Thyroid hormone responsiveness in N-Tera-2 cells

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Abstract

N-TERA-2 cl/D1 (NT2) cells, a human embryonal cell line with characteristics of central nervous system precursor cells, were utilised to study thyroid hormone action during early neuronal growth and differentiation. Undifferentiated NT2 cells expressed mRNAs encoding thyroid hormone receptors (TRs) α1, α2 and β1, iodothyronine deiodinases types 2 (D2) and 3 (D3) (which act as the pre-receptor regulators), and the thyroid hormone-responsive genes myelin basic protein (MBP) and neuroendocrine specific protein A (NSP-A). When terminally differentiated into post-mitotic neurons (hNT), TRα1 and TRβ1 mRNA expression was decreased by 74% (P=0·05) and 95% (P<0·0001) respectively, while NSP-A mRNA increased 7-fold (P<0·05). However, mRNAs encoding TRα2, D2, D3 and MBP did not alter significantly upon neuronal differentiation and neither did activities of D2 and D3. With increasing 3,5,3′-triiodothyronine (T_3) concentrations, TRβ1 mRNA expression in cultured NT2 cells increased 2-fold at 10 nM T_3 and 1·3-fold at 100 nM T_3 (P<0·05) compared with that in T_3-free media but no change was seen with T_3 treatment of hNT cells. D3 mRNA expression in NT2 cells also increased 3-fold at 10 nM T_3 (P=0·01) and 2·4-fold at 100 nM T_3 (P<0·05) compared with control, but there was no change in D3 enzyme activity. In contrast there was a 20% reduction in D3 mRNA expression in hNT cells at 10 nM T_3 (P<0·05) compared with control, with accompanying reductions in D3 activity with increasing T_3 concentrations (P<0·05). There was no significant change in the expression of the TRα isoforms, D2, MBP and NSP-A with increasing T_3 concentrations in either NT2 or hNT cells. Undifferentiated NT2 and differentiated hNT cells show differing patterns of T_3-responsiveness, suggesting that there are different regulatory factors operating within these cell types.

Journal of Endocrinology (2003) **178,** 159–167

Introduction

The importance of thyroid hormones in the development of the human central nervous system (CNS) is well established (Rovet 1999, Chan & Kilby 2000) but the specific molecular mechanisms involved during thyroid hormone-dependent effects on early CNS development are poorly understood. Before the onset of endogenous thyroid hormone production (Fisher & Klein 1981), the first trimester human fetus is exposed to physiologically relevant levels of maternally derived thyroid hormones (Calvo *et al.* 2002), which exert their effects through interaction with thyroid hormone receptors (TRs) expressed in specific fetal tissues (Chan *et al.* 2002). In rats, relatively large alterations in maternal thyroid hormone status at a stage of gestation before the onset of fetal thyroid hormone production have influenced the expression of thyroid hormone-responsive genes within the fetal CNS (Dowling & Zoeller 2000, Dowling *et al.* 2001). These findings provide a possible molecular basis for the observed epidemiological link in humans between maternal hypothyroxinaemia in early pregnancy and neurodevelopmental deficits in children (Man *et al.* 1991, Haddow *et al.* 1999, Pop *et al.* 1999). To explore this link further, we have used NTERA-2 cl/D1 (NT2) cells as an *in vitro* human model for investigating thyroid hormone action in neuronal cells of embryonic origin.

NT2 cells are a pluripotent (Ferrari *et al.* 2000) embryonal cell line identified from a human teratocarcinoma (Andrews *et al.* 1984) and they exhibit similar biochemical and developmental characteristics to CNS precursor cells in the very early embryo (Martin 1980, Hartley *et al.* 1999, Satoh & Kuroda 2000). They differentiate in response to retinoic acid (RA) into post-mitotic neurons (hNT), confirmed by the expression of neurofilament (Lee & Andrews 1986) and MAP2 (Bani-Yaghoub *et al.* 1999) protein.

Intracellular thyroid hormone action requires the pre-receptor delivery of the active ligand, 3,5,3′-triiodothyronine (T_3), in part determined by the actions of

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iodothyronine deiodinases, as well as the expression of functional TR isoforms. TRs are nuclear transcription factors encoded by the Erb-A α and β genes, each producing two major isoforms (Oppenheimer & Schwartz 1997). TRα1, TRβ1 and TRβ2 all bind T_3 to form complexes which can interact with thyroid response elements (TREs) in the promoter regions of thyroid hormone-responsive genes in order to regulate their transcription. The non-ligand binding α2 isoform, in contrast, may modulate the actions of TRs through competitive inhibition (Lazar *et al.* 1989, Lazar 1993). Our group has previously reported the presence of TRα and β isoforms in the human fetal CNS from as early as 7 weeks of gestation (Chan *et al.* 2002), with the strongest expression found within cortical pyramidal neurons and Purkinje cells of the cerebellum (Kilby *et al.* 2000).

