Thyroid hormones promote cell differentiation and up-regulate the expression of the *seladin-1* gene in *in vitro* models of human neuronal precursors

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Abstract

Thyroid hormones (TH) play an important role in the development of human brain, by regulating the expression of specific genes. *Selective Alzheimer's disease indicator-1* (*seladin-1*) is a recently discovered gene with neuroprotective properties, which has been found to be down-regulated in brain regions affected by Alzheimer's disease. Seladin-1 has anti-apoptotic properties mainly due to the inhibition of the activation of caspase 3. The aim of this study was to determine whether *seladin-1* may be regarded as a new mediator of the effects of TH in the developing brain. In order to demonstrate this hypothesis, the effects of TH both on cell differentiation and on the expression of *seladin-1* were assessed in two different cell models, i.e. fetal human neuroepithelial cells (FNC) and human mesenchymal stem cells (hMSC), which can be differentiated into neurons. 3,3',5-Triiodothyronine (T₃) determined

different biological responses (inhibition of cell adhesion, induction of migration, and increase in the expression of the neuronal marker neurofilament-M and Na⁺ and Ca²⁺ channel functionality) in both FNC and hMSC, which express TH receptors. Then, we showed that TH significantly increase the expression levels of *seladin-1*, and that T₃ effectively prevents camptothecin-induced apoptosis. However, in hMSC-derived neurons the expression of *seladin-1* was not affected by TH. Our results demonstrated for the first time that *seladin-1* is a novel TH-regulated gene in neuronal precursors. In view of its anti-apoptotic activity, it might be hypothesized that one of the functions of the increased *seladin-1* levels in the developing brain may be to protect neuronal precursor cells from death.

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Introduction

Thyroid hormones (TH) play a fundamental role in fetal life, particularly in promoting brain development. TH affect the expression of several genes, which are related to cell migration (i.e. reelin, laminin, tenascin C), myelination (i.e. myelin basic protein, proteolipid protein, myelin-associated glycoprotein), and neuronal differentiation (i.e. nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF); König & Neto 2002, Santisteban & Bernal 2005). In addition, a time-regulated activity of type II and III iodothyronine deiodinases (D2 and 3) in different brain areas is essential during brain development (Kester *et al.* 2004). Accordingly, it has been shown that early maternal hypothyroxinemia alters fetal brain histogenesis and cytoarchitecture in rats (Lavado-Autric *et al.* 2003), and unrecognized hypothyroidism in women in the first trimester

of pregnancy may adversely affect the neuropsychological development of the progeny (Haddow *et al.* 1999, Pop *et al.* 2003, Morreale de Escobar *et al.* 2004). These observations raised the attention of the scientific community on the benefit of screening programs for assessing thyroid function in pregnant women (Allan *et al.* 2000, de Escobar 2001, Redmond 2002, Surks *et al.* 2004).

Selective Alzheimer's disease indicator-1 (*seladin-1*) is a gene which has been identified a few years ago and which has been found to be down-regulated in brain regions affected by Alzheimer's disease (AD). Conversely, *in vitro* overexpression of *seladin-1* conferred protection against β -amyloid-mediated toxicity and from oxidative stress (Greeve *et al.* 2001, Kuehnle *et al.* 2008). The neuroprotective effect of seladin-1 appears to be due, at least in part, to the inhibition of caspase 3 activity, a key mediator of apoptosis (Greeve *et al.* 2001). We have

demonstrated that estrogen and the estrogen receptor modulators, raloxifene and tamoxifen, protect fetal human neuroepithelial cells (FNC) from β -amyloid and H_2O_2 toxicity by increasing the expression of *seladin-1* (Benvenuti *et al.* 2005, Luciani *et al.* submitted). These cells were isolated, cloned, and propagated previously from fetal olfactory epithelium (Vannelli *et al.* 1995). FNC cells show unique features, because they synthesize both neuronal proteins and olfactory markers and respond to odorant stimuli, suggesting their origin from the stem cell compartment that generates mature olfactory receptor neurons. In addition, we have recently demonstrated that high levels of expression of *seladin-1* were detectable in human mesenchymal stem cells (hMSC), whereas a significant reduction was observed in hMSC-derived neurons (hMSC-n; Benvenuti *et al.* 2006). We have also detected high levels of *seladin-1* transcript in the adult hippocampus (Benvenuti *et al.* 2006), one of the brain areas affected in AD (Selkoe 2001), in which stem cells with a defined neurogenic potential are located in the adult brain (Lie *et al.* 2004). These findings suggest that *seladin-1* is a predominant product of multipotent cells and that the defective *seladin-1* expression detected in AD-vulnerable brain regions may be linked to an impaired neuronal stem cell compartment, which could be a potential risk factor for the development of this disease. Interestingly, there is evidence both in embryonic and in adult mammals that TH plays a key role in the development and maintenance of basal forebrain cholinergic neurons typically involved in AD (Patel *et al.* 1987, Calzà *et al.* 1997).

