Thyroid hormone regulates hippocampal neurogenesis in the adult rat brain

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We have examined the influence of thyroid hormone on adult hippocampal neurogenesis, which encompasses the proliferation, survival and differentiation of dentate granule cell progenitors. Using bromodeoxyuridine (BrdU), we demonstrate that adult-onset hypothyroidism significantly decreases hippocampal neurogenesis. This decline is predominantly the consequence of a significant decrease in the survival and neuronal differentiation of BrdU-positive cells. Both the decreased survival and neuronal differentiation of hippocampal progenitors could be rescued by restored euthyroid status. Adult-onset hyperthyroidism did not influence hippocampal neurogenesis, suggesting that the effects of thyroid hormone may be optimally permissive at euthyroid levels. Our in vivo and in vitro results revealed that adult hippocampal progenitors express thyroid receptor isoforms. The in vitro studies demonstrate that adult hippocampal progenitors exhibit enhanced proliferation, survival and glial differentiation in response to thyroid hormone. These results support a role for thyroid hormone in the regulation of adult hippocampal neurogenesis and raise the possibility that altered neurogenesis may contribute to the cognitive and behavioral deficits associated with adult-onset hypothyroidism.

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Introduction

Thyroid hormone plays a critical role in neurodevelopment. In the developing nervous system, thyroid hormone exerts significant influences on both neuronal and glial progenitors, regulating their proliferation, survival and differentiation (Billon et al., 2001; Porterfield and Hendrich, 1993; Jones et al., 2003). Deficiency of thyroid hormone during critical periods of brain development is associated with profound, and often irreversible, morphological defects that contribute to severe cognitive and neurological impairment (Porterfield and Hendrich, 1993). Although the developmental effects of thyroid hormone have been well established, its influence on the adult brain is relatively poorly understood. The adult mammalian brain does not exhibit the severe morphological defects associated with developmental hypothyroidism, however, hypothyroidism in adulthood has been clearly linked to cognitive dysfunction and depressed mood (Dugbartey, 1998; Jackson, 1998). For example, adult-onset hypothyroidism in humans is associated with impairment in learning, verbal fluency and spatial tasks (Baldini et al., 1997; Osterweil et al., 1992), as well as an increased susceptibility to depression (Haggerty et al., 1993). Adult hypothyroid rats show deficits in learning tasks (Fundaro, 1989) and also exhibit increased immobility in the Porsolt forced swim test, mimicking animal models of depression (Kulikov et al., 1997). This suggests the possibility that compromised thyroid status in the adult may result in morphological changes in brain regions strongly implicated in learning, memory and mood, such as the hippocampus.

The hippocampus exhibits morphological plasticity well into adulthood. It is one of the few brain regions that retain the ability to make new neurons throughout adult life in several mammalian species including humans (Eriksson et al., 1998; Kempermann and Gage, 2000). Newborn neurons generated from progenitors residing in the subgranular zone (SGZ) of the dentate gyrus migrate into the granule cell layer (GCL), integrate within the circuitry, receiving afferents and sending out functional efferents (van Praag et al., 2002). The generation of newborn neurons within the adult hippocampus has been suggested to play an important role in hippocampal function (Schinder and Gage, 2004). Several studies have indicated a role for adult hippocampal neurogenesis in hippocampal-dependent learning and memory (Gould et al., 1999; Schinder and Gage, 2004; Shors et al., 2001), though there still remains controversy on the exact contribution of neurogenesis to this process (Prickaerts et al., 2004). The generation of new neurons in the adult hippocampus is reduced in animal models of
depression (Duman, 2004), and enhanced neurogenesis has been implicated in the behavioral effects of antidepressants (Santarelli et al., 2003).

Thyroid hormone exerts profound effects on precursor cells during development. The effects of thyroid hormone are pleiotrophic and vary based on the precursor population examined (Anderson et al., 2003). Thyroid hormone is known to reduce proliferation of oligodendrocyte precursors (Bass et al., 1997) and is critical in the timing of oligodendrocyte differentiation (Tokumoto et al., 2002). During development, blockade of thyroid hormone receptor α inhibits neurogenic precursor proliferation (Lezoualc’h et al., 1995). In addition, thyroid hormone enhances the postmitotic survival of neuronal progenitors (Muller et al., 1995) and also affects their migration, arborization and expression of specific phenotypic markers (Anderson et al., 2003; Gould et al., 1991a,b). In addition, thyroid hormone has been shown to regulate the morphology and maturation of developing astrocytes (Farwell and Dubord-Tomasetti, 1999; Gould et al., 1990a,b). The influence of thyroid hormone on different precursor populations has been studied predominantly during development, and its effects on progenitors in the adult brain are poorly understood. The role of thyroid hormone in regulating adult hippocampal neurogenesis is at present unknown. Given the deficits that arise in hippocampal function as a consequence of adult hypothyroidism (Smith et al., 2003), as well as the influence of thyroid hormone on neuronal progenitors and hippocampal morphology during development (Madeira et al., 1991; Porterfield and Hendrich, 1993), we hypothesized that the process of adult hippocampal neurogenesis may be sensitive to thyroid hormone status. We have examined the effects of adult-onset hypothyroidism and hyperthyroidism on dentate granule cell progenitors in vivo and have also evaluated the effects of thyroid hormone on cultured progenitor cells isolated from the adult hippocampus. Our in vivo and in vitro results indicate that thyroid hormone plays a role in regulating adult hippocampal neurogenesis.

**Results**

**Hypothyroid status does not alter the proliferation of adult dentate granule cell progenitors**

The effect of hypothyroid status on the proliferation of adult dentate granule cell progenitors was assessed using the mitotic marker BrdU to label dividing progenitors. MMI-treated hypothyroid animals did not show any change in the number of BrdU-positive cells within the SGZ/GCL (Fig. 1). BrdU-positive cells were predominantly localized to the SGZ at the border of the hilus and the GCL in both control and MMI groups (Figs. 1A–D) and were often observed in clusters. Although no change was seen in SGZ/GCL BrdU-positive cell number, a significant reduction (53%) in the number of BrdU-positive cells localized to the hilar region of the dentate gyrus was observed in MMI-treated animals as compared to the control (Fig. 1E). In addition, PTU-treated hypothyroid animals also showed a significant reduction (36%) in the hilar BrdU-positive cell number (Control = 262.67 ± 24.48; PTU = 170 ± 19.97; P < 0.05) but no change in the BrdU-positive cell number within the SGZ/GCL (data not shown; P > 0.05).