Deiodinases are selenocysteine-containing enzymes and are pre-receptor modulators of local thyroid hormone action (Kohrle 2000). The predominant circulating thyroid hormone, thyroxine (T_4), is converted to T_3 by type I (D1) or type II (D2) deiodinases (Visser *et al.* 1983, 1988). Conversely, type III (D3) deiodinase primarily converts T_4 and T_3 into inactive metabolites (Visser 1996).

D1 activity is undetectable in human fetal and adult cerebral cortices (Campos-Barros *et al.* 1996, Chan *et al.* 2002) and is unlikely to play a significant role in the human brain. In contrast, D2 and D3 enzymes, with K_m values in the nanomolar range (Visser *et al.* 1983, 1988, Salvatore *et al.* 1996), are thought to be crucial in the homeostatic regulation of intracellular T_3 concentrations (Bates *et al.* 1999) especially in the brain (Santini *et al.* 2001), where approximately 80% of T_3 is derived locally through D2 activity (Crantz *et al.* 1982).

In rodent models, hypothyroxinaemia induced by subtotal maternal thyroid ablation results in enhanced D2 activity and decreased D3 activity in the fetal brain (Calvo *et al.* 1990), while in cultures of rat astrocytes depleted of T_3 similar alterations in D2 and D3 activities are also described (Cavalieri *et al.* 1986, Esfandiari *et al.* 1992).

We have assessed the transcription of two genes known to be expressed in the early mammalian CNS, myelin basic protein (MBP) (Zecevic *et al.* 1998, Tosic *et al.* 2002) and neuroendocrine specific protein A (NSP-A) (Dowling *et al.* 2001). A positively regulated TRE in the MBP promoter has been described in rat oligodendrocytes and neuronal cells (Farsetti *et al.* 1992, Jeannin *et al.* 1998). In rats, NSP-A down-regulation in specific regions of the fetal CNS has been observed when a maternal injection of T_4 is given in early gestation. Since a specific TRE has not yet been characterised for NSP-A, this gene may be indirectly down-regulated by thyroid hormones (Dowling *et al.* 2001).

We hypothesise that CNS precursor cells are thyroid hormone-responsive and demonstrate homeostatic T_3 regulation through changes in deiodinase activity even before they terminally differentiate into neurons. In this study, we aimed to show that NT2 cells express TRs and deiodinases, then determine whether NT2 cells demonstrate altered expression of mRNA encoding the TR isoforms, deiodinase subtypes, MBP and NSP-A, as well as altered D2 and D3 activities, when differentiated into neurons and also in response to increasing concentrations of T_3.

Materials and Methods

Cell culture

Undifferentiated NT2 cells (Stratagene, La Jolla, CA, USA) were maintained in complete D-MEM/F12 media containing Dulbecco's Eagle's medium NUT F12 (Ham), supplemented with 10% fetal bovine serum (FBS), 1 × L-glutamine and 1% penicillin/streptomycin (pen/strep) (Life Technologies, Grand Island, NY, USA) as previously described (Andrews *et al.* 1984). Cells were passaged and split 1:4 twice weekly, and cultured at 37 °C and 5% CO_2.

Differentiation of NT2 precursor cells to hNT neurons was conducted as previously described (Pleasure *et al.* 1992). NT2 cells ($2{\cdot}5 \times 10^6$) were plated in each T75 tissue culture flask 24 h before beginning RA induction with complete D-MEM/F12 + 10 μM RA (Sigma-Aldrich) media, which was fed to the cells three times a week for 5–6 weeks.

Replate 1 Following washes with sterile PBS, Dulbecco A (pH 7·3) (Oxoid, Hants, UK) and Versene (1 × 1:5000; Life Technologies), the cells were detached with trypsin–EDTA (0·05% trypsin, 0·53 mM EDTA.4Na; Gibco/Invitrogen) and replated in complete D-MEM/F12 at a density of 30–40 × 10^6 cells per T75 flask. After 48 h this complete D-MEM/F12 medium was stored as conditioned medium at 4 °C. The cells were then fed with D-MEM/F12 containing 5% FBS, 1% pen/strep, 10 μM 5-fluoro-2′-deoxyuridine, 10 μM uridine and 1 μM cytosine β-D-arabinofuranoside (all mitotic inhibitors from Sigma-Aldrich) four times over 9–10 days followed by a single feed of conditioned medium without mitotic inhibitors 24 h before replate 2.

Replate 2 (neuron harvesting) Cells were washed with PBS + 0·9 mM Ca^{2+} and 0·5 mM Mg^{2+}. Only differentiated post-mitotic neurons (hNT) were detached (leaving behind the undifferentiated NT2 cells) with trypsin (without EDTA; Gibco) and plated onto poly-D-lysine- and laminin- (Sigma-Aldrich) coated flasks and tissue culture plates for the experiments. hNT neurons were passaged in 50:50 conditioned media (i.e. equal amounts of complete and conditioned media) every 3–4 days for 1 week, thus yielding a 99% pure population of neurons.