Therefore, we hypothesized that *seladin-1* may be one of the mediators of the effects of TH during the development of the nervous system. According to this hypothesis, in the present study, we first determined the role of TH on cell differentiation in FNC and hMSC, by addressing their effects on cell adhesion, invasiveness, expression of the neuronal marker neurofilament-M, Na^+ and Ca^{2+} channels functionality as evaluated by their current density, and activation and inactivation voltage dependence. Thereafter, we analyzed whether TH affect the expression of *seladin-1* in these cells and prevent apoptosis. In order to clarify whether *seladin-1* may be targeted as a TH-regulated gene selectively in neuronal precursors, the effect of TH on *seladin-1* expression was also determined in hMSC-n.

Materials and Methods

Materials

Media and sera for cell cultures were purchased from Euroclone (Wetherby, West York, UK) and tissue plasticware was obtained from GreinerBio-One (Frickenhäusen, Germany). The neurobasal medium was obtained from Life Technologies (Invitrogen). Other reagents for cell cultures were obtained from Sigma. The flow cytometry buffer (CellWASH) was obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Monoclonal antibodies for flow cytometry immunophenotyping were obtained from BD Pharmingen

(San Diego, CA, USA) and Ancell (St N Bayport, MN, USA), whereas antibodies for immunocytochemistry were from Chemicon (Temecula, CA, USA). For RNA extraction, the Nucleospin RNAII kit was purchased from Macherey-Nagel (Duren, Germany). Reagents for RT-PCR studies were from Applied Biosystem Inc. (Foster City, CA, USA). TH ($3,3',5$ -triiodothyronine, T_3 and L-thyroxine, T_4) and camptothecin were purchased from Sigma. Molecular weight marker GeneRuler 100 and 50 bp DNA Ladder were from Fermentas (Burlington, Ontario, Canada).

Cell cultures

FNCs were isolated from human fetal olfactory neuroepithelium, as described previously (Vannelli *et al.* 1995). Long-term FNC cell cultures were propagated in Coon's-modified Ham's F12, supplemented with 10% fetal calf serum (FCS) and antibiotics (growth medium). The B4 clone, showing the highest level of the expression of neuronal and olfactory markers, was used in this study. hMSC were isolated from the iliac crest of normal donor marrow aspirates. Informed consent was obtained from all donors and the institutional ethical committee approved all the procedures. hMSC were isolated and characterized as described previously (Benvenuti *et al.* 2006). The induction of a neuronal phenotype in hMSC was performed according to the method of Woodbury *et al.* (2000), with some modifications, as described previously (Benvenuti *et al.* 2006).

RT-PCR for TH receptors (TR) and type I and II deiodinases (D1 and D2)

Total RNA was isolated from FNC, hMSC, and hMSC-n. RT-PCR was performed using SuperScript III One-Step RT-PCR with Platinum Taq kit (Invitrogen Life Technologies Inc.) with 1 μ g total RNA as template. Specific oligonucleotides were used as upstream and downstream primers, as described previously (Gittoes *et al.* 1997, de Souza Meyer *et al.* 2005). All reactions were performed in duplicate. After amplification, 10 μ l aliquots of the PCR products were analyzed on a 2% ethidium bromide agarose gel and visualized under u.v. light using Kodak Image Station 140 CF.

Cell adhesion assay

For cell adhesion assay, the cells were plated in 96-well plates at a density of 5×10^4 (hMSC) cells/well and 6×10^4 (FNC) cells/well with or without 1 nM T_3 in medium with 1% FCS for 24 h. At the end of the incubation, the cell monolayer was washed twice with PBS in order to remove the non-adherent cells by aspiration and 100 μ l Rose Bengal stain (0.25% in PBS) was added to each well for 5 min at room temperature (Gamble & Vadas 1988). After aspiration of the stain, each well was washed twice in PBS and the stain was released by adding 200 μ l/well of an ethanol/PBS (1:1) solution for 30 min. The samples were analyzed using an ELISA plate

reader (Seac-Radim) at 570 nm wavelength and the results were expressed as optical density (OD)/well (mean $\pm$ S.E.M.). The experiments were performed in hexaplicate and were repeated at least three times.

Quantitative cell migration assay

hMSC and FNC cells were passaged twice prior to the assay and were washed with PBS. Successively, the cells were detached using a harvesting buffer (0.05% trypsin in Hank's balanced salt solution containing 25 mM HEPES), and a quenching medium (serum-free Dulbecco's modified minimum essential medium (DMEM) containing 5% BSA) was added to the dishes. After centrifugation, the cells were resuspended in the quenching medium and 500 μ l cell suspension, with or without 1 nM T_3 , was added to both collagen I- and BSA-coated Boyden chambers (top). The same number of cells was plated in collagen I-coated wells for cell adhesion and morphology control. After 20 h incubation, the cells were stained using crystal violet according to the manufacturer's instructions. Finally, 300 μ l extraction buffer was added to each membrane for 15 min, then a 50 μ l stained solution was removed and placed in 96-multiwell plates, and the absorbance was read at 540 nm.

Immunocytochemistry

hMSC were cultured in four-chamber slides in a growth medium. Subsequently, the cells were treated with 1 nM T_3 for 4 days in a serum-free medium. Then, they were washed twice with PBS, fixed in 4% paraformaldehyde and 0.1% glutaraldehyde, and were immunostained for neurofilament-M (NF-M) using a rabbit anti-NF-M C-terminal antibody (1:1000). The cells were incubated overnight at 4 °C with the primary antibody, followed by a 1 h incubation with a peroxidase-conjugated secondary antibody. Finally, the cells were exposed to Vectastain ABC and AEC reagents (Vector Laboratories Inc., Burlingame, CA, USA) and counterstained with hematoxylin. Immunopositive cells/field were counted in ten 40 $\times$ fields for each experiment ($n=3$) and the results were expressed as mean $\pm$ S.E.M.

Electrophysiological studies

The electrophysiological records were obtained on FNC and hMSC (control and T_3 -treated) adherent on glass cover slips using the whole-cell patch-clamp technique under voltage-clamp conditions. The experimental setup, microelectrodes, pulse protocols of stimulation, data acquisitions, and analysis were as previously described in detail (Benvenuti *et al.* 2006). When outward K^+ currents had to be suppressed, parallel experiments were performed using a 20 mM TEA solution containing 122.5 mM NaCl, 2 mM $CaCl_2$, 20 mM TEA-OH, and 10 mM HEPES. To test the high-voltage-activated Na^+ channels' sensitivity, TTX (1 μ M) was used. In order to have Ca^{2+} as the only permeant cation, Ca^{2+} currents were recorded in a TEA- Ca^{2+} bath solution, 10 mM $CaCl_2$,

145 mM TEABr, and 10 mM HEPES. In turn, 10 μ M nifedipine or 100 μ M Cd^{2+} , added as $CdSO_4$, were used to block L-type Ca^{2+} channels and all but T-type Ca^{2+} channels respectively. The steady-state ionic current activation was evaluated using

$$I_a(V) = G_{max}(V - V_{rev})/\{1 + \exp((V_a - V)/k_a)\} \quad (1)$$

and the steady-state inactivation using

$$I_h(V) = I/\{1 + \exp(-(V_h - V)/k_h)\} \quad (2)$$

where G_{max} is the maximal conductance for I_a , V_{rev} is the apparent reversal potential, V_a and V_h are the potentials that elicit the half-maximal size, and k_a and k_h are the steepness factors. For the activation curve evaluation, the cell was held at -80 mV and step pulses (10 ms or 1 s for Na^+ current, I_{Na} , and Ca^{2+} current, I_{Ca} , recording respectively) ranging from -70 to 50 mV were applied in increments of 10 mV. The inactivation curve was evaluated on the current elicited by a voltage step at 0 mV, pre-pulsed by voltage steps as those used for activation with an inter-pulse period of 2 or 20 ms for I_{Na} and I_{Ca} recording respectively. Data are expressed as mean $\pm$ S.E.M.

Quantitative real-time RT-PCR

The quantification of seladin-1, integrin subunits αV , $\beta 3$, and $\alpha 2b$ mRNA, was performed by real-time RT-PCR, based on TaqMan technologies. The total RNA to be subjected to RT was extracted from FNC, hMSC, and hMSC-n (basal conditions and after T_3 or T_4 treatment for 72 h in serum-free medium). Total RNA isolation and cDNA synthesis were obtained as reported previously (Luciani *et al.* 2004). The primers and probe for the integrin subunits αV , $\beta 3$ and $\alpha 2b$ were Assay-On-Demand gene expression products (Applied Biosystems); the PCR mixture (25 μ l final volume) consisted of 1 $\times$ final concentration of Assay-On-Demand mix, 1 $\times$ final concentration of Universal PCR Master Mix (Applied Biosystems), and 20 ng cDNA. Each measurement was carried out in triplicates and three experiments were performed. The mRNA quantification was based on the comparative Ct method according to the manufacturer's instructions (Applied Biosystems) and data were normalized to ribosomal 18S RNA expression. The results were expressed as percentage mRNA versus control. The conditions used for seladin-1 mRNA measurement were described previously (Luciani *et al.* 2004).

TUNEL analysis for the determination of apoptotic cells

Apoptosis was determined by TUNEