these mutant mice. Images show representative photomicrographs of BrdU⁺ cells at ×200 from TRα1^{-/-} (A) and TRα2^{-/-} (B) mice (right images), along with respective wild-type controls (left images). BrdU⁺ cells were predominantly observed in clusters within the SGZ, at the border of the hilus and the GCL. Left graphs: stereological analysis revealed no change in number of BrdU⁺ cells within the SGZ/GCL in TRα1^{-/-} (A) and TRα2^{-/-} (B) mice, as compared to their respective wild-type controls. Right graphs: to examine ongoing adult hippocampal progenitor proliferation, we also used immunohistochemical analysis of an endogenous marker of cell proliferation, PCNA. Quantitative analysis did not indicate any change in numbers of PCNA⁺ cells within the SGZ/GCL of TRα1^{-/-} (A) and TRα2^{-/-} (B) mice, as compared to respective wild-type controls. Results are expressed as the mean ± SE number of BrdU⁺ cells in the SGZ/GCL (*n*=3–5/group), or number of PCNA⁺ cells per section.

a significant increase in the number of DCX⁺ cells/section within the SGZ/GCL (Fig. 4A, right panel), whereas TRα2^{-/-} mice had reduced DCX⁺ cell numbers as compared to their wild-type controls (Fig. 4B, right panel). Taken together, the BrdU and DCX results indicate an increased hippocampal neurogenesis in TRα1^{-/-} mice in contrast to the decrease seen in the TRα2^{-/-} strain.

DCX immunohistochemistry marks both the cell body and the dendritic arbors of newborn neurons (Fig. 4A, B). As immature neurons undergo a morpho-

logical maturation, the dendritic tree becomes more elaborate with the presence of complex tertiary dendrites. As described previously (26), we subcategorized DCX⁺ cells as those with or without tertiary dendrites (Fig. 4C, left panels). While numbers of DCX⁺ cells were clearly different in TRα1^{-/-} and TRα2^{-/-} mice, we did not observe any change in the morphological maturation of these DCX⁺ cells, as the percentage of DCX⁺ cells with complex tertiary dendrites was unaltered in the mutants as compared to their controls (Fig. 4C, right panels).

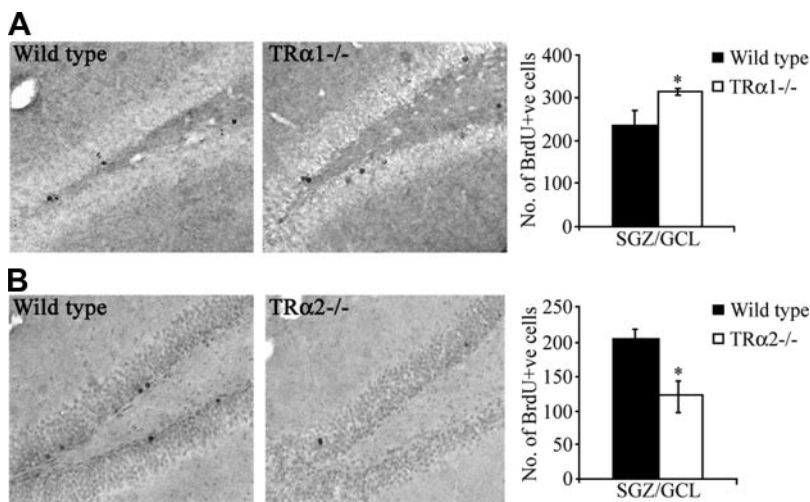


Figure 3. TRα1^{-/-} mice exhibit an increase in survival of BrdU⁺ adult hippocampal progenitors, whereas TRα2^{-/-} mice show a decrease in survival of BrdU⁺ progenitors. TRα1^{-/-} and TRα2^{-/-} mice and respective wild-type littermate controls received BrdU (150 mg/kg) once daily by intraperitoneal injection for 3 d and were sacrificed 28 d after final BrdU injection. Images show representative photomicrographs of BrdU⁺ cells from TRα1^{-/-} (A) and TRα2^{-/-} (B) mice (right panels), along with respective wild-type littermate controls (left panels) at ×200. BrdU⁺ cells within the SGZ and the GCL were ovoid in morphology and rarely observed in clusters. Right panels: quantitative stereological analysis showed that TRα1^{-/-} knockout mice had significantly higher numbers of surviving BrdU⁺ cells in the SGZ/GCL (A), whereas TRα2^{-/-} knockout mice showed a significant

decrease in numbers of BrdU⁺ cells in the SGZ/GCL (B). Results are expressed as the mean ± SE number of BrdU⁺ cells in the SGZ/GCL (*n*=3–5/group). **P* < 0.05 vs. wild-type control (Student's *t* test).

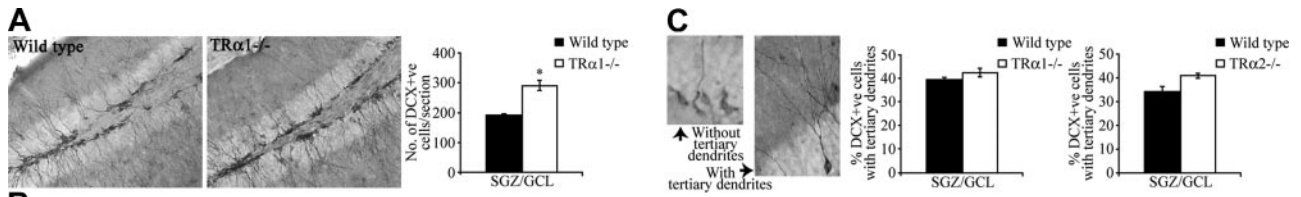


Figure 4. TR $\alpha 1^{-/-}$ mice exhibit an increase in the number of DCX $^{+}$ immature neurons in the adult hippocampus, whereas TR $\alpha 2^{-/-}$ mice show a decline in the numbers of hippocampal DCX $^{+}$ immature neurons. Immunohistochemistry for DCX was carried out to examine the survival of postmitotic adult hippocampal progenitors. *A, B*) Representative photomicrographs of DCX $^{+}$ immature neurons at $\times 200$ within the SGZ/ GCL in the DG subfield in TR $\alpha 1^{-/-}$ (*A*) and TR $\alpha 2^{-/-}$ (*B*) mice (right images), along with respective wild-type controls (left images). Quantitative analysis revealed that TR $\alpha 1^{-/-}$ mice exhibit a significant increase in the number of DCX $^{+}$ cells in the SGZ/GCL, as compared to wild-type controls (*A*, right panel). In striking contrast, TR $\alpha 2^{-/-}$ mice showed a significant decline in the number of DCX $^{+}$ cells in the SGZ/GCL, as compared to wild-type controls (*B*, right panel). *C*) Analysis of the dendritic morphology of DCX $^{+}$ cells revealed no change in the morphological maturation of DCX $^{+}$ cells in TR $\alpha 1^{-/-}$ and TR $\alpha 2^{-/-}$ mice as compared to their wild-type littermate controls. Left panels: representative images of DCX $^{+}$ cells at $\times 400$, categorized as with or without tertiary dendrites. Right panels: Quantitative analysis of percentage distribution of DCX $^{+}$ cells with tertiary dendrites in the SGZ/GCL revealed no change in TR $\alpha 1^{-/-}$ (left graph) and TR $\alpha 2^{-/-}$ (right graph) mice, as compared to wild-type controls. Results are expressed as mean \pm SE number of DCX $^{+}$ cells per section in the SGZ/GCL, or mean \pm SE percentage of DCX $^{+}$ cells with tertiary dendrites in the SGZ/GCL ($n=3-5$ /group). * $P < 0.05$ vs. wild-type control (Student's *t* test).