The identity of the hNT cells was confirmed by the presence of neurofilament protein (Hens *et al.* 1998,

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Table 1 The quantitative primer and probe sequences. Each pair of primers was designed to span exon–intron boundaries to prevent genomic amplification, except for deiodinase type 3, which is a single exon gene. The quantitative primer and probe sequences used for the deiodinases and thyroid hormone receptors are as previously described (Chan *et al.* 2002). The MBP primers and probes were designed to span the region crossing the exon 3 and 4 boundary that would amplify all four isoforms of MBP. The NSP-A primers and probes spanned the region crossing the first part of exon 1 and the start of exon 2, which is unique to this isoform of NSP. Tests on negative reverse transcriptase controls showed no genomic amplification

Gene	Forward primer	TaqMan probe	Revers primer
D1	TTGGGAGTTTATGCAAGGTAATAGG	TCAGGTACAACTTCCAAAATTCAGCACCAGTG	TGGAACTAAAGTCTTCAATAAG CCTCTT
D2	CTGCGCTGCGTCTGGAA	CCCAATTTCACCTGTTTGTAGGCATCGAG	GAGACATGCACCACACTGGAAT
D3	GCTTCCAGAGCCAGCACATC	TCGACTACGCGCAAGGGAACCG	GCTGCCGAAATTGAGAACCA
TRα1	GCTGCAGGCTGTGCTGCTA	CACCTTCATCAGCAGCTTGGGCC	CGATCATGCGGAGGTCAGT
TRα2	CAAACACAACATTCCGCACTTC	CTCTGCACTTCTCTCTCCTTCATCAGCAGC	GCCCCCTTGTACAGAATCGA
TRβ1	GTGTCTCAAGTGCCCAGACCTT	TGGGGATGTACCCTTTACATTTCTTCTCCTCC	CACAGAGCTCGTCCTTGTCTAA GTAA
TRβ2	AAGTGCAGTCGCCATCGTATT	TCAAAAAAAAGGGTACATCCCCAGTTACTT AGACAAG	GGCTTTGTCACCACACACTACAC
MBP	TCCCTGCCCCAGAAGTCA	CGGACCCAAGATGAAAACCCCGTAGTC	GTGCGAGGCGTCACAATGT
NSP-A	GCCCGTTGCCATGGAAA	AACACCTGCCACACCTGTGGATGCA	CGATCCTTCCCCATCTTTTGA

Bani-Yaghoub *et al.* 1999) on a Western blot performed as follows. NT2 and hNT cells were plated in six-well plates as above. Protein was prepared using 1% SDS (Sigma-Aldrich). Protein concentration was measured by the Bradford assay with BSA as standard. Soluble protein (20 µg) was denatured (5 min, 95 °C) in loading buffer (Lamelli (Bio-Rad Laboratories, Richmond, CA, USA) plus β-mercaptoethanol (Sigma) in the ratio 4:1) and separated by electrophoresis in 10% SDS polyacrylamide gels, transferred to polyvinylidene fluoride membranes, incubated in 5% non-fat milk in PBS with 0·1% Tween for 2 h at room temperature, followed by incubation with a mouse monoclonal antibody to neurofilament 200 (RT97 clone; Serotec, UK) at 1:500 for 16 h at 4 °C. After washing in PBS plus 0·1% Tween, blots were incubated with anti-mouse secondary antibodies conjugated to horseradish peroxidase (Dako A/S, Copenhagen, Denmark) at 1:2000 for 1 h at room temperature. After further washes, antigen–antibody complexes were visualised by the ECL-plus chemiluminescence detection system (Amersham Pharmacia Biotech). The light chain of neurofilament protein (68 kDa) was detected in differentiated hNT neurons but not in undifferentiated NT2 cells when exposed to three different doses of T_3 (0, 10, 100 nM) (data not shown).

RNA extraction and quantitative RT-PCR

Approximately 2–3 × 10^5 undifferentiated NT2 cells or hNT cells were plated into each well of a six-well plate and passaged in complete medium the day before the experiment. For 6 h immediately prior to experimentation, cells were treated with medium supplemented with charcoal-stripped FBS (First Link UK Ltd, Midlands, UK), then treated with T_3 (0, 10 nM or 100 nM; n=7 each dose) (Sigma-Aldrich) for 16 h before total RNA extraction using a single-step acid guanidinium phenol–chloroform (Tri-reagent; Sigma-Aldrich) extraction method (Chomczynski & Sacchi 1987). Preliminary experiments had demonstrated these T_3 doses and the treatment time chosen to be optimal for the detection of changes in gene expression (data not shown). One millilitre of tri-reagent was added to each well, and the resulting total RNA dissolved in nuclease-free water and quantified. One microgram of RNA was reverse transcribed in a total reaction volume of 20 µl, containing 0·25 µg random hexamer primers, 2 µl 10 × AMV reverse transcriptase buffer, 10 pmol deoxynucleotide triphosphate (dNTP), 10 units ribonuclease inhibitor and 7·5 units AMV reverse transcriptase (all reagents from Promega, Madison, WI, USA). Upon completion of the RT reaction the RT mixture was diluted 1:1 in nuclease-free water.

Relative expression of the mRNA encoding the gene of interest was calculated using the ABI PRISM 7700 Sequence Detection System, which employs TaqMan chemistry. Target genes were labelled with the reporter dye FAM while the housekeeping gene, 18S, used as an internal reference to allow for differences in RT efficiency, was labelled with VIC. Details of the methods have been previously described (McTernan *et al.* 2001, Chan *et al.* 2002, McCabe *et al.* 2002). Briefly, quantitative RT-PCR was carried out in 25 µl volumes in 96-well plates, in a reaction buffer containing 1 × TaqMan Universal PCR Master Mix, 3 mM $Mn(OAc)_2$, 200 µM dNTPs, 1·25 units AmpliTaq Gold polymerase, 1·25 units AmpErase UNG, 100–200 nM TaqMan probe and 900 nM primers. The quantitative primer and probe sequences are given in Table 1. All reactions, except for the TRs and D1, were multiplexed with a set of probe and primers for 18S

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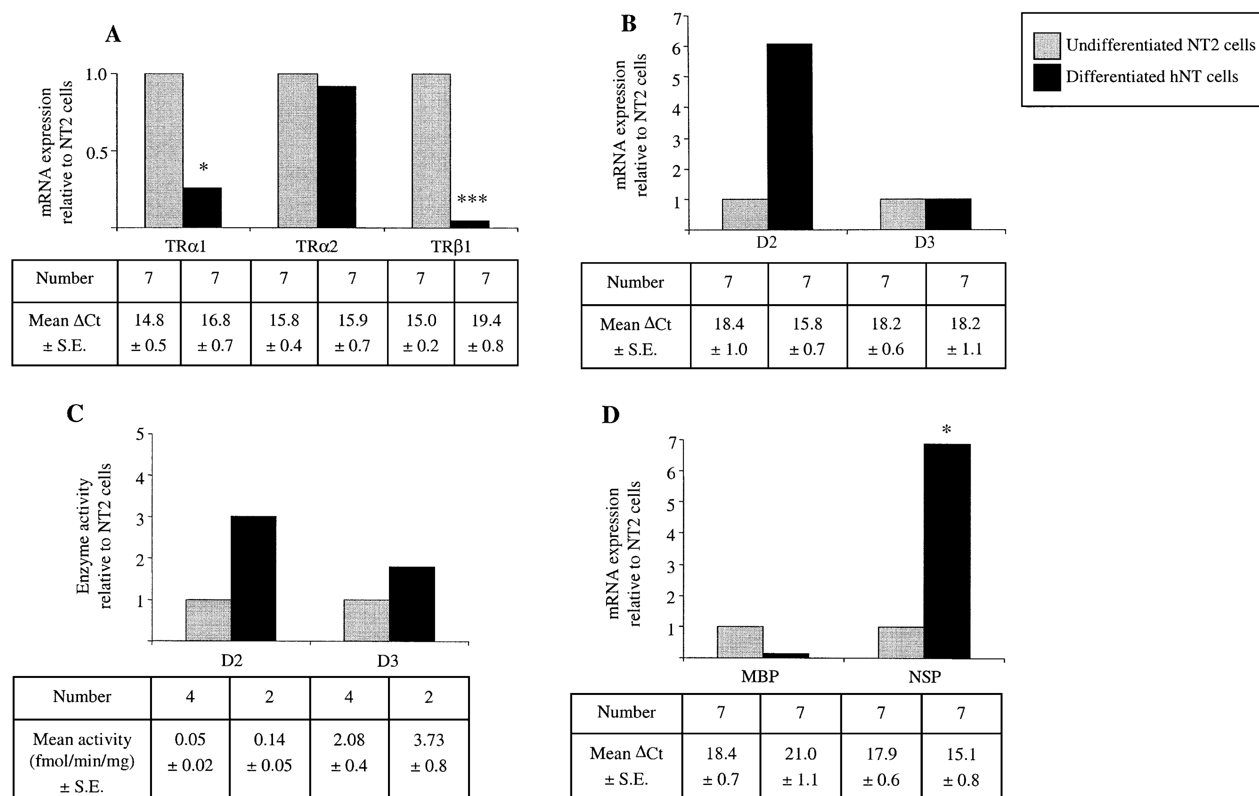

Number	7	7	7	7	7	7
Mean ΔCt ± S.E.	14.8 ± 0.5	16.8 ± 0.7	15.8 ± 0.4	15.9 ± 0.7	15.0 ± 0.2	19.4 ± 0.8

Number	7	7	7	7
Mean ΔCt ± S.E.	18.4 ± 1.0	15.8 ± 0.7	18.2 ± 0.6	18.2 ± 1.1

Number	4	2	4	2
Mean activity (fmol/min/mg) ± S.E.	0.05 ± 0.02	0.14 ± 0.05	2.08 ± 0.4	3.73 ± 0.8

Number	7	7	7	7
Mean ΔCt ± S.E.	18.4 ± 0.7	21.0 ± 1.1	17.9 ± 0.6	15.1 ± 0.8

Figure 1 (A) The relative expression of mRNA encoding the TR isoforms α1, α2 and β1 in differentiated hNT cells compared with undifferentiated NT2 cells, given an arbitrary value of 1. Statistically significant differences were $*P<0{\cdot}05$, $***P<0{\cdot}0001$. The number of samples in the experiment and the mean ΔCT values (± S.E.M.) obtained using quantitative RT-PCR for each group are given in the corresponding columns in the table below the graph. (B) The relative expression of mRNA encoding the iodothyronine deiodinases type 2 (D2) and type 3 (D3) in differentiated hNT cells compared with undifferentiated NT2 cells, given an arbitrary value of 1. The number of samples expressing the mRNA and the mean ΔCT values (± S.E.M.) obtained using quantitative RT-PCR for each group are given in the corresponding columns in the table below the graph. (C) The relative activity of iodothyronine deiodinases type 2 (D2) and type 3 (D3) in differentiated hNT cells compared with undifferentiated NT2 cells, given an arbitrary value of 1. The number of samples investigated and the mean (± S.E.M.) enzyme activity are given in the corresponding columns in the table below the graph. (D) The relative expression of mRNA encoding myelin basic protein (MBP) and neurospecific protein A (NSP-A) in differentiated hNT cells compared with undifferentiated NT2 cells, given an arbitrary value of 1. Statistically significant difference was $*P<0{\cdot}05$. The number of samples and the mean ΔCt values (± S.E.M.) obtained using quantitative RT-PCR for each group are given in the corresponding columns in the table below the graph.

ribosomal RNA, provided as a pre-optimised, primer-limited control system (Applied Biosystems, Warrington, Cheshire, UK). The TR and D1 reactions were single-plexed to avoid potential interference with 18S reactions indicated by preliminary experiments.

As previously published (Chan *et al.* 2002), values were expressed as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values (ΔCt=Ct of the target gene (e.g. D2) minus Ct of the housekeeping gene 18S). These values were averaged and transformed through the equation $2^{-\Delta\Delta Ct}$ to give fold changes in gene expression.

Deiodinase activity assays

Approximately $1{\cdot}5$–$2{\cdot}5 \times 10^6$ undifferentiated NT2 cells or hNT cells were plated in T75 flasks and passaged in complete medium the day before the experiment. Treatment with T_3 at 0, 10 and 100 nM concentrations (NT2: $n=4$ per dose; hNT: $n=2$ at 0 nM, $n=3$ each at 10 and 100 nM) was carried out as described above. After treatment for 16 h, cells in each T75 flask were washed with ice-cold PBS and scraped into a cell suspension then centrifuged down into a cell pellet.

Activities of specific deiodinase subtypes were estimated using methods described previously (Visser *et al.* 1983, Yoshida *et al.* 1985, Koopdonk-Kool *et al.* 1996). The cell pellets were resuspended in ten volumes of 0·1 M phosphate (pH 7·2), 2 mM EDTA and 1 mM dithiothreitol (P100E2D1 buffer). D2 activity was assayed by measurement of the release of radioiodide from the outer-ring labelled T_4, while D3 activity was assayed by HPLC analysis of the formation of radioactive T_2 and 3′-iodothyronine from outer-ring labelled T_3. Deiodination

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in the presence of the cells (~1 mg protein/ml) was corrected for non-enzymatic deiodination in the absence of cells.

D2 activity assay Incubations were carried out for 120 min at 37 °C with 1 nM (10^5 c.p.m.) [3′,5′-^{125}I]T_4 in the presence of 100 nM T_3 to block D3 and in the absence or presence of 100 nM T_4 to saturate D2 in 0·1 ml P100E2D25 buffer. Deiodinase activity was ascribed to D2 if inhibited by excess unlabelled T_4. HPLC analysis demonstrated that in cell suspensions with significant D2 activity, labelled T_4 was converted under these conditions to equivalent amounts of radioactive T_3 and iodide (Visser *et al.* 1983).

D3 activity assay Incubations were carried out for 60 min at 37 °C with 1 nM (2×10^5 c.p.m.) [3′-^{125}I]T_3 in the absence or presence of 100 nM T_3 to saturate D3 in 0·1 ml P100D2D50 buffer. Deiodinase activity was ascribed to D3 if inhibited by excess unlabelled T_3 (Yoshida *et al.* 1985, Koopdonk-Kool *et al.* 1996).

Statistics

Comparisons between hNT and NT2 cells were performed on ΔCt values using the unpaired *t*-test. Where a significant difference in variance was found, the Kruskal–Wallis non-parametric test was used instead. The T_3 dose–response comparisons were analysed using the Kruskal–Wallis multiple comparisons test on the mRNA fold changes and deiodinase activites obtained within each experiment.

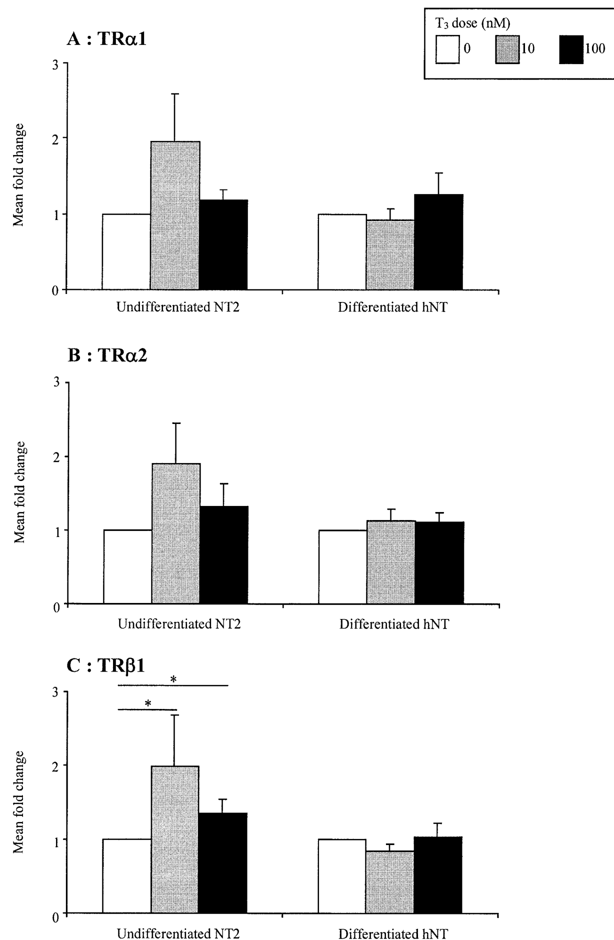

Figure 2 The mean fold changes (± S.E.M.) of mRNA encoding the TR isoforms α1 (A), α2 (B) and β1 (C) in undifferentiated NT2 cells (n=7) and differentiated hNT cells (n=7) exposed to increasing T_3 doses: 0 (given an arbitrary value of 1), 10 and 100 nM. The fold change for each T_3 dose within each experiment was calculated as described in the Methods. Statistically significant difference was *$P<0·05$.

Results

Changes in TR, deiodinase, MBP and NSP-A expression when undifferentiated NT2 cells are differentiated into post-mitotic hNT neurons

TRα1 and TRβ1 mRNA expression decreased by 74% (P=0·05) and 95% ($P<0·0001$) respectively but mRNA encoding α2 did not change significantly (Fig. 1A).

There was a non-significant 6-fold rise in D2 mRNA (P=0·06); D3 mRNA did not change upon differentiation (Fig. 1B). Similarly there was no significant difference in basal activities of D2 and D3 between hNT and NT2 cells (Fig. 1C). In both NT2 and hNT cells D2 activity was negligible (<0·35 fmol/min per mg).

There was a non-significant 84% reduction in MBP mRNA upon differentiation (P=0·06) but NSP-A mRNA increased significantly by 7-fold upon differentiation ($P<0·05$) (Fig. 1D).

Neither undifferentiated NT2 nor differentiated hNT cells expressed mRNA encoding TRβ2 or D1.

Changes in TR, deiodinase, MBP and NSP-A expression with increasing T_3 concentrations

TRs mRNAs encoding both α1 and α2 isoforms showed no significant change with increasing T_3 concentrations in NT2 and hNT cells (Fig. 2A and B). In undifferentiated NT2 cells TRβ1 mRNA expression increased by 2-fold at 10 nM T_3 and 1·3-fold at 100 nM T_3 ($P<0·05$) compared with that in T_3-free media. However, there was no significant change in mRNA expression between 10 and 100 nM T_3. In contrast, differentiated hNT cells showed no change in TRβ1 mRNA expression in response to increasing T_3 concentrations (Fig. 2C).

Deiodinases D2 showed no response to increasing T_3 concentrations in NT2 and hNT cells at both mRNA (Fig. 3A) and activity (data not shown) levels.

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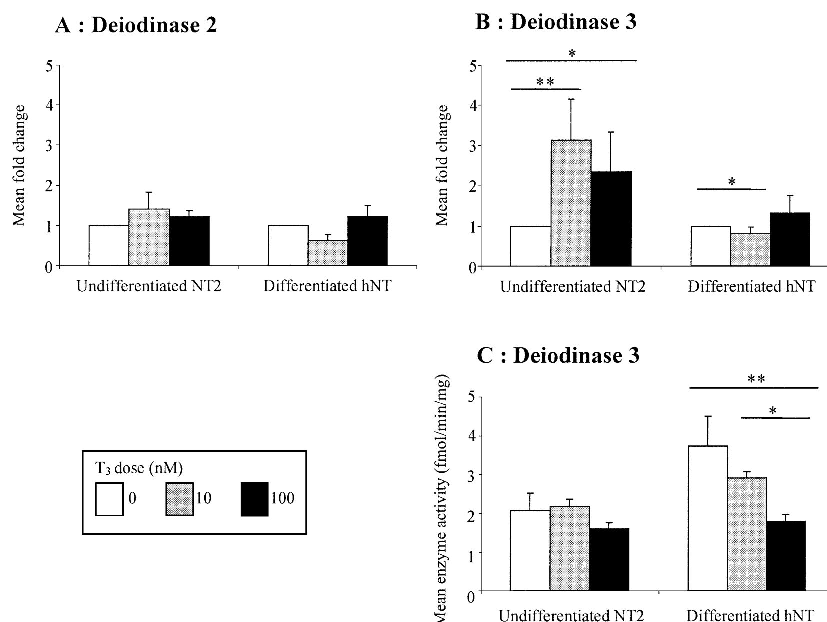