To address whether hypothyroid status influences the proliferation of progenitors that reside in the subventricular zone lining the lateral ventricles, the number of BrdU-positive cells/mm² in this region was examined. The number of BrdU-positive cells/mm² was not significantly influenced by hypothyroid status (Control = 5031.33 ± 619.75; PTU = 5424.17 ± 166.12; mean ± SEM; n = 3/group, P > 0.05). Serum T3 levels in the MMI and PTU-treated animals confirmed their experimentally hypothyroid status (Control = 57 ± 9; MMI = 15 ± 3; Control = 66 ± 5; PTU = 10 ± 4; results are expressed as ng/dl and are the mean ± SEM, P > 0.05). In addition, the levels of circulating corticosterone hormone were also determined in MMI- and PTU-treated hypothyroid animals (Control = 100 ± 8.22; MMI = 238.83 ± 35.29; P < 0.05; Control = 100 ± 18.91; PTU = 49.39 ± 16.18; P > 0.05; results are expressed as percent of control and are the mean ± SEM).

![Fig. 1. Hypothyroidism does not influence the proliferation of adult dentate granule cell progenitors.](image-url)
Hypothyroid status decreases the survival of adult dentate granule cell progenitors

To assess the survival of a newborn pool of BrdU-positive progenitors when exposed to hypothyroidism, we used a BrdU-labeling schedule in which BrdU treatment was first given to drug-naive animals followed by treatment with the goitrogens MMI and PTU. The survival of dentate granule cell progenitors in the SGZ/GCL was significantly reduced in both the MMI-(55%) and PTU (60%)-treated groups in comparison to their respective controls (Fig. 2). The BrdU-positive cells in the survival experiments had an ovoid or round shape, were often localized to the GCL and were not observed in tight clusters (Figs. 2C–F). No change was observed in the hilir BrdU-positive cell number in the MMI- and PTU-treated groups as compared to their respective controls (Fig. 2). All goitrogen-treated animals were confirmed to be experimentally hypothyroid through the measurement of circulating T3 hormone levels.

The absolute number of BrdU-positive cells that persists in the control groups 21 or 28 days after BrdU labeling is itself significantly decreased relative to the starting pool of BrdU progenitors 24 h after BrdU injection (number of BrdU-positive cells at 24 h, SGZ/GCL = 5640.80 ± 576.79, mean ± SEM; n = 5; P < 0.05). Consistent with previous reports (Malberg et al., 2000), approximately 60% and 40% of the BrdU-labeled progenitors persist in control groups sacrificed 21 (Fig. 2B) and 28 days (Fig. 2A) after BrdU injection respectively. In contrast, the number of newborn BrdU-positive cells that persists in animals rendered hypothyroid following BrdU injection and then sacrificed at 21 days (PTU, Fig. 2B) or 28 days (MMI, Fig. 2A) is approximately 25% and 20% respectively. This indicates that the number of BrdU-positive dentate granule cell progenitors which normally declines in number following proliferation exhibits a significant enhancement of this decline in survival in hypothyroid animals. In addition, we analyzed the distribution of BrdU-positive cells within the GCL, 4 weeks after BrdU labeling. Although there were considerably fewer BrdU-positive cells in hypothyroid animals, they did not differ in their percent distribution in the inner versus outer compartments of the GCL (data not shown; P > 0.05), suggesting that migration may be broadly normal. The hypothyroidism-induced decrease in the number of surviving progenitors cannot be attributed to any changes in hippocampal volume, as volumetric analyses of the hippocampus and GCL revealed no significant differences between control and hypothyroid groups (Table 1).

We used the TUNEL assay to determine whether the decreased survival of dentate granule cell progenitors in hypothyroid animals is likely to result from enhanced apoptosis. Newborn neurons are predominantly present in the inner compartment of the GCL that is directly in contact with the SGZ in contrast to the outer region that contains more mature neurons. We observed a significant increase in TUNEL-positive cells in the inner layer of MMI-treated hypothyroid animals in comparison to controls (Fig. 3). In contrast, the outer compartment of the GCL showed the same numbers of TUNEL-positive cells in both groups. This indicates increased apoptosis in hypothyroid animals which is restricted to the inner compartment of the GCL that contains the newborn neurons.

Hypothyroid status decreases the neuronal differentiation of adult dentate granule cell progenitors

To address whether the surviving BrdU-positive progenitors in the SGZ/GCL show any difference in acquisition of cell fate in

Table 1

<table>
<thead>
<tr>
<th>Control</th>
<th>MMI</th>
<th>PTU</th>
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<tbody>
<tr>
<td>Hippocampus (mm³)</td>
<td>18.69 ± 0.80</td>
<td>16.63 ± 1.28</td>
</tr>
<tr>
<td>GCL (mm³)</td>
<td>0.96 ± 0.01</td>
<td>0.90 ± 0.03</td>
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Adult-onset hypothyroidism does not influence hippocampal or granule cell layer (GCL) volume. Animals received the goitrogens MMI (28 days) or PTU (21 days) in drinking water in two separate experiments with distinct control groups and were sacrificed on the final day of goitrogens treatment. Values represent mean ± SEM (n = 5/group).
control versus hypothyroid groups, we examined the percentage of BrdU cells that acquire a neuronal (NeuN) or glial (GFAP) phenotype 28 days after BrdU labeling. Confocal analysis revealed that, in control animals, most BrdU-positive cells in the SGZ/GCL acquire a neuronal phenotype (~80%) (Fig. 4). In contrast, the percentage of BrdU-positive cells that differentiate into mature neurons is significantly reduced in hypothyroid animals with 50% and 69% of the BrdU-positive cells showing a neuronal phenotype in MMI- and PTU-treated hypothyroid animals respectively (Fig. 4). In both control and hypothyroid animals, very few BrdU-positive cells in the SGZ/GCL were observed to colocalize with the glial marker GFAP (Fig. 4). It is unclear whether the non-colocalized BrdU-positive cells seen in hypothyroid animals represent quiescent undifferentiated cells, a delay in neuronal differentiation or cells eventually to be targeted for death. Taken together, these data indicate a significant decrease in the neuronal, but not glial differentiation, of dentate granule cell progenitors in the SGZ/GCL. In contrast to the predominantly neuronal differentiation of BrdU-positive cells in the SGZ/GCL, hilar BrdU-labeled cells in both control and hypothyroid animals were found to colocalize with GFAP with no observed differences between the groups (data not shown; \( P > 0.05 \)).

Fig. 3. Hypothyroidism results in a significant increase in apoptotic cells in the inner layer of the dentate gyrus granule cell layer. Shown are representative fluorescence photomicrographs from an MMI-treated animal of a TUNEL-positive cell (B, C—arrowhead) observed within the inner portion of the granule cell layer of a DAPI-stained section (A). Quantitative analysis indicated that the number of TUNEL-positive cells/section in the inner layer (IL), but not the outer layer (OL), of the dentate gyrus granule cell layer in MMI-treated animals was significantly increased as compared to controls (D). The results are expressed as the mean ± SEM (n = 4/group) TUNEL-positive cells/section. \( *P < 0.05 \) indicates significantly different from control (Student’s \( t \) test).

Fig. 4. Hypothyroidism results in a significant decrease in the neuronal differentiation of adult dentate granule cell progenitors within the subgranular zone (SGZ)/granule cell layer (GCL). Drug-naive rats first received BrdU injections followed by goitrogen treatment with MMI or PTU for 28 days in separate experiments with distinct control groups. Colocalization of BrdU-positive cells (A, D) with the neuronal marker NeuN (B, E) is indicated by arrowheads in the merged images (C, F) in representative confocal images from a control (A–C) and an MMI-treated hypothyroid (D–F) animal. The arrow (F) indicates the lack of colocalization of a BrdU-positive cell with NeuN in the representative image from an MMI animal (D–F). Animals rendered hypothyroid using MMI (G) or PTU (H) showed a significant decrease in the percent colocalization of BrdU-positive cells in the SGZ/GCL with the neuronal marker NeuN as compared to controls. Very few BrdU-positive cells colocalized with GFAP in the SGZ/GCL. The results are expressed as the mean ± SEM (n = 5/group) percent colocalization of BrdU-positive cells with NeuN or GFAP in the SGZ/GCL. \( *P < 0.05 \) indicates significantly different from control (Student’s \( t \) test).
The values are expressed as the mean ± SEM (n = 5/group).

* P < 0.05 (Student’s t test) indicates significantly different from control.

**P** < 0.05 indicates significantly different from control and#

**P** < 0.05 indicates significantly different from MMI group (one-way ANOVA; post-hoc Bonferroni test).

**Table 2**

<table>
<thead>
<tr>
<th>Blood levels of T3 (ng/100 ml)</th>
<th>Control</th>
<th>MMI</th>
<th>MMI + T3/T4</th>
<th>T3/T4</th>
</tr>
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<tr>
<td></td>
<td>44 ± 4</td>
<td>10 ± 3*</td>
<td>36 ± 3</td>
<td>41 ± 7</td>
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Restored euthyroid status rescues the decrease in survival and neuronal differentiation of adult dentate granule cell progenitors in hypothyroid animals

We next examined whether the effects of hypothyroidism on the survival and neuronal differentiation of granule cell progenitors could be rescued by restoration of euthyroid status. MMI-treated animals were restored to euthyroid status by combined T3/T4 replacement treatment (Table 2). MMI-treated animals restored to euthyroid status did not show the MMI-induced significant reduction in the survival of BrdU-positive cells in the SGZ/GCL and were found to be no different from the control group (Fig. 5). In addition, the decrease in the neuronal differentiation of BrdU-labeled cells observed in MMI-treated animals was restored to control levels in MMI-treated animals that received T3/T4 replacement treatment (Fig. 6). The percent colocalization of BrdU/NeuN in the control and MMI + T3/T4 groups did not differ from each other but were significantly greater than the MMI group (Fig. 6). The low-dose T3/T4 treatment itself did not influence either the survival or neuronal differentiation of adult dentate granule cell progenitors (Figs. 5, 6).

Hyperthyroid status does not influence the proliferation, survival and differentiation of adult dentate granule cell progenitors

To examine the influence of hyperthyroid status on adult hippocampal neurogenesis, we addressed whether animals treated with T3 exhibited any change in the proliferation, survival or differentiation of adult dentate granule cell progenitors. In the proliferation experiment, the number of BrdU-positive cells within the SGZ/GCL, as well as in the hilus, was not influenced by T3 treatment (Fig. 7A). In addition, the proliferation of adult subventricular zone progenitors was also not altered by hyperthyroidism (Fig. 7C). Significantly elevated serum T3 levels confirmed the hyperthyroid status of T3-treated animals (Control = 39 ± 10.5, T3 = 135.20 ± 7.31; results are expressed as ng/dl and are the mean ± SEM, P < 0.05). To examine the influence of hyperthyroidism on the survival and eventual phenotype choice made by newborn BrdU-positive cells, drug-naïve animals first received BrdU before being administered either vehicle or T3 treatment. The number of BrdU-positive cells that persists at 21 days in both control and T3-treated hyperthyroid rats did not significantly differ in either the SGZ/GCL or the hilus (Fig. 7B). In addition, the neuronal (NeuN) and glial (GFAP) differentiation of BrdU-positive cells in the SGZ/GCL was not altered by hyperthyroidism (Fig. 7C). Significantly elevated serum T3 levels confirmed the hyperthyroid status of T3-treated animals (Control = 39 ± 10.5, T3 = 135.20 ± 7.31; results are expressed as ng/dl and are the mean ± SEM, P < 0.05). In contrast to thyroid hormone deficiency, hyperthyroidism did not significantly
alter the proliferation, survival or differentiation of adult dentate granule cell progenitors.

Expression of thyroid receptor isoforms by adult hippocampal progenitors in vivo and in vitro

To address the possibility that the effects of thyroid hormone may be mediated via direct influences on adult hippocampal progenitors, we examined whether these progenitors both in vivo and in vitro expressed thyroid receptor isoforms (TRα, TRβ1 and TRβ2). In tissue sections, BrdU-positive progenitor cells, which are observed in tight clusters in the SGZ 2 h after BrdU labeling, were found to express the alpha and beta thyroid receptor isoforms (Fig. 8A). We observed that, while about 40% of SGZ BrdU-positive cells colocalize with TRα immunoreactivity, the extent of colocalization of BrdU-labeled cells with TRβ1 (~80%) and TRβ2 (~75%) immunoreactivity is substantially higher (Fig. 8B). Our studies at present do not reveal what fraction of BrdU-positive cells in the SGZ express more than one TR isoform. However, in colocalization studies with a cocktail of all TR antibodies in tissue sections, we did observe a few BrdU-positive cells in the SGZ that did not express any TR isoform (data not shown).