TR $\alpha 1^{-/-}$ and TR $\alpha 2^{-/-}$ mice show an altered expression of markers of the neurogenic niche

Immunohistochemical analyses were performed to determine whether TR $\alpha 1^{-/-}$ and TR $\alpha 2^{-/-}$ mice exhibit changes in expression of additional stage-specific markers for adult hippocampal neurogenesis, such as PSA-NCAM and stathmin, which have previously been shown to colocalize with DCX expression (13, 27, 28). Similar to what was seen with DCX, TR $\alpha 1^{-/-}$ mice exhibited a significant increase, whereas TR $\alpha 2^{-/-}$ mice showed a significant decline in the number of cells positive for PSA-NCAM (TR $\alpha 1^{-/-}$: Fig. 5*A*, TR $\alpha 2^{-/-}$: Fig. 5*B*) and stathmin (TR $\alpha 1^{-/-}$: Fig. 5*C*, TR $\alpha 2^{-/-}$: Fig. 5*D*) as compared to their wild-type controls. While PSA-NCAM expression (Fig. 5*A, B*) was noted in both

the cell body, as well as the dendritic arbor similar to DCX expression, stathmin expression (Fig. 5*C, D*) was predominantly observed in the cell body. Taken together, our results demonstrate that TR $\alpha 1^{-/-}$ mice show a significant increase, while TR $\alpha 2^{-/-}$ mice exhibit a decline, in the number of DCX-, PSA-NCAM- and stathmin-positive cells within the adult hippocampal neurogenic niche.

We also sought to address whether the TR $\alpha 1^{-/-}$ and TR $\alpha 2^{-/-}$ mice exhibit any gross changes in hippocampal and GCL volume using modified stereological approaches (10). We observed no significant change in either the total hippocampal volume or in the volume of the GCL in the DG hippocampal subfield in the TR $\alpha 1^{-/-}$ or TR $\alpha 2^{-/-}$ mice, as compared to their respective littermate wild-type controls (Supplemental

Figure 5. TR $\alpha 1^{-/-}$ and TR $\alpha 2^{-/-}$ mice display altered expression of neurogenic niche markers. Numbers of PSA-NCAM and stathmin-immunopositive cells in TR $\alpha 1^{-/-}$ and TR $\alpha 2^{-/-}$ mice were assessed using immunofluorescence. Panels show representative immunofluorescence images for PSA-NCAM (*A, B*) and stathmin (*C, D*) from TR $\alpha 1^{-/-}$ (*A, C*) and TR $\alpha 2^{-/-}$ mice (*B, D*) and respective wild-type controls at $\times 200$. Quantitative analysis indicated a significant increase in numbers of both PSA-NCAM- and stathmin-positive cells in the SGZ/GCL of the TR $\alpha 1^{-/-}$ mice vs. wild-type controls (*A, C*). In contrast, a significant decline in numbers of both PSA-NCAM- and stathmin-positive cells per section was observed in TR $\alpha 2^{-/-}$ mice vs. wild-type controls (*B, D*). Results are expressed as mean \pm SE number of PSA-NCAM- or stathmin-positive cells per section in the SGZ/GCL ($n=3-5$ /group). * $P < 0.05$ vs. wild-type control (Student's *t* test).

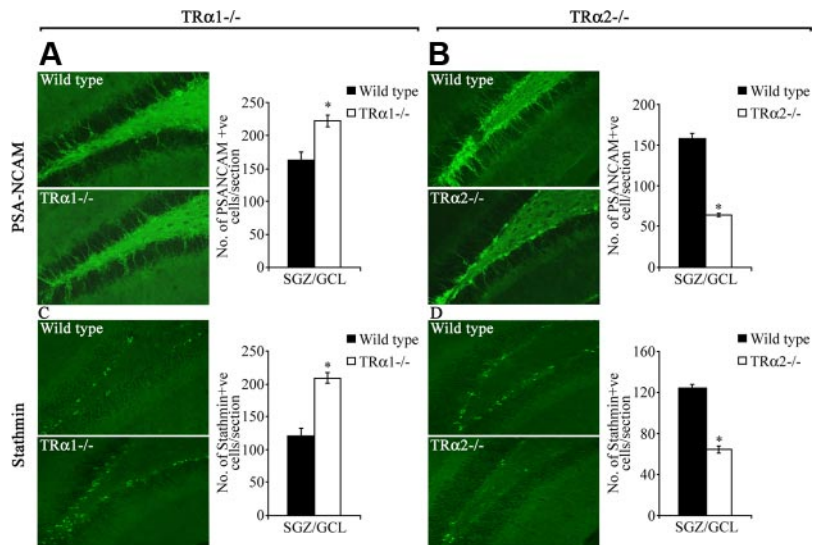


Fig. 1). Further, granule cell neuron marker (Prox1) expression, mossy fiber bundle innervation of the CA3, and GFAP-immunopositive glial cell numbers in the DG subfield were found to be unaltered in the TR α 1^{-/-} or TR α 2^{-/-} mice as compared to their respective littermate wild-type controls (Supplemental Figs. 2 and 3).

Differentiation of newborn progenitors is not perturbed in TR α 1^{-/-} and TR α 2^{-/-} mice

To address whether cell fate acquisition by newborn adult hippocampal progenitors is altered in TR α 1^{-/-} and TR α 2^{-/-} mice, we examined the percentage of colocalization of BrdU with the neuronal marker NeuN or the glial marker GFAP, 28 d after BrdU administration using triple immunofluorescence and confocal microscopy. Quantitative analysis, based on confocal z-stack analysis to detect colocalization, revealed no change in the percentage of colocalization of BrdU with NeuN or GFAP in both TR α 1^{-/-} and TR α 2^{-/-} mice, as compared to the wild-type controls (Fig. 6B, C). In wild type, as well as TR α 1^{-/-} and TR α 2^{-/-} mice, more BrdU⁺ cells acquired a neuronal, rather than a glial, fate. BrdU⁺ cells that do not colocalize with either NeuN or GFAP may represent as yet undifferentiated cells.

Thyroid hormone administration to TR α 2^{-/-} mice rescues the decline in the number of DCX⁺ immature neurons

We hypothesized that the decreased survival of BrdU⁺ progenitors and the decline in DCX, PSA-NCAM, and stathmin cell numbers in TR α 2^{-/-} mice may reflect a limited availability to ligand for the overexpressed TR α 1, thus causing an aporeceptor effect in the neurogenic niche similar to those described previously for other tissues (18). To test this hypothesis, we treated the TR α 2^{-/-} mice with excess thyroid hormone. Ani-

mals were divided into 4 groups, with TR α 2^{-/-} mice and their wild-type controls receiving either vehicle or thyroid hormone in drinking water for 16 d prior to sacrifice. We then assessed whether the hormone treatment could rescue the decrease in number of hippocampal progenitors expressing the endogenous marker, DCX, in the TR α 2^{-/-} mice. Thyroid hormone treatment was capable of completely rescuing the decline in DCX⁺ cell number in the SGZ/GCL of TR α 2^{-/-} mice (Fig. 7B). Interestingly, thyroid hormone treatment also significantly enhanced the number of DCX⁺ cells in the SGZ/GCL of wild-type animals. These results support the hypothesis that the decrease in progenitor survival observed in TR α 2^{-/-} mice, is a consequence of a TR α 1 aporeceptor effect, which can be ameliorated through the restoration of an appropriate ligand-receptor ratio *via* thyroid hormone treatment.

Rescue of survival and neuronal differentiation of adult hippocampal progenitors in TR α 1^{+m} mice by thyroid hormone administration

To further address the hypothesis that an unliganded TR α 1 results in a decline in adult hippocampal neurogenesis, we used mice expressing a mutant TR α 1 with dominant-negative activity. These heterozygous mice (TR α 1^{+m}) were generated by introducing a point mutation in the TR α 1 gene (TR α 1R384C) that lowers the affinity for thyroid hormone 10-fold (20). We first studied the effects of reduced ligand-binding in TR α 1^{+m} mice on adult hippocampal progenitor proliferation. Wild-type controls and TR α 1^{+m} mice received a single BrdU injection and were sacrificed 2 h later. BrdU immunohistochemistry followed by stereological quantitative analysis showed that mutant mice did not differ from wild-type animals in the number of BrdU⁺ cells; these results were corroborated by PCNA immunohistochemistry (Fig. 8A, B).

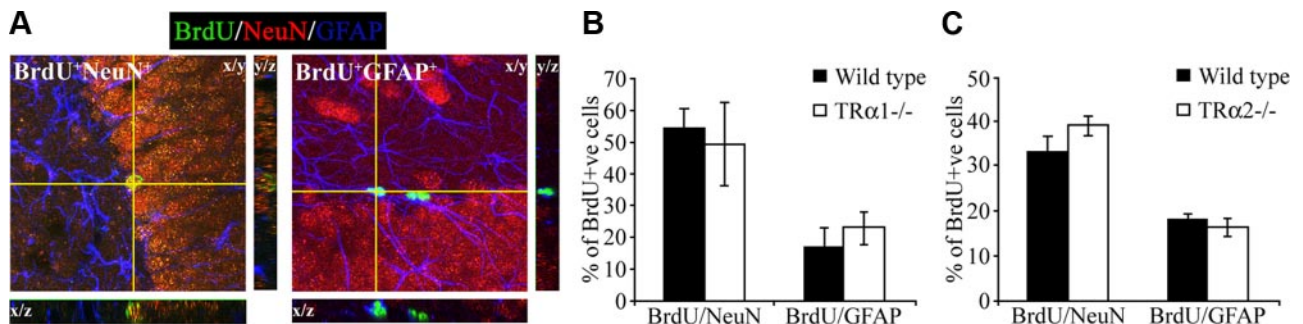
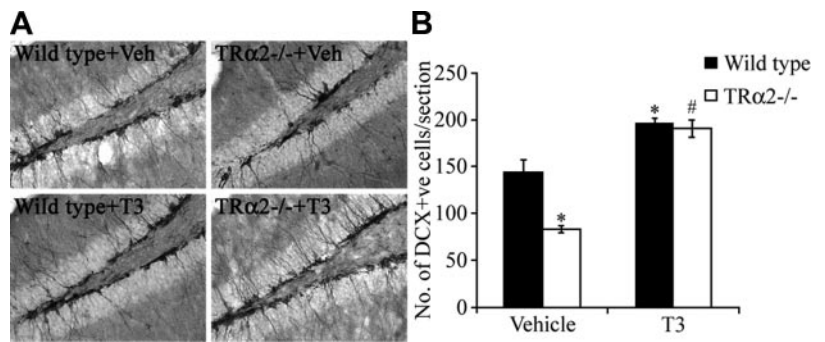


Figure 6. Differentiation of adult hippocampal progenitors into NeuN⁺ neurons or GFAP⁺ glia is unaffected in TR α 1^{-/-} and TR α 2^{-/-} mice. TR α 1^{-/-} and TR α 2^{-/-} mice, along with their respective wild-type littermate controls, received BrdU (150 mg/kg) once daily by intraperitoneal injection for 3 d and were sacrificed 28 d after the last injection. To examine the neuronal and glial differentiation of newborn adult hippocampal progenitors within the SGZ/ GCL triple immunofluorescence for BrdU, the mature neuronal marker, neuronal nuclei (NeuN), and the glial marker, GFAP was performed. A) Representative merged confocal z-stack images of BrdU colocalization with NeuN (BrdU⁺NeuN⁺) or GFAP (BrdU⁺GFAP⁺), with the cell at the crosshairs demonstrating colocalization (\times 600). B, C) Confocal z-stack analysis indicated no change in colocalization of BrdU with either NeuN or GFAP in the TR α 1^{-/-} (B) or the TR α 2^{-/-} (C) mice *vs.* wild-type controls. Results are expressed as mean \pm SE percentage colocalization of BrdU⁺ cells with NeuN or GFAP in the SGZ/GCL ($n=3-5$ /group).

Figure 7. Thyroid hormone treatment rescues the decrease in number of DCX⁺ immature neurons in TR α 2^{-/-} mice. Wild-type and TR α 2^{-/-} mice were divided into 4 experimental groups (wild type + veh, wild type + T3, TR α 2^{-/-} + veh, and TR α 2^{-/-} + T3). The groups receiving thyroid treatment were given T3 (0.5 μ g/ml) in drinking water for 16 d prior to sacrifice. **A**) Representative photomicrographs (\times 200) of DCX immunohistochemistry in the SGZ/GCL of vehicle- and T3-treated wild-type and TR α 2^{-/-} mice. **B**) Stereological quantitative analysis revealed a significant decline in the number of cells expressing DCX in the vehicle-treated TR α 2^{-/-} mice *vs.* vehicle-treated wild-type controls. This decrease was rescued by thyroid hormone treatment, as the number of DCX⁺ cells in the T3-treated TR α 2^{-/-} mice was significantly higher than vehicle-treated TR α 2^{-/-} mice. There was also a significant increase in the number of DCX⁺ cells in the SGZ/GCL of T3-treated wild-type mice *vs.* vehicle treated wild-type controls. Results are expressed as mean \pm SE number of DCX⁺ cells per section in the SGZ/GCL ($n=4$ or 5 /group). * $P < 0.05$ *vs.* vehicle-treated wild-type control group; # $P < 0.05$ *vs.* vehicle-treated TR α 2^{-/-} group (ANOVA and Bonferroni *post hoc* test).



To examine the survival of BrdU⁺ progenitors in the hippocampus, wild-type controls and TR α 1^{+m} mice were injected with BrdU once daily for 3 d and sacrificed 30 d after the last BrdU injection. As compared to wild-type mice, a significant decrease in the number of surviving BrdU⁺ cells was seen in TR α 1^{+m} mice. Thyroid hormone treatment of TR α 1^{+m} mice normalized the numbers of surviving BrdU⁺ cells as compared to vehicle-treated wild-type controls (Fig. 8D). Interestingly, thyroid hormone treatment to wild-type controls itself resulted in an increased number of BrdU⁺ cells that persisted 30 d after BrdU injection. Taken together, with the results obtained from the TR α 2^{-/-} mice, the data indicate that an unliganded TR α 1 receptor could function as an inhibitor of adult hippocampal neurogenesis through an effect on the survival of newborn hippocampal progenitors.

To determine whether the unliganded TR α 1 influences the differentiation of BrdU⁺ progenitors into mature neurons, double-labeling studies were performed using antibodies to BrdU and NeuN. Confocal analysis revealed a significant reduction in the percentage of BrdU⁺ cells that are immunopositive for the neuronal marker NeuN (Fig. 8E) indicating that fewer progenitor cells acquire a neuronal phenotype in TR α 1^{+m} mice. Thyroid hormone treatment ameliorated the decrease in neuronal differentiation observed in TR α 1^{+m} mutant mice (Fig. 8E). However, thyroid hormone treatment to wild-type mice did not influence neuronal differentiation. These results suggest that unliganded TR α 1 influences both hippocampal progenitor cell survival and neuronal differentiation.

Given the decline observed in BrdU/NeuN colocalization in TR α 1^{+m} mice, we next sought to address whether the expression of calretinin in BrdU⁺ postmitotic hippocampal progenitors is also altered in TR α 1^{+m} mice. Calretinin, a calcium-binding protein, is transiently expressed in the postmitotic stages of hippocampal progenitor cell development (29), and calretinin-immunopositive cells represent a fraction of immature, postmitotic adult hippocampal progenitors that are destined for neuronal differentiation. Calretinin expression is thought

to precede the expression of the mature neuronal marker NeuN. Confocal analysis revealed a significant decline in the colocalization of BrdU with calretinin in the TR α 1^{+m} mice, which was partially normalized by thyroid hormone treatment (Fig. 8F).