Figure 3 The mean fold changes (± S.E.M.) of mRNA encoding the iodothyronine deiodinase type 2 (A) and type 3 (B) in undifferentiated NT2 cells (n=7) and differentiated hNT cells (n=7) exposed to increasing T_3 doses: 0 (given an arbitrary value of 1), 10 and 100 nM. The fold change for each T_3 dose within each experiment was calculated as described in the Methods. The mean (± S.E.M.) enzyme activity of iodothyronine deiodinases type 3 (C) in undifferentiated NT2 cells and differentiated hNT cells exposed to increasing T_3 doses: 0, 10 and 100 nM. Statistically significant differences were $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$.

In undifferentiated NT2 cells, D3 mRNA increased by 3-fold at 10 nM T_3 (P=0·01) and 2·4-fold at 100 nM T_3 ($P<0{\cdot}05$) compared with cells in T_3-free media (Fig. 3B) but there was no significant change in D3 activity in response to increasing T_3 concentrations (Fig. 3C).

In differentiated hNT cells, there was a 20% reduction in D3 mRNA expression at 10 nM T_3 ($P<0{\cdot}05$) compared with that in T_3-free media (Fig. 3C). D3 activity in differentiated hNT cells also decreased with T_3 treatment, with a significant decrease seen between control and 100 nM T_3 ($P<0{\cdot}01$) and between 10 and 100 nM T_3 ($P<0{\cdot}05$) (Fig. 3D).

Thyroid hormone-responsive genes In order to investigate the possibility of downstream effects of thyroid hormone on transcription, we assessed the expression of mRNA encoding MBP and NSP-A. There was no significant change in mRNA encoding MBP (Fig. 4A) and NSP-A (Fig. 4B) with increasing T_3 concentrations between 0, 10 and 100 nM T_3 in both NT2 and hNT cells.

Discussion

The expressions of mRNAs encoding TR isoforms and iodothyronine deiodinases are described in NT2 and hNT cells. Our findings indicate that these cells are potentially thyroid hormone-responsive and possess mechanisms for pre-receptor regulation of thyroid hormone action. The absence of TRβ2 and D1 expression in NT2 cells is expected since TRβ2 expression is largely confined to the pituitary gland while D1 activity has previously not been detected in the human brain (Campos-Barros *et al.* 1996, Chan *et al.* 2002).

The reduction in mRNAs encoding TRα1 and TRβ1 upon neuronal differentiation reported here in NT2 cells is in contrast to that observed in studies of primary cultures of cortical neurons from fetal rats in which a constant TRα1 mRNA expression, an increased TRβ1 mRNA and either an increased or decreased TRα2 mRNA expression have been reported (Castiglia *et al.* 1992, Leonard *et al.* 1994). These apparent differences may reflect comparison of our findings in terminally differentiated neurons with those from a homogeneous culture of pluripotent CNS precursor cells, while the rat experiments described had compared neuronal findings with differentiating neurons and mixed CNS cultures. Furthermore, we have used a more sensitive and accurate method of quantifying mRNA than those described for the rat experiments. It is possible that pluripotent CNS cells have the highest level of TR mRNA expression, with both up-regulation and down-regulation of TR expression occurring during the course of differentiation into post-mitotic neurons.

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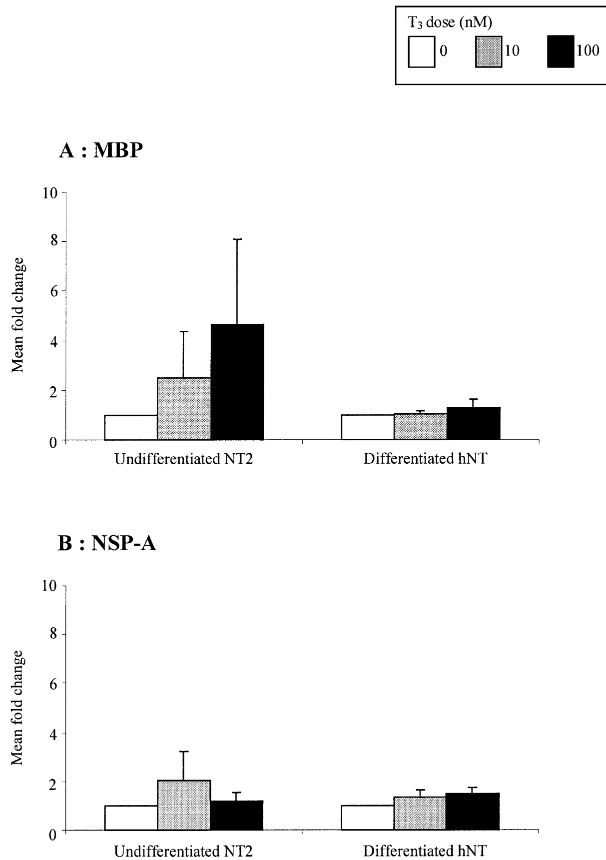