Immunofluorescent studies using a cocktail of TR receptor isofrom antisera (TRα, β1 and β2) revealed that the nuclei of all nestin-positive hippocampal progenitors in vitro were brightly stained in a clear punctate pattern (Fig. 8C). In particular, the individual staining observed with TRα (α1 and α2) and TRβ1 antiserum resulted in intense punctate immunofluorescence within the nuclei of all nestin-positive hippocampal progenitors (Fig. 8C). In contrast, the expression of TRβ2 isofrom revealed by immunostaining had a diffuse nuclear expression (Fig. 8C), lacking both the clear punctate signal and intensity observed with the TRα and TRβ1 immunofluorescence. Our expression studies suggest that in vitro all hippocampal progenitor cells appear to contain nuclear thyroid hormone receptors and suggest the possibility of differences in levels of expression of specific TR isoforms.

Influence of thyroid hormone on adult hippocampal progenitors in vitro

To address the effects of thyroid hormone on the proliferation of adult hippocampal progenitors in culture, cells were exposed to 20 nM or 50 nM of thyroid hormone for 2 weeks prior to a BrdU pulse for 2 h to assess the number of cells entering the S-phase of the cell cycle. Thyroid hormone at 20 nM significantly increased the incorporation of BrdU by 15% as compared to controls (Fig. 9A). A higher dose of thyroid hormone (T3, 50 nM) did not significantly alter the percentage of BrdU-positive cells observed (Control = 21.39 ± 2.62; T3 (50 nM) = 26.69 ± 2.22; mean ± SEM; n = 3, P > 0.05). To study whether thyroid hormone influences the survival of adult hippocampal progenitors in vitro, we examined the number of cells undergoing apoptosis in control and T3-treated (20 nM and 50 nM) cultures using a TUNEL assay. A significant reduction in the percentage of TUNEL-positive cells detected was observed in cultures treated with 20 nM of thyroid hormone (Fig. 9B). The higher dose of thyroid hormone (50 nM) in contrast did not alter the percentages of apoptotic cells detected (Control = 4.42 ± 1.45; T3 (50 nM) = 7.69 ± 1.50; mean ± SEM; n = 3, P > 0.05), indicating a dose-dependence to the effects of thyroid hormone on the survival of adult hippocampal progenitors.

To address the effects of thyroid hormone on the differentiation of adult hippocampal progenitors, cells were exposed to differentiation medium in the presence or absence of thyroid hormone (20 nM, 50 nM or 100 nM). Following 2 weeks of treatment, cells were fixed and then stained with markers specific to neurons and glia and evaluated for the effects of thyroid hormone. Thyroid hormone did not alter the percentage of cells that differentiated into beta-tubulin (TuJ1)-positive neurons. The number of TuJ1-positive neurons was 12.3 ± 0.8% in controls and 10.73 ± 1.04% in T3 (20 nM) (Fig. 9C), 8.99 ± 1.08% in T3 (50 nM) and 10.27 ± 1.25% in T3 (100 nM) (P > 0.05). The percentage of GFAP-positive cells was observed to be significantly increased at all doses of thyroid hormone examined. The number of GFAP-immunoreactive cells significantly increased from 2.44 ± 0.55% in controls to 4.44 ± 0.5%, 4.48 ± 0.75% and 4.41 ± 0.44% (P < 0.05) for adult hippocampal progenitors treated with 20 nM (Fig. 8C), 50 nM and
100 nM of T3, respectively. Taken together, these results suggest that thyroid hormone treatment increases the differentiation of adult hippocampal progenitor cells into astrocytes but does not alter neuronal differentiation of progenitors in vitro.

**Discussion**

The role of thyroid hormone as a critical epigenetic signal in neurodevelopment is well established (Bernal et al., 2003). To our knowledge, our study provides the first evidence that thyroid hormone plays a role in hippocampal neurogenesis in the adult mammalian brain. Adult hippocampal neurogenesis, consisting of the proliferation of hippocampal progenitors, their survival and differentiation into neurons or glia, is susceptible to regulation at all of these stages. Adult-onset hypothyroidism decreases the survival and neuronal differentiation of dentate granule cell progenitors. This effect can be completely rescued by restored euthyroid status, indicating that it results from decreased thyroid hormone levels. Hyperthyroidism in contrast does not alter adult hippocampal neurogenesis, suggesting that though thyroid hormone may be required for normal hippocampal neurogenesis, the effects of thyroid hormone on adult progenitors may already be optimally permissive at euthyroid levels itself. Our in vitro studies with adult hippocampal progenitor cells, as well as the expression of TR isoforms by hippocampal progenitors, provide evidence to support a direct effect of thyroid hormone on progenitors. The increased proliferation and survival of adult hippocampal progenitors in vitro in response to thyroid hormone was observed at the lower dose of T3 (20 nM) used, raising the possibility of a dose range in which thyroid hormone may

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**Fig. 8.** Adult hippocampal progenitors express thyroid hormone receptor isoforms in vivo and in vitro. The colocalization of BrdU-positive cells with thyroid receptor α, thyroid receptor β1 and thyroid receptor β2 in tissue sections is indicated by arrows in representative confocal laser scanning images (A). Non-colocalized BrdU-positive cells are indicated by arrowheads (A). Quantitative analysis indicated a higher percent colocalization of BrdU-positive cells in vivo with TRβ1 and TRβ2 isoforms as compared to TRα (B). Epifluorescent images from adult hippocampal progenitors in vitro revealed the presence of individual thyroid receptor isoforms on all nestin-positive cells (C). A cocktail of all thyroid receptor antisera revealed clear, punctate nuclear staining in all nestin-positive hippocampal progenitors (C).
mediate effects on proliferation and survival of adult progenitors. In contrast, all doses of T3 tested in vitro resulted in an increase in the percentage of astrocytic, but not neuronal, differentiation. Taken together, our in vivo and in vitro studies provide evidence that thyroid hormone regulates adult hippocampal neurogenesis.

In the developing nervous system, thyroid hormone influences the mitotic activity of neuronal progenitors, including neonatal dentate granule cell progenitors (Lezoualc'h et al., 1995; Madeira et al., 1991). Adult dentate granule cell progenitors residing in the SGZ, the predominant site of hippocampal neurogenesis, do not show any changes in proliferation in response to altered thyroid hormone status in vivo. Interestingly, we did observe decreased hilar BrdU-positive cell number in response to adult-onset hypothyroidism. In the dentate gyrus, most of the BrdU-positive cells are found in the SGZ with a small fraction being observed in the hilus. In the proliferation experiment, only a few of these hilar BrdU-positive cells were found to colocalize with the astrocytic marker GFAP. Although these cells could express GFAP at a later time point, the present data do not allow us to conclude any specific effect on astrocytic turnover. Our in vitro results indicate that adult hippocampal progenitors do exhibit increased proliferation in response to specific doses of thyroid hormone. This finding could be due to the fact that the in vitro progenitors include cells from both the SGZ and the hilus. This raises the possibility that distinct populations of dividing cells in the adult hippocampus may respond differentially to thyroid hormone status. Taken together, these results suggest that, while thyroid hormone is capable of directly influencing the proliferation of progenitors, the proliferative phase of adult hippocampal neurogenesis in vivo does not appear to be altered by thyroid hormone perturbations.

Our results indicate that, while the birth of newborn cells in the SGZ of hypothyroid animals is unaffected, their subsequent survival is substantially compromised, supporting a role for thyroid hormone in the postmitotic survival of adult dentate granule cell progenitors. This decline in survival of dentate progenitors is likely to arise from apoptotic cell death, as we see an increase in TUNEL-positive cells within the inner compartments of the GCL where newly generated cells tend to reside. Our in vitro studies support a direct role for thyroid hormone in the survival of adult hippocampal progenitors, as we observed a significant reduction in TUNEL-positive progenitors following thyroid hormone treatment. Interestingly, this effect of thyroid hormone in vitro was observed with the lower dose of T3 tested and was absent at the higher dose. In vivo, while hypothyroidism decreases adult hippocampal progenitor survival, hyperthyroid status does not appear to influence survival. The in vivo and in vitro results indicate the possibility that the effects of thyroid hormone on adult hippocampal progenitors may be critically dependent on thyroid hormone levels. During neonatal development, thyroid hormone has been implicated in the survival of both hippocampal and cerebellar granule cells (Madeira et al., 1991; Muller et al., 1995). In the adult brain, a single study indicates that thyroxine augments naturally occurring cell death of progenitors in the high vocal center of the zebra finch brain, a site of lifelong neuronal

Fig. 9. Influence of thyroid hormone treatment on the proliferation, survival and differentiation of adult hippocampal progenitors in vitro. Thyroid hormone (T3, 20 nM) resulted in a significant increase in the percentage of BrdU-positive cells among the total pool of cells counted (DAPI) as compared to controls (A). Exposure of adult hippocampal progenitors to thyroid hormone (T3, 20 nM) resulted in a significant reduction in the percentage of TUNEL-positive cells detected (B) indicating an increase in survival. The results are expressed as the mean ± SEM (n = 3/group) percent of BrdU-positive or TUNEL-positive cells of the total pool counted (DAPI) (*P < 0.05, Student's t test). The influence of thyroid hormone on neuronal and glial differentiation of adult hippocampal progenitors in vitro was assessed using the neuronal marker beta-tubulin (Tuj1) and the astrocytic marker glial fibrillary acidic protein (GFAP). Thyroid hormone (T3, 20 nM) did not alter the percentage of cells that differentiated into Tuj1-positive neurons but significantly increased the expression of GFAP-positive cells (C). The results are expressed as the mean ± SEM (n = 3/group) percent colocalization of Tuj1-positive cells or GFAP-positive cells from the total pool counted (DAPI) (*P < 0.05, Student's t test). Shown are representative epifluorescent images from control (D) and thyroid-hormone-treated (E) adult hippocampal progenitors showing staining for Tuj1 (red), GFAP (green) and the nuclear stain DAPI (blue).
replacement (Tekumalla et al., 2002). Our studies indicate that the role of thyroid hormone in adult hippocampal neurogenesis may be particularly relevant during the phase of recruitment of newly generated cells into the GCL.

Not only do newly generated BrdU-positive cells in the SGZ/GCL of adult-onset hypothyroid animals survive less, the newborn cells that do persist show a significant reduction in neuronal differentiation. In hypothyroid animals, ~60% of the progenitors formed NeuN-positive neurons in contrast to the controls where ~80% formed NeuN-positive neurons. The rescue of this decline by thyroid hormone replacement suggests that euthyroid levels may be important for the normal neuronal differentiation of adult dentate progenitors. Our in vitro studies did not show any effect of thyroid hormone on the differentiation of adult hippocampal progenitors into TuJ-1 positive neurons. In contrast, we observed a significant increase in astrocytic differentiation of adult hippocampal progenitors treated with thyroid hormone. The differences we see in the influence of thyroid hormone on neuronal differentiation in vivo and on astrocytic differentiation in vitro could arise due to several reasons. One of these is the difference in the local in vivo milieu and in vitro culture conditions in which thyroid hormone mediates its effects. Second, the effects of thyroid hormone appear to be dose-dependent, and it is possible that the dose of thyroid hormone in vitro and that observed by hippocampal progenitors in vivo may differ. Third, the in vitro population of progenitors includes cells from both the SGZ and hilus, whereas in vivo it is mainly the SGZ progenitors that differentiate into NeuN-positive granule cell neurons. Our results provide evidence that the maturation of adult hippocampal progenitors into astrocytes in vitro is sensitive to thyroid hormone. Previous studies have indicated that thyroid hormone regulates astrocytic development within the cortex, hippocampus and cerebellum (Faivre-Sarrailh et al., 1991; Gould et al., 1990a,b). Given the critical role of hippocampal astrocytes in generating a neurogenic niche (Song et al., 2002), it is possible that the effects we observe of hypothyroidism on decreased neuronal differentiation in vivo may be mediated via effects on the astrocyte-responsive neurogenic niche. Previous studies have suggested that hypothyroidism delays, rather than permanently blocks, neurodevelopment (Bernal et al., 2003). Whether the decline in neuronal differentiation of the surviving BrdU-positive cells in hypothyroid animals represents a delay or a block in neuronal differentiation or whether undifferentiated BrdU-positive cells may be targeted for cell death is at present unclear and requires further study. Several previous studies have clearly indicated a role for thyroid hormone in oligodendrocytic differentiation, however, for the purpose of our study, we have focused on the differentiation of adult hippocampal progenitors into neurons, as progenitors in the SGZ predominantly differentiate into granule cell neurons.

The process of adult hippocampal neurogenesis that results in a SGZ progenitor cell forming a granule cell neuron involves several stages. Although the precise lineage followed by adult hippocampal progenitors is not at present understood, certain milestones have been proposed to occur in the process of neuronal development in the adult hippocampus (Kempermann et al., 2004). Starting with the division of a putative stem cell, SGZ progenitors then pass through at least three developmental stages of transiently amplifying progenitor cells which can be distinguished by morphology and marker expression. The critical decision of long-term survival and recruitment into terminal differentiation of SGZ progenitors is thought to predominantly occur once the progenitors are postmitotic (Kempermann et al., 2004). Based on our results, thyroid hormone is likely to exert its effects on postmitotic SGZ progenitors at a time point when the decision of long lasting survival occurs. It has been suggested that, during this transient postmitotic stage, initial arborizations and synaptic connections appear, which may be important for the maintenance or elimination of these immature neurons. During development, thyroid hormone has been reported to influence the formation of dendritic arbors (Gould et al., 1991a,b) and regulate spine density (Gould et al., 1990a,b). This suggests the possibility that thyroid hormone through its effects on maturation of immature neurons may play a key role in the decision for long-term survival and hence formation of terminally differentiated granule cell neurons. Future studies using a panel of key expression markers to distinguish the different stages of neuronal development in the adult hippocampus will be important in the further characterization of the effects of thyroid hormone on adult hippocampal neurogenesis.

A recent study indicates that thyroid hormone regulates the proliferation of adult subventricular zone progenitors (Fernandez et al., 2004). However, this effect of thyroid hormone on proliferation was not observed in neural stem cells cultured from the subventricular zone of control, hypo- and hyperthyroid animals (Fernandez et al., 2004). Our studies did not reveal any influence of thyroid hormone status on subventricular zone progenitor proliferation in vivo. One possible reason for this discrepancy may be the clear difference in age of the animals used.

The mechanisms that underlie the influence of thyroid hormone on adult dentate progenitors are at present unknown, but several possibilities can be proposed. Thyroid hormone may exert direct effects via thyroid receptors generated from two genes, TRa and TRβ that encode several isoforms. Of these TRa1, TRβ1 and TRβ2 bind thyroid hormone and regulate transcription, whereas TRa2 does not bind thyroid hormone. Our in vivo studies indicate that 40% of BrdU-positive granule cell progenitors are immunopositive for TRa, while TRβ1 and TRβ2 isoforms appear to be expressed by about 75% of the BrdU-positive progenitors, 2 h post BrdU labeling. Although it is unclear whether these subsets are distinct or overlapping, it raises the intriguing possibility that TR isoforms may mark adult granule cell progenitors at different stages in their lineage. Our in vitro studies revealed that the nuclei of all nestin-positive hippocampal progenitors are immunopositive for TR isoforms and suggested the possibility of differences in levels of expression with lower TRβ2 expression. Further characterisation of the role of TR isoforms, if any, in the lineage of adult dentate progenitors warrants an independent study. TR receptors could regulate the transcription of target genes such as bcl-2, sonic hedgehog, BMP-4, neurotrophins and NeuroD (Chantoux and Françon, 2002; Giordano et al., 1992; Martel et al., 2002; Muller et al., 1995; Stolow and Shi, 1995) that may then influence adult hippocampal neurogenesis. Our in vitro studies provide support to the hypothesis that thyroid hormone mediates its effects on adult hippocampal neurogenesis through direct effects on the progenitors.

The decreased neurogenesis that results from hypothyroidism could also involve effects on the local microenvironment or possible indirect influences. Hypothyroidism is known to influence locomotor activity (Sala-Roca et al., 2002) and corticosterone levels (Lo et al., 1998; Sabeur et al., 1993), both of which regulate adult hippocampal neurogenesis (Gould et al., 1991a,b; van Praag et al., 1999). Given that previous reports indicate no change, or if at all an increase, in activity in hypothyroid rats, the hypothyroidism-
induced decrease in neurogenesis is unlikely to result from deficits in locomotor activity (Crockert et al., 1986; Tamasy et al., 1986). The decreased neurogenesis in hypothyroidism is unlikely to be attributable to altered corticosterone since the goitrogens MMI and PTU increased and did not alter corticosterone respectively, but both decreased neurogenesis.

The functional significance of decreased hippocampal neurogenesis observed in adult-onset hypothyroidism is at present unclear. One can speculate that the hypothyroidism-induced deficits in hippocampal-dependent cognitive functions such as learning and memory (Osterweil et al., 1992) may involve at least in part decreased hippocampal neurogenesis. Adult hypothyroidism-induced learning and memory deficits can be reversed by thyroid hormone replacement (Dugbartey, 1998) correlating well with our results that euthyroid status replacement rescues deficits in adult hippocampal neurogenesis. Adult-onset hypothyroidism has also been associated with depressive symptoms in animal models and humans (Bauer and Whybrow, 2001). It is known that animal models of depression exhibit decreased neurogenesis (Malberg and Duman, 2003) suggesting the possibility that the effects of hypothyroidism on mood may involve decreased adult hippocampal neurogenesis (Bauer and Whybrow, 2001). In addition, clinical studies have suggested that thyroid hormone can augment and accelerate the effects of antidepressants (Altschuler et al., 2003; Bauer and Whybrow, 2001). It is possible that the effects of thyroid hormone on adult hippocampal neurogenesis may be relevant in this context. The results of our study indicate that thyroid hormone regulates adult hippocampal neurogenesis and motivates future experiments to address the contribution of adult hippocampal neurogenesis to the cognitive and behavioral deficits associated with adult-onset hypothyroidism.

Experimental methods

Animal treatment paradigms and BrdU labeling

Male Sprague–Dawley rats (250–270 gm) bred in our animal-breeding colony were used for all experiments. All animal procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the TIFR Animal Ethics Committee. Animals were maintained on a 12 h light–dark cycle with access to food and water ad libitum. To examine the influence of thyroid status on adult hippocampal neurogenesis, animals were rendered hypothyroid using the goitrogens 2-mercapto-1-methylimidazole (MMI, Sigma, USA) or 6-n-propyl-2-thiouracil (PTU, Sigma) and were rendered hyperthyroid using 3,5,3'–triiodothyronine (T3). To restore euthyroid status to hypothyroid animals, a combination replacement therapy of triiodothyronine (T3, Sigma) and thyroxine (T4, Sigma) was utilized.

For experiments to examine the influence of hypothyroidism on the proliferation of adult dentate granule cell progenitors, animals received MMI (0.025%) or PTU (0.05%) in the drinking water for 28 or 21 days respectively, with the control groups receiving normal drinking water. To examine the influence of hyperthyroidism on the proliferation of adult dentate progenitors, animals received subcutaneous (s.c.) injections of T3 (1 mg/kg) or vehicle (0.02 N NaOH) daily for 9 days. All animals in the proliferation experiments received intraperitoneal (i.p.) injections of the mitotic marker 5-bromo-2'-deoxyuridine (BrdU, 50 mg/kg; Sigma) twice daily on the last 2 days of treatment and were sacrificed 24 h following the last BrdU injection. To determine the expression of thyroid receptor isoforms by adult dentate granule cell progenitors, animals received a single BrdU (50 mg/kg) injection and were sacrificed 2 h later.

In experiments to determine the influence of hypothyroidism on the survival and differentiation of adult dentate granule cell progenitors, drug-naive animals first received BrdU twice daily for two consecutive days before commencing treatments. To determine the starting number of BrdU-positive cells, one group of animals was sacrificed 24 h after the last BrdU injection. For the survival experiment, animals received either MMI (28 days) or PTU (21 days) in drinking water, with respective controls receiving normal drinking water. For the differentiation experiment, animals received MMI or PTU for 28 days each. All animals were sacrificed on the last day of goitrogen treatment. To examine the influence of hypothyroid status on the number of apoptotic cells, MMI-treated animals and controls were sacrificed at an earlier time point of 21 days after BrdU treatment. To address the influence of hyperthyroidism on the survival and differentiation of adult dentate progenitors, drug-naive animals first received BrdU twice daily for two consecutive days. Following the last BrdU administration, animals received daily injections of T3 (1 mg/kg; s.c.) or vehicle from day 3 to day 21 and were sacrificed 21 days after the last BrdU injection.

Experiments examining the influence of restored euthyroid status in hypothyroid animals had four experimental groups: Control, MMI, MMI + T3/T4 and T3/T4. Drug-naive animals first received BrdU twice daily for two consecutive days. Control and T3/T4 groups then received normal drinking water, whereas the MMI and MMI + T3/T4 animals received MMI in drinking water for 28 days. The T3/T4 and MMI + T3/T4 groups received daily s.c. injections of T3 (10 μg/kg) and T4 (10 μg/kg) for the duration of goitrogen treatment. Control and MMI groups received daily s.c. injections of the vehicle (0.02 N NaOH). The combination of T3/T4 used in this study was based on a previous report indicating that combined T3/T4 treatment is the most suitable for restoring euthyroid status in all tissues (Escobar-Morreale et al., 1996). All animals were sacrificed 28 days after the last BrdU injection.

Tissue culture and thyroid hormone treatment

Dissociated neural progenitor cell cultures were established from dissected hippocampi of adult Sprague–Dawley rats using a previously established method (Palmer et al., 1999). Hippocampal progenitor cells isolated using this method were cultured in DMEM/F-12 with N2 supplement (Gibco, USA) and 40 ng/ml FGF-2 (R&D Systems, USA) on poly-ornithine (Sigma) and laminin (Sigma) coated tissue culture plates. Adult hippocampal progenitor cells were serially passaged and observed to form enriched populations of nestin-positive progenitors in growth medium containing FGF-2. Prior to each experiment, cells in culture were transferred into 16-well polystyrene and laminin coated Labtak chamber slides (Nunc, USA) at a density of 5 × 10^5 cells/well and maintained in the growth medium (DMEM/F-12 + N2 + 20 ng/ml FGF-2) overnight before transfer to experimental medium the next day. To address the influence of thyroid hormone on the proliferation and survival of hippocampal progenitor cells, growth medium was used with or without added thyroid hormone (T3; 20 nM or 50 nM). To study the effects of thyroid hormone on the differentiation of hippocampal progenitor cells, growth medium...
was replaced with differentiation medium (DMEM/F-12 + N2) containing lowered FGF-2 (1 ng/ml), 1% fetal calf serum (Gibco) and triiodothyronine (20 nM, 50 nM or 100 nM). In all experiments, medium was replaced every other day for the duration of the experiments namely 2 weeks. Control wells contained the appropriate medium without thyroid hormone. In the proliferation experiment, progenitor cells were exposed to a pulse of BrdU (40 μM, Sigma) for 2 h. At the end of all experiments, hippocampal progenitor cultures were fixed by exposure to 4% paraformaldehyde (PFA) for 15 min prior to further processing.

**Immunohistochemistry and immunofluorescence**

Rats were sacrificed through transcardial perfusion with 4% PFA, and serial coronal sections (50 μm) through the rostro-caudal extent of the hippocampus were generated using a vibratome. Free-floating sections were processed for BrdU immunohistochemistry as described previously (Kulkarni et al., 2002). In brief, following DNA denaturation and acid hydrolysis, sections were incubated overnight with mouse anti-BrdU antibody (1:500, Boehringer Mannheim, USA) and then exposed to secondary antibody (biotinylated anti-mouse IgG, 1:500, Vector Laboratories, USA). Signal amplification was carried out with an avidin–biotin complex (Vector) and was detected with diaminobenzidine (Sigma). For BrdU immunohistochemistry on hippocampal progenitor cell cultures in 16-well chamber slides, following fixation with 4% PFA and acid hydrolysis, cells were incubated overnight with mouse anti-BrdU antibody (1:300, Boehringer Mannheim) and then exposed to secondary antibody (Cy3-conjugated anti-mouse IgG, 1:1000, Molecular Probes, USA). Cells were counterstained with DAPI (10 μg/ml, Sigma) and then mounted with Fluoromount (Sigma).