Decrease in NeuroD⁺ cells in the hippocampal neurogenic niche of TR α 2^{-/-} and TR α 1^{+m} mice

The transcription factor NeuroD is required for the differentiation of dentate granule cells of the hippocampus (30) and is expressed in adult hippocampal progenitors (31, 32). We examined by immunohistochemistry the number of NeuroD⁺ cells within the SGZ/GCL of TR α 2^{-/-} and TR α 1^{+m} mice. In wild-type mice, NeuroD was strongly expressed in the SGZ where adult hippocampal progenitors reside, whereas the expression was substantially lower in the GCL (Fig. 9A, C). In both mutant mouse strains we observed a significant reduction in the number of NeuroD-immunopositive cells within the SGZ/GCL as compared to their respective wild-type controls (Fig. 9). Thyroid hormone treatment normalized the number of NeuroD⁺ cells in TR α 1^{+m} mice (Fig. 9D). In the TR α 2^{-/-} mice, thyroid hormone treatment showed a trend to increase the number of NeuroD⁺ cells/section; however, this did not reach significance ($P=0.06$, ANOVA and Bonferroni *post hoc* test; Fig. 9B). Interestingly, thyroid hormone treatment resulted in a significant increase in NeuroD⁺ cell number in wild-type control littermates from both the TR α 2^{-/-} and TR α 1^{+m} experiments (Fig. 9B, D).

DISCUSSION

Our aim in this study was to address the role of TR α 1 in adult hippocampal neurogenesis using TR α 1^{-/-}, TR α 2^{-/-}, and TR α 1^{+m} mutant mice. We provide novel evidence that TR α 1 regulates adult hippocampal neurogenesis, through effects on progenitor survival and neuronal differentiation. The striking similarity

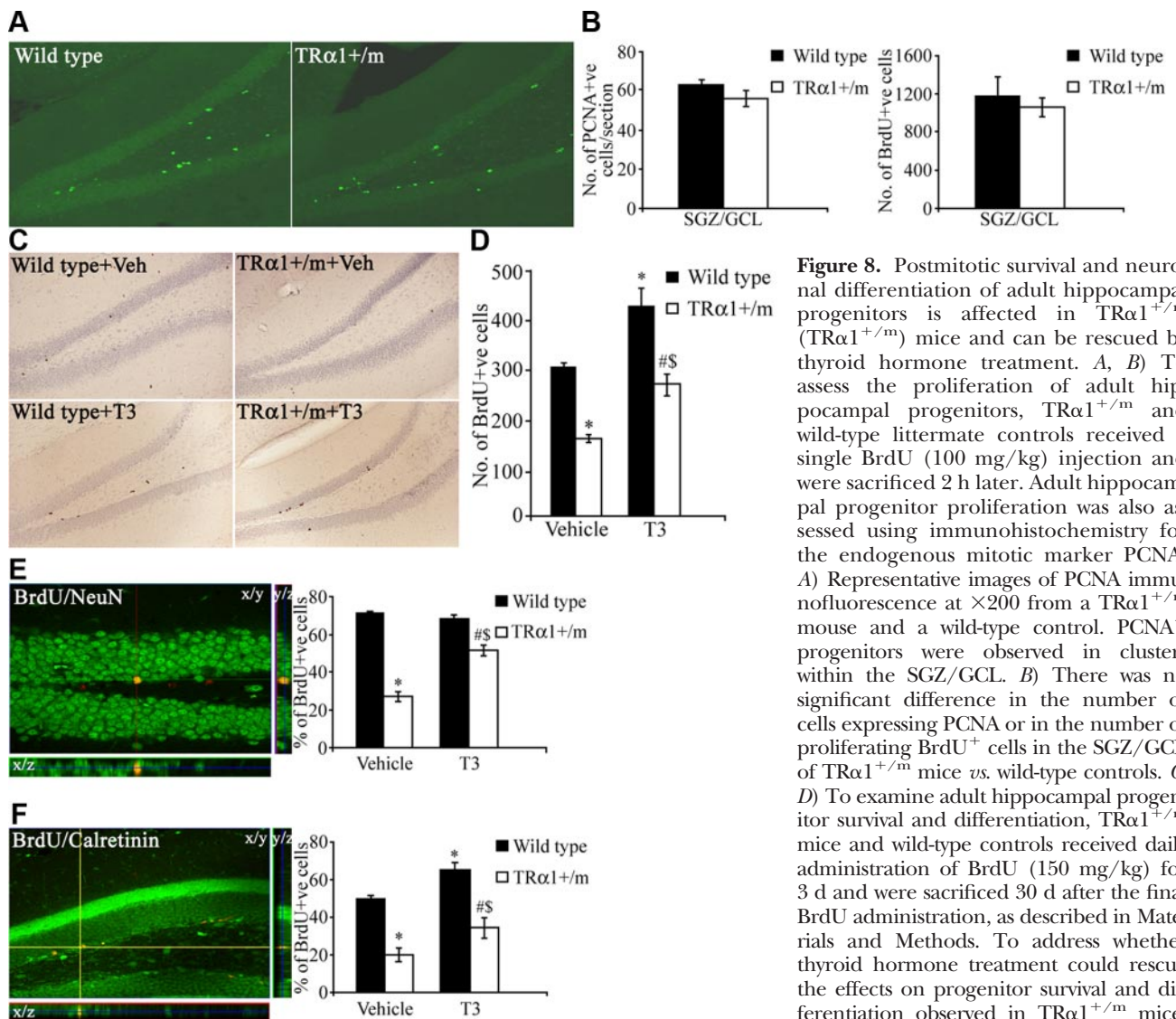


Figure 8. Postmitotic survival and neuronal differentiation of adult hippocampal progenitors is affected in $TR\alpha 1^{+/m}$ ($TR\alpha 1^{+/m}$) mice and can be rescued by thyroid hormone treatment. *A, B*) To assess the proliferation of adult hippocampal progenitors, $TR\alpha 1^{+/m}$ and wild-type littermate controls received a single BrdU (100 mg/kg) injection and were sacrificed 2 h later. Adult hippocampal progenitor proliferation was also assessed using immunohistochemistry for the endogenous mitotic marker PCNA. *A*) Representative images of PCNA immunofluorescence at $\times 200$ from a $TR\alpha 1^{+/m}$ mouse and a wild-type control. PCNA⁺ progenitors were observed in clusters within the SGZ/GCL. *B*) There was no significant difference in the number of cells expressing PCNA or in the number of proliferating BrdU⁺ cells in the SGZ/GCL of $TR\alpha 1^{+/m}$ mice *vs.* wild-type controls. *C, D*) To examine adult hippocampal progenitor survival and differentiation, $TR\alpha 1^{+/m}$ mice and wild-type controls received daily administration of BrdU (150 mg/kg) for 3 d and were sacrificed 30 d after the final BrdU administration, as described in Materials and Methods. To address whether thyroid hormone treatment could rescue the effects on progenitor survival and differentiation observed in $TR\alpha 1^{+/m}$ mice,

animals were divided into 4 experimental groups (wild type + veh, $TR\alpha 1^{+/m}$ + veh, wild type + T3, and $TR\alpha 1^{+/m}$ + T3). Thyroid hormone treatment was administered in drinking water as described in Materials and Methods. *C*) Representative photomicrographs of BrdU immunohistochemistry at $\times 200$ in the SGZ/GCL of vehicle and T3-treated wild-type and $TR\alpha 1^{+/m}$ mice. *D*) Number of BrdU⁺ cells that persist 30 d after BrdU administration in the SGZ/GCL of $TR\alpha 1^{+/m}$ mice was significantly lower than that observed in vehicle-treated wild-type controls. T3-treatment restored the decrease in postmitotic survival in the $TR\alpha 1^{+/m}$ mice and also significantly increased the number of BrdU⁺ cells in the SGZ/GCL of wild-type mice compared to vehicle-treated wild-type controls. *E, F*) Neuronal differentiation was assessed by examining the colocalization of BrdU with two markers of neuronal differentiation, NeuN and calretinin. Microphotos show representative merged confocal z-stack images of BrdU-NeuN colocalization (*E*) and BrdU-calretinin colocalization (*F*) at $\times 400$. Crosshairs indicate examples of BrdU⁺ cells that colocalize with NeuN (*E*) and calretinin (*F*), with orthogonal sections confirming colocalization in the z plane. Percentage colocalization of BrdU with NeuN (*E*) and with calretinin (*F*) was significantly reduced in $TR\alpha 1^{+/m}$ mice *vs.* wild-type controls. Thyroid hormone treatment was capable of partially rescuing the decline in BrdU-NeuN and BrdU-calretinin colocalization observed in $TR\alpha 1^{+/m}$ mice. Results are expressed as mean \pm SE number of BrdU⁺ cells (*B, D*), number of PCNA⁺ cells per section (*B*), or percentage colocalization of BrdU⁺ cells with NeuN (*E*) or calretinin (*F*) in the SGZ/GCL ($n=4$ or 5 /group). * $P < 0.05$ *vs.* vehicle-treated wild-type controls; # $P < 0.05$ *vs.* T3-treated wild-type controls; \$ $P < 0.05$ *vs.* vehicle treated $TR\alpha 1^{+/m}$ group (ANOVA and Bonferroni *post hoc* test).

between the decreased adult neurogenesis observed in hypothyroidism (9, 10), and our results of the neurogenic phenotype in mutant mice with $TR\alpha 1$ aporeceptor activity, strongly suggest that an unliganded $TR\alpha 1$ contributes to the neurogenic decline in adult-onset hypothyroidism.

While the requirement for thyroid hormone during neurodevelopment is well established (33, 34), it is only

recently that the effects of thyroid hormone on adult neurogenesis have been reported (9, 10, 11, 35). Impaired adult hippocampal neurogenesis is implicated as a key structural correlate that contributes to the functional deficits in hippocampus-dependent learning and mood-related behaviors observed in rodents and humans with adult-onset hypothyroidism (4, 11, 36). However, the role of individual TRs in adult neurogenesis

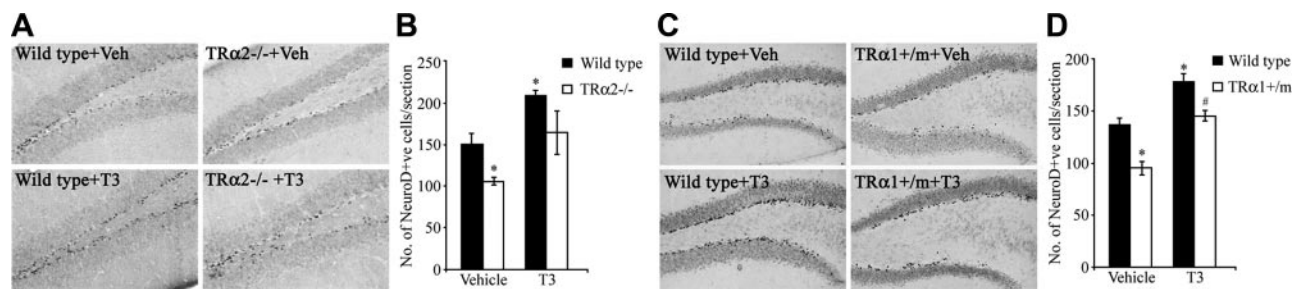


Figure 9. TRα2^{-/-} and TRα1^{+/m} mice exhibit a significant reduction in number of NeuroD⁺ cells within the SGZ/GCL. To assess the effect of thyroid hormone treatment on the number of NeuroD⁺ cells in TRα2^{-/-} and TRα1^{+/m} mice, treatment groups received T3 (0.5 μg/ml) in drinking water, as described in Materials and Methods. A, C) Representative photomicrographs of NeuroD-expressing cells at ×200 in the SGZ/ GCL of vehicle- and T3-treated TRα2^{-/-} mice (A) and vehicle- and T3-treated TRα1^{+/m} mice (C) and respective wild-type controls. B, D) There was a significant decrease in the number of NeuroD⁺ adult hippocampal progenitors in the SGZ/GCL of the TRα2^{-/-} knockout mice *vs.* wild-type controls, which was partially rescued by thyroid hormone treatment but did not reach significance ($P=0.06$, Bonferroni *post hoc* test) (B). Similarly, there was a significant decline in number of NeuroD-expressing cells in the SGZ/GCL of vehicle-treated TRα1^{+/m} mice *vs.* vehicle-treated wild-type controls, which was rescued by thyroid treatment (D). T3-treated wild-type mice also showed a significant increase in the number of NeuroD⁺ cells compared to vehicle-treated wild-type controls (B, D). Results are expressed as the mean ± SE number of NeuroD⁺ cells per section ($n=3-5$ /group). * $P < 0.05$ *vs.* vehicle-treated wild-type control; # $P < 0.05$ *vs.* T3-treated wild-type control (ANOVA and Bonferroni *post hoc* test).

remains largely unidentified, with a single report (35) suggesting a role for TRα isoforms in the regulation of progenitor cell cycling in the subventricular zone lining the lateral ventricles.

Approximately 75% of thyroid hormone binding by TRs in the adult rodent brain is accounted for by TRα1 (16, 37). Immunohistochemical evidence indicates TRα isoform expression in adult hippocampal progenitors (10), but their stage-specific expression is unknown. Our colocalization studies of the TRα1-GFP chimeric protein with stage-specific progenitor markers indicate TRα1 expression in NeuroD and DCX-immunopositive progenitors, but not in proliferating BrdU⁺ progenitors. Our unpublished results with the TRα1-GFP-knock-in mice indicate that TRα1 expression is restricted to postmitotic stages of neuronal progenitor development. Further, proliferation was not altered in TRα1^{-/-} mice, TRα2^{-/-} mice, or TRα1^{+/m} mutant mice. Taken together, these results imply that neither TRα1 nor TRα2 influence adult hippocampal progenitor proliferation. This is interesting in light of previous evidence (9, 10) that indicate no change in progenitor turnover following hypothyroidism or hyperthyroidism, suggesting a role for thyroid hormone in the postmitotic stages of adult hippocampal progenitor development.

TRα1^{-/-} mice exhibited an increased survival of adult hippocampal progenitors, in striking contrast to the TRα1-overexpressing TRα2^{-/-} mice that show a significant decline in progenitor survival. It could be argued that the decrease in postmitotic progenitor survival in the TRα2^{-/-} mice is a consequence of TRα1 overexpression, or TRα2 loss, or a combination of both. However, we hypothesized, on the basis of the previously described phenotype of the TRα2^{-/-} mice (18) that the overexpression of TRα1 may result in aporeceptor activity due to limited availability to ligand, and therefore reduced postmitotic survival of adult hip-

poampal progenitors. Strong support for this hypothesis comes from our results with the TRα1^{+/m} mice that express a mutant TRα1 with aporeceptor activity, which show a robust decline in adult hippocampal progenitor survival. Both the TRα2^{-/-} and TRα1^{+/m} mice show a phenotype similar to hypothyroidism with decreased hippocampal progenitor survival, adding credence to the hypothesis that a TRα1 aporeceptor contributes to the deleterious consequences of hypothyroidism on adult hippocampal neurogenesis (Fig. 10). Interestingly, TRα1 deficiency enhances hippocampal progenitor survival suggesting the possibility that in the TRα1^{-/-} mice, besides the loss of liganded TRα1, the removal of a strong repressor activity of unliganded TRα1 may underlie the increased progenitor survival.

The cellular mechanisms for the effects of TRα1 on hippocampal progenitor survival are at present unclear. However, it is tempting to draw parallels with the proposed mechanisms for the actions of TRα1 on embryonic neuronal progenitors. Unliganded TRα1 has been suggested to arrest neuronal progenitors at specific stages of development until ligand availability allows them to progress further into neuronal differentiation (38). Adult hippocampal progenitors may recapitulate the effects of TRα1 aporeceptors observed in their developmental counterparts, with the TRα1 aporeceptor affecting expression of genes relevant for the progression of adult hippocampal progenitors to the next developmental stage. Indeed in both the TRα2^{-/-} and TRα1^{+/m} mice, the decline in progenitor survival suggests that TRα1 aporeceptors may result in postmitotic progenitors being arrested during maturation, with an eventual cell loss in the absence of a rescue by the ligand. Further support for this idea comes from our observations that thyroid hormone administration to TRα2^{-/-} and TRα1^{+/m} mice rescues the decreased survival observed using either an endogenous marker (DCX) or exogenous label (BrdU). The data also

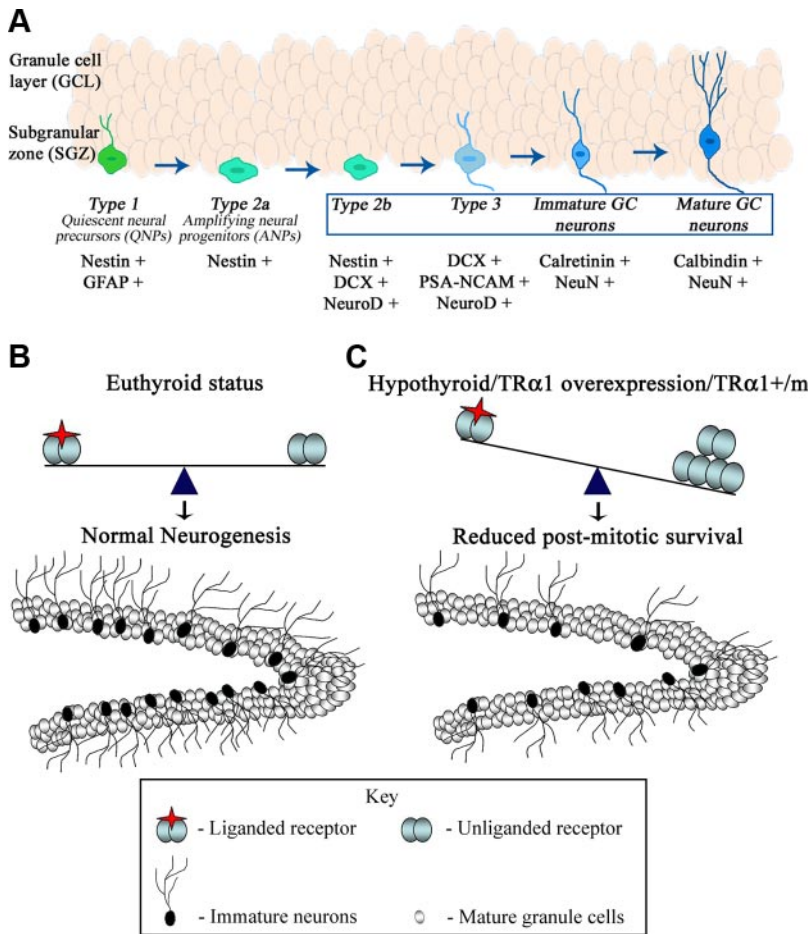


Figure 10. A) Schematic of the developmental stage-specific expression of markers by adult hippocampal progenitors. Type 1 quiescent neural precursors (QNPs) express nestin and GFAP and are the putative stem cells in the adult hippocampus. Type 2a cells, the transiently amplifying neural progenitors (ANPs), express Nestin alone, while Type 2b cells express nestin along with DCX and NeuroD. Type 3 neuroblasts express DCX, PSA-NCAM, and NeuroD, while immature neurons express calretinin and NeuN. Mature granule cell neurons express calbindin and NeuN. Boxed area denotes the stages of hippocampal progenitor development that are sensitive to perturbations of TR α 1.

B, C) Schematics of adult hippocampal neurogenesis in an euthyroid animal and in conditions that result in unliganded TR α 1 receptor. Schematics show the DG subfield of the hippocampus, containing mature granule cell neurons (white) within the GCL, the hilus, and the neurogenic niche, namely the SGZ at the border of the hilus and the GCL, which contains the adult hippocampal progenitors. Postmitotic neuroblasts (black) in the SGZ arise from cell division of adult hippocampal progenitors and eventually differentiate into mature granule cells (white) within the GCL of the DG. *B*) Schematic of normal, ongoing hippocampal neurogenesis, which occurs in euthyroid conditions. *C*) Mice with conditions such as hypothyroidism, TR α 2^{-/-} mice that overexpress TR α 1, and dominant-negative TR α 1 (TR α 1^{+/m}) mutant mice with a 10-fold reduction in affinity for thyroid hormone all show a decline in adult hippocampal neurogenesis, through a decrease in the survival of postmitotic neuro-

blasts. These effects are thought to arise as a consequence of repressor effects on the postmitotic survival of adult hippocampal progenitors induced by a TR α 1 aporeceptor. A change in the ratio of unliganded and liganded TR α 1 receptors may contribute *via* TR α 1 aporeceptor effects to the impaired adult hippocampal neurogenesis observed in adult-onset hypothyroidism.

indicate that thyroid hormone treatment to wild-type mice increases hippocampal progenitor survival. Our results lead us to speculate that the effects of thyroid hormone on adult hippocampal neurogenesis may largely involve an alleviation of the TR α 1 aporeceptor effect. A question that remains at present is whether the actions of TR α 1, either liganded or unliganded, on adult hippocampal progenitor survival involve cell autonomous or noncell autonomous effects *via* modulation of the neurogenic niche. We have previously shown that thyroid hormone does exert direct effects on hippocampal progenitors *in vitro* (10); however, the *in vitro* effects differ substantially from the effects of perturbed thyroid hormone levels *in vivo*, indicating that dispersed progenitor cultures do not recapitulate the *in vivo* neurogenic niche vis a vis responses to thyroid hormone. Future studies that address the influence of TR α 1 on neurogenic niche trophic factor expression, deiodinase expression in the hippocampal neurogenic niche and on astrocytic release of regulatory factors would further our mechanistic understanding of the effects of TR α 1 on hippocampal neurogenesis.

Besides its effects on hippocampal progenitor survival, adult-onset hypothyroidism also significantly decreases neuronal differentiation, an effect relieved by

thyroid hormone treatment (9, 10). The TR α 1^{+/m} mice exhibit a significant decline in the survival and neuronal differentiation of hippocampal progenitors, both of which can be rescued by thyroid hormone. This strong phenotypic similarity suggests that unliganded TR α 1 may account for all of the changes observed in hippocampal neurogenesis in hypothyroidism. However, it is important to note that TR α 2^{-/-} mice, that overexpress TR α 1, despite a robust decline in progenitor survival show normal neuronal differentiation of hippocampal progenitors, indicating a difference from the TR α 1^{+/m} mice. This discrepancy is likely to be due to the weak aporeceptor activity of TR α 1 in the TR α 2^{-/-} mice, as caused by the receptor overexpression, as compared to the relatively much stronger aporeceptor activity caused by the point mutation in the TR α 1R384C mutant receptor. Other contributing factors may include the differences in TR stoichiometry in these mutant mice. Future studies addressing the regulation of key target genes that modulate hippocampal progenitor survival and neuronal differentiation may help to resolve these issues. The TR α 1^{-/-} mice that show enhanced hippocampal progenitor survival exhibit normal neuronal differentiation, suggesting that deficiency of this receptor may not be capable of

further enhancing the neuronal differentiation of adult hippocampal progenitors.

We further addressed the early stages of postmitotic hippocampal progenitor development using two different markers, calretinin and NeuroD. Calretinin is a transient marker of postmitotic hippocampal progenitors destined for neuronal differentiation (29). The TR α 1^{+/^m} mice show decreased BrdU-calretinin percentage colocalization, which can be rescued by thyroid hormone. Thyroid hormone administration to wild-type mice enhances the percentage of BrdU-labeled progenitors that are calretinin-immunopositive. Since we observed an influence of TR α 1 aporeceptors on the calretinin-positive stage of neuronal differentiation in adult hippocampal progenitors, we examined whether an earlier stage of fate choice, namely, the acquisition of neuronal fate through the transcription factor NeuroD is also influenced. We observed a robust decline in NeuroD⁺ cell numbers within the hippocampal neurogenic niche of both TR α 1^{+/^m} and TR α 2^{-/⁻} mice, which could be rescued by thyroid hormone administration. NeuroD is expressed in adult hippocampal progenitor cells (31) and is essential for progenitor survival and neuronal differentiation (30, 39). Furthermore, NeuroD expression is regulated by thyroid hormone, and hypothyroidism reduces NeuroD expression in the developing rat cerebellum (40) and the adult hippocampus (unpublished results). There are strong parallels to the effects of thyroid hormone on myogenic differentiation, where the TR α 1 aporeceptor represses the transcription of the myogenic transcription factor, MyoD (41). Our results suggest that TR α 1 aporeceptor activity may hold hippocampal progenitors at the early postmitotic stage prior to the acquisition of NeuroD⁺ identity and the commitment to a neuronal fate. The ligand thyroid hormone could act to allow progression of neuronal differentiation, and on the basis of reports that thyroid hormone cooperates with neurotrophins during hippocampal development (42) may also modulate the sensitivity of these postmitotic progenitors to neurotrophins within the niche.

Previous reports demonstrate that TR α 1^{+/^m} mice also exhibit depressive behavior (43), anxiety, and memory impairments that are rescued by thyroid hormone treatment (22). Given the role of adult hippocampal neurogenesis in hippocampal-dependent memory (7, 44), depression (8), and anxiety behavior (45), this highlights the possibility that decreased hippocampal neurogenesis as a consequence of TR α 1 aporeceptor activity may contribute to the behavioral deficits observed in both hypothyroidism and TR α 1^{+/^m} mutant mice. In contrast, the TR α 1^{-/⁻} mice exhibit enhanced fear-associated learning, a behavior known to require hippocampal neurogenesis (46). The increased neurogenesis that we observed in the TR α 1^{-/⁻} mice may contribute to the improved fear of learning. However, the TR α 1^{-/⁻} mice also exhibit enhanced anxiety behavior, which has a more controversial association with neurogenesis, with increased anxiety behavior observed both following a decline or induction in

hippocampal neurogenesis (47, 48). Our results motivate studies to determine the contribution of the neurogenic changes observed in the TR α 1 mutants to the behavioral phenotypes observed in these mice.

In summary, we provide definitive evidence that TR α 1 regulates adult hippocampal neurogenesis. Further, our data strongly indicate that TR α 1 aporeceptor activity mediates the impaired survival and neuronal differentiation of adult hippocampal progenitors in adult-onset hypothyroidism. Our results highlight the requirement for future studies to identify the target genes that are regulated by both TR α 1 apo- and holo-receptor activity, and their contribution to the decreased hippocampal neurogenesis and cognitive/behavioral deficits observed in adult-onset hypothyroidism. EJ

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REFERENCES

1. Bernal, J. (2002) Action of thyroid hormone in brain. *J. Endocrinol. Invest.* **25**, 268–288
2. Bernal, J. (2007) Thyroid hormone receptors in brain development and function. *Nat. Clin. Pract. Endocrinol. Metab.* **3**, 249–259
3. Osterweil, D., Syndulko, K., Cohen, S. N., Pettler-Jennings, P. D., Hershman, J. M., Cummings, J. L., Tourtellotte, W. W., and Solomon, D. H. (1992) Cognitive function in non-demented older adults with hypothyroidism. *J. Am. Geriatr. Soc.* **40**, 325–335
4. Dugbartey, A. T. (1998) Neurocognitive aspects of hypothyroidism. *Arch. Intern. Med.* **158**, 1413–1418
5. Fundaro, A. (1989) Behavioral modifications in relation to hypothyroidism and hyperthyroidism in adult rats. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **13**, 927–940
6. Kulikov, A., Torrèani, J., and Jeanningros, R. (1997) Experimental hypothyroidism increases immobility in rats in the forced swim paradigm. *Neurosci. Lett.* **234**, 111–114
7. Kitabatake, Y., Sailor, K. A., Ming, G. L., and Song, H. (2007) Adult neurogenesis and hippocampal memory function: new cells, more plasticity, new memories? *Neurosurg. Clin. N. Am.* **18**, 105–113
8. Vaidya, V. A., Fernandes, K., and Jha, S. (2007) Regulation of adult hippocampal neurogenesis: relevance to depression. *Expert Rev. Neurother.* **7**, 853–864
9. Ambrogini, P., Cuppini, R., Ferri, P., Mancini, C., Ciaroni, S., Voci, A., Gerdoni, E., and Gallo, G. (2005) Thyroid hormones affect neurogenesis in the dentate gyrus of adult rat. *Neuroendocrinology* **81**, 244–253
10. Desouza, L. A., Ladiwala, U., Daniel, S. M., Agashe, S., Vaidya, R. A., and Vaidya, V. A. (2005) Thyroid hormone regulates hippocampal neurogenesis in the adult rat brain. *Mol. Cell. Neurosci.* **29**, 414–426
11. Montero-Pedrazuela, A., Venero, C., Lavado-Autric, R., Fernández-Lamo, I., García-Verdugo, J. M., Bernal, J., and Guadaño-Ferraz, A. (2006) Modulation of adult hippocampal neurogenesis by thyroid hormones: implications in depressive-like behavior. *Mol. Psychiatry* **11**, 361–371
12. Ming, G. L., and Song, H. (2005) Adult neurogenesis in the mammalian central nervous system. *Annu. Rev. Neurosci.* **28**, 223–250
13. Jin, K., Mao, X. O., Cottrell, B., Schilling, B., Xie, L., Row, R. H., Sun, Y., Peel, A., Childs, J., Gendeh, G., Gibson, B. W., and Greenberg, D. A. (2004) Proteomic and immunochemical char-