Figure 4 The mean fold changes (± S.E.M.) of mRNA encoding myelin basic protein (MBP) (A) and neurospecific protein A (NSP-A) (B) in undifferentiated NT2 cells (n=7) and differentiated hNT cells (n=7) exposed to increasing T_3 doses: 0 (given an arbitrary value of 1), 10 and 100 nM. The fold change for each T_3 dose within each experiment was calculated as described in the Methods.

The T_3 regulation of TR mRNA expression in rodents is both isoform- and cell type-dependent (Hodin *et al.* 1990, Yen 2001). The pluripotent NT2 cells respond to T_3 by increasing TRβ1 expression like rat astrocytes and oligodendrocytes (Lebel *et al.* 1993, Baas *et al.* 1998) but following neuronal differentiation into post-mitotic hNT cells this response is lost and the cells behave similarly to rat neurons, showing no change in TR expression with T_3 exposure (Castiglia *et al.* 1992, Lebel *et al.* 1993). T_3-induced changes in TRα isoform mRNA expression may be an acquired characteristic upon differentiation into non-neuronal cell types (Hodin *et al.* 1990), hence the lack of change in TRα isoform mRNA expression seen in both NT2 and hNT cells.

The presence of D2 and D3 mRNA and activity in our 99% pure cultures of hNT neurons refutes the conventional belief that deiodinases may be exclusively expressed in glial cells within the CNS (Asteria 1998). In fact, more recent *in vivo* studies in rats have revealed the presence of D2 transcripts in some interneurons of the cerebral cortex (Guadano-Ferraz *et al.* 1999) and a distribution of D3 transcripts suggestive of predominant neuronal localisation (Tu *et al.* 1999).

Rat astrocytes have been reported to show a compensatory increase in D2 activity and a decrease in D3 activity when cultured in serum-free medium (i.e. absent thyroid hormones) (Cavalieri *et al.* 1986), and conversely a decreased D2 activity and increased D3 activity when exposed to thyroid hormones (Esfandiari *et al.* 1992). The fact that we did not see any T_3-induced D2 response at either transcriptional (mRNA) or post-translational (activity) levels in the NT2 cell type at two different stages of differentiation suggests that these cells may not be the critical cell type within which homeostatic regulation of thyroid hormone action occurs within the CNS.

In contrast, our data show an appropriate up-regulation of D3 mRNA in undifferentiated NT2 cells when exposed to T_3, suggesting the operation of transcriptional regulation of D3 in CNS precursor cells. There appears to be a limit to this compensatory mechanism as at a supraphysiological dose of T_3 (100 nM) a larger magnitude of D3 up-regulation was not observed compared with 10 nM T_3. This is akin to the response observed in rat astrocyte cultures where a maximal up-regulation in D3 activity was observed at a concentration of 10 nM T_3 treatment (Esfandiari *et al.* 1992). However, D3 activity in NT2 cells failed to reflect the changes in mRNA encoding D3. This could be due either to delay in the synthesis of functional protein following the induction of mRNA expression or to translational regulation of the D3 enzyme, a finding suggested by our previous study of human fetal cerebral cortices (Chan *et al.* 2002). Upon neuronal differentiation, the up-regulatory D3 response to T_3 treatment was lost and replaced by unexpected down-regulations of D3 mRNA observed with 10 nM T_3 treatment and of D3 activity seen with increasing T_3 concentrations. Thus it appears that with regard to deiodinase expression neuronal cells may respond differently to T_3 exposure compared with other CNS cell types.

The presence of mRNA encoding MBP in NT2 cells is in accord with *ex vivo* data indicating MBP expression in the early fetal nervous system (Zecevic *et al.* 1998, Tosic *et al.* 2002). However, we failed to observe MBP up-regulation by T_3 in a dose-dependent manner as reported in rat CNS models (Tosic *et al.* 1992), which may be due in part to species differences in the regulation of MBP expression.

Our finding of increased NSP-A mRNA expression with differentiation is in agreement with the report of a significant increase in NSP-A protein expression upon NT2 differentiation into neurons described previously (Hens *et al.* 1998). However, a reduction in NSP-A expression does not appear to occur with T_3 treatment in

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either NT2 or hNT cells, as might be predicted from *in vivo* rat studies (Dowling *et al.* 2001).

It is notable that undifferentiated NT2 and differentiated hNT cells show differing patterns of T_3-responsiveness in terms of TRβ1 and D3 expression, suggesting that there are different regulatory factors operating at different stages of neuronal differentiation.

Acknowledgements

This work has been funded by the Medical Research Council (UK), the Scientific Projects Committee, University of Birmingham and the Masons Trust.

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Received 25 March 2003
Accepted 10 April 2003

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