For double-label immunofluorescence experiments to examine the neuronal or glial differentiation of adult dentate granule cell progenitors and to detect thyroid hormone receptor isoform expression by these progenitors, tissue sections were processed as above prior to exposure to the following antibody cocktails: (1) rat anti-BrdU (1:500, Accurate Biochemicals, USA) with mouse anti-neuronal nuclei (NeuN) (1:1000, Chemicon, USA) or rabbit anti-glial fibrillary acidic protein (GFAP) (1:500, Chemicon), (2) rat anti-BrdU (1:500, Accurate) with one of the goat anti-thyroid hormone receptor antibodies (c-Erb-A α, c-Erb-A β1 or c-Erb-A β2; 1:100, Santa Cruz Biotechnology, USA) overnight. The c-Erb-A α antibody does not distinguish between the TRα1 and TRα2 receptor isoforms. Sections were incubated with cocktails of secondary antibodies: (1) biotinylated anti-rat IgG (1:500, Chemicon) with rhodamine-conjugated anti-mouse or anti-rabbit IgG, (1:500, Chemicon), (2) biotinylated anti-goat IgG (1:500, Chemicon) and rhodamine-conjugated anti-rat IgG (1:500, Chemicon) followed by incubation with fluorescein-conjugated streptavidin (1:500, Vector). Immunofluorescence colocalization was determined using confocal Z-plane sectioning (0.5 μm steps) with a Biorad MRC 1024 confocal microscope. Immunofluorescent double-labeling of hippocampal progenitors in culture was performed to examine their expression of thyroid receptor isoforms, as well as the neuronal and glial differentiation of T3-treated or untreated cells. Cells were incubated overnight with individual goat anti-thyroid hormone receptor antibodies or a cocktail mixture (c-Erb-A α, c-Erb-A β1 or c-Erb-A β2; 1:150, Santa Cruz Biotechnology) and the mouse anti-nestin antibody (1:10, DSHB, USA). To determine glial and neuronal differentiation, cells were incubated with rabbit anti-beta tubulin antibody (1:2000, Covance, USA) and mouse anti-glial fibrillary acidic protein (1:300, Chemicon) overnight. Cells were then incubated with cocktails of secondary antibodies: (1) biotinylated anti-goat IgG (1:500, Chemicon) and Cy3-conjugated anti-mouse IgG (1:1000, Molecular Probes) followed by incubation with fluorescein-conjugated streptavidin (1:500, Vector) or (2) Cy3-conjugated anti-rabbit IgG (1:1000, Molecular Probes) and Alexa 488-conjugated anti-mouse IgG (1:300, Molecular Probes). Cells were counterstained with DAPI (10 μg/ml, Sigma) and were then mounted with Fluoromount (Sigma) and imaged using an epifluorescent Zeiss (Axioskop 2 plus) microscope.

**TUNEL assay**

The TdT-mediated dUTP nick end labeling (TUNEL) assay was performed as per the instructions using an in situ cell death detection kit (Boehringer Mannheim). Animals were sacrificed by rapid decapitation, and brains were frozen and stored at −70°C. Apoptotic TUNEL-positive cells in the dentate gyrus region were quantitated on cryostat cut (14 μm) sections. Sections were counterstained with DAPI (10 μg/ml, Sigma) to aid visualization of the granule cell layer (GCL). The number of TUNEL positive cells/section (9 sections/animal) was counted as being in the inner layer when they were directly touching the subgranular zone (SGZ) or within it and was counted as being in the outer layer of the GCL when they were further than two cell body widths from the SGZ. The TUNEL assay was also performed on cultured hippocampal progenitors in 16-well chamber slides followed by counterstaining with DAPI (10 μg/ml, Sigma) and mounted using Fluoromount.

**Serum T3 and corticosterone assays**

Trunk blood was collected from all experimental animals at the time of sacrifice. Serum was separated out and stored at −20°C. Serum T3 levels were determined in all animals using the commercially available radioimmunoassay kit (RIAK-4/4A, BRIT, Mumbai, India) based upon a previously described protocol (Joseph et al., 1996). Serum corticosterone levels were determined using a commercially available ELISA kit (R&D Systems).

**Volume measurement**

Every fourth section through the hippocampus of each animal was stained with cresyl violet (Sigma) and was used to determine the total volume of the GCL and the hippocampus in control and hypothyroid animals. Using the Macintosh-based Scion Image software (Scion, USA), the area occupied by the GCL and hippocampus was determined by outlining the region using boundary criteria established by Paxinos and Watson, 1998. The total volume ($V$) of the GCL and hippocampus was calculated as per the formula $V = \Sigma A \times T \times 4$, where $\Sigma A$ represents the total of area measurements, $T$ is the section thickness, and 4 is the sample periodicity.

**Cell counting**

Quantitation of the BrdU-positive cells in tissue sections was carried out using a previously described modified, unbiased stereology protocol (Malberg et al., 2000) on a Zeiss Axioskop microscope. Quantitation was done on coded sections by an
To determine whether effects are restricted to hippocampal granule cell progenitors, every third striatal section was processed for Brdu immunohistochemistry to label subventricular zone (SVZ) progenitors. Using a stereology-based protocol (Sailor et al., 2003), Brdu-positive cells present on the lateral face of the lateral ventricle were counted (eight sections/animal; Bregma 1.60 to 0.20; n = 3/group). The area in which the cells were counted was traced and measured in mm² using NIH image 1.62 software (Scion image, USA) to yield the cells per mm².

The percent colocalization of Brdu labeling with cell-specific markers was determined in the SGZ and granule cell layer in eight sections (250 µm apart) per animal from the differentiation experiments. A minimum of 50 Brdu-positive cells were analyzed per animal (n = 4–5/group) using Z-plane sectioning (0.5 µm) to confirm the colocalization of Brdu with the neuronal marker NeuN or the glial marker GFAP.

Quantitation of the effects of T3 on cultured adult rat hippocampal progenitor cells was done using an epifluorescent Zeiss microscope under blinded conditions. To determine the effects of T3 on proliferation, the percentage of Brdu-positive cells following T3 treatment was determined and DAPI staining was used to determine the total cells per field of view. To address the influence of T3 on survival, the percentage of TUNEL-positive cells from the total number of cells counted (DAPI) was determined. The number of Brdu-positive cells for the proliferation experiment and TUNEL-positive cells for the survival experiment was counted in more than 600 cells/well (3 random, non-overlapping individual fields per well) and 3 wells were scored per condition. To determine the influence of T3 treatment on the neuronal and glial differentiation of adult hippocampal progenitor cells in vitro, the percentage of cells expressing the neuronal marker beta-tubulin (Tuj1) or the glial marker GFAP from the total number of cells counted (DAPI) was examined. The percentage of Tuj1 positive and GFAP-positive cells was scored by counting more than 600 cells from 3 non-overlapping individual fields per well and 3 wells per condition.

Statistical analysis

Results were subjected to statistical analysis using the program Prism (Graphpad, 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 four groups were subjected to statistical analyses using one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test, with significance determined at P values < 0.05.

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References