- acterization of a role for stathmin in adult neurogenesis. *FASEB J.* **18**, 287–299
14. Kempermann, G., Jessberger, S., Steiner, B., and Kronenberg, G. (2004) Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* **27**, 447–452
 15. Yen, P. M. (2001) Physiological and molecular basis of thyroid hormone action. *Physiol. Rev.* **81**, 1097–1142
 16. Schwartz, H. L., Strait, K. A., Ling, N. C., and Oppenheimer, J. H. (1992) Quantitation of rat tissue thyroid hormone binding receptor isoforms by immunoprecipitation of nuclear triiodothyronine binding capacity. *J. Biol. Chem.* **267**, 11794–11799
 17. Koenig, R. J., Lazar, M. A., Hodin, R. A., Brent, G. A., Larsen, P. R., Chin, W. W., and Moore, D. D. (1989) Inhibition of thyroid hormone action by a non-hormone binding c-erbA protein generated by alternative mRNA splicing. *Nature* **337**, 659–661
 18. Saltó, C., Kindblom, J. M., Johansson, C., Wang, Z., Gullberg, H., Nordström, K., Mansén, A., Ohlsson, C., Thorén, P., Forrest, D., and Vennström, B. (2001) Ablation of TR α 2 and a concomitant overexpression of α 1 yields a mixed hypo- and hyperthyroid phenotype in mice. *Mol. Endocrinol.* **15**, 2115–2128
 19. Wikström, L., Johansson, C., Saltó, C., Barlow, C., Campos Barros, A., Baas, F., Forrest, D., Thorén, P., and Vennström, B. (1998) Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor α 1. *EMBO J.* **17**, 455–461
 20. Tinnikov, A., Nordström, K., Thorén, P., Kindblom, J. M., Malin, S., Rozell, B., Adams, M., Rajanayagam, O., Pettersson, S., Ohlsson, C., Chatterjee, K., and Vennström, B. (2002) Retardation of post-natal development caused by a negatively acting thyroid hormone receptor α 1. *EMBO J.* **21**, 5079–5087
 21. Wallis, K., Dudazy, S., van Hogerlinden, M., Nordstrom, K., Mittag, S., and Vennstrom, B. (2010) The thyroid hormone receptor α 1 protein is expressed in embryonal post mitotic neurons and persists in most adult neuronal cells. *Mol. Endocrinol.* In press
 22. Venero, C., Guadaño-Ferraz, A., Herrero, A. I., Nordström, K., Manzano, J., de Escobar, G. M., Bernal, J., and Vennström, B. (2005) Anxiety, memory impairment, and locomotor dysfunction caused by a mutant thyroid hormone receptor α 1 can be ameliorated by T3 treatment. *Genes Dev.* **19**, 2152–2163
 23. Seki, T., and Arai, Y. (1993) Highly polysialylated neural cell adhesion molecule (NCAM-H) is expressed by newly generated granule cells in the dentate gyrus of the adult rat. *J. Neurosci.* **13**, 2351–2358
 24. Malberg, J. E., Eisch, A. J., Nestler, E. J., and Duman, R. S. (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J. Neurosci.* **20**, 9104–9110
 25. Paxinos, G., and Franklin, K. B. J. (2004) *The Mouse Brain in Stereotaxic Coordinates*, Academic Press, San Diego, CA, USA
 26. Wang, J. W., David, D. J., Monckton, J. E., Battaglia, F., and Hen, R. (2008) Chronic fluoxetine stimulates maturation and synaptic plasticity of adult-born hippocampal granule cells. *J. Neurosci.* **28**, 1374–1384
 27. Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H. G., and Aigner, L. (2005) Doublecortin expression levels in adult brain reflect neurogenesis. *Eur. J. Neurosci.* **21**, 1–14
 28. Nacher, J., Crespo, C., and McEwen, B. S. (2001) Doublecortin expression in the adult rat telencephalon. *Eur. J. Neurosci.* **14**, 629–644
 29. Brandt, M. D., Jessberger, S., Steiner, B., Kronenberg, G., Reuter, K., Bick-Sander, A., von der Behrens, W., and Kempermann, G. (2003) Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. *Mol. Cell. Neurosci.* **24**, 603–613
 30. Liu, M., Pleasure, S. J., Collins, A. E., Noebels, J. L., Naya, F. J., Tsai, M. J., and Lowenstein, D. H. (2000) Loss of BETA2/NeuroD leads to malformation of the dentate gyrus and epilepsy. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 865–870
 31. Seki, T. (2002) Expression patterns of immature neuronal markers PSA-NCAM, CRMP-4 and NeuroD in the hippocampus of young adult and aged rodents. *J. Neurosci. Res.* **70**, 327–334
 32. Hodge, R. D., Kowalczyk, T. D., Wolf, S. A., Encinas, J. M., Rippey, C., Enikolopov, G., Kempermann, G., and Hevner, R. F. (2008) Intermediate progenitors in adult hippocampal neurogenesis: Tbr2 expression and coordinate regulation of neuronal output. *J. Neurosci.* **28**, 3707–3717
 33. Calzà, L., Aloe, L., and Giardino, L. (1997) Thyroid hormone-induced plasticity in the adult rat brain. *Brain Res. Bull.* **44**, 549–557
 34. Gilbert, M. E., and Paczkowski, C. (2003) Propylthiouracil (PTU)-induced hypothyroidism in the developing rat impairs synaptic transmission and plasticity in the dentate gyrus of the adult hippocampus. *Brain Res. Dev. Brain Res.* **145**, 19–29
 35. Lemkine, G. F., Raj, A., Alfama, G., Turque, N., Hassani, Z., Alegria-Prévot, O., Samarut, J., Levi, G., and Demeneix, B. A. (2005) Adult neural stem cell cycling in vivo requires thyroid hormone and its α receptor. *FASEB J.* **19**, 863–865
 36. Baldini, I. M., Vita, A., Mauri, M. C., Amodei, V., Carrisi, M., Bravin, S., and Cantalamessa, L. (1997) Psychopathological and cognitive features in subclinical hypothyroidism. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **21**, 925–935
 37. Ercan-Fang, S., Schwartz, H. L., and Oppenheimer, J. H. (1996) Isoform-specific 3,5,3'-triiodothyronine receptor binding capacity and messenger ribonucleic acid content in rat adenohipophysis: effect of thyroidal state and comparison with extrapituitary tissues. *Endocrinology* **137**, 3228–3233
 38. Muñoz, A., Wrighton, C., Seliger, B., Bernal, J., and Beug, H. (1993) Thyroid hormone receptor/c-erbA: control of commitment and differentiation in the neuronal/chromaffin progenitor line PC12. *J. Cell Biol.* **121**, 423–438
 39. Gao, Z., Ure, K., Ables, J. L., Lagace, D. C., Nave, K. A., Goebbels, S., Eisch, A. J., and Hsieh, J. (2009) Neurod1 is essential for the survival and maturation of adult-born neurons. *Nat. Neurosci.* **12**, 1090–1092
 40. Chantoux, F., and Francon, J. (2002) Thyroid hormone regulates the expression of NeuroD/BHFL during the development of rat cerebellum. *Mol. Cell. Endocrinol.* **194**, 157–163
 41. Daury, L., Busson, M., Casas, F., Cassar-Malek, I., Wrutniak-Cabello, C., and Cabello, G. (2001) The triiodothyronine nuclear receptor c-ErbA α 1 inhibits avian MyoD transcriptional activity in myoblasts. *FEBS Lett.* **508**, 236–240
 42. Clos, J., and Legrand, C. (1990) An interaction between thyroid hormone and nerve growth factor promotes the development of hippocampus, olfactory bulbs and cerebellum: a comparative biochemical study of normal and hypothyroid rats. *Growth Factors* **3**, 205–220
 43. Pilhatsch, M., Winter, C., Nordström, K., Vennström, B., Bauer, M., and Juckel, G. (2010) Increased depressive behaviour in mice harboring the mutant thyroid hormone receptor α 1. *Behav. Brain Res.* **214**, 187–192
 44. Becker, S., and Wojtowicz, J. M. (2007) A model of hippocampal neurogenesis in memory and mood disorders. *Trends Cogn. Sci.* **11**, 70–76
 45. Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., and Hen, R. (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* **301**, 805–809
 46. Guadaño-Ferraz, A., Benavides-Piccione, R., Venero, C., Lancha, C., Vennström, B., Sandi, C., DeFelipe, J., and Bernal, J. (2003) Lack of thyroid hormone receptor α 1 is associated with selective alterations in behavior and hippocampal circuits. *Mol. Psychiatry* **8**, 30–38
 47. Jessberger, S., Clark, R. E., Broadbent, N. J., Clemenson, G. D., Jr., Consiglio, A., Lie, D. C., Squire, L. R., and Gage, F. H. (2009) Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. *Learn Mem.* **16**, 147–154
 48. Fuss, J., Ben Abdallah, N. M., Vogt, M. A., Touma, C., Pacifici, P. G., Palme, R., Witzemann, V., Hellweg, R., and Gass, P. (2010) Voluntary exercise induces anxiety-like behavior in adult C57BL/6J mice correlating with hippocampal neurogenesis. *Hippocampus* **20**, 364–376

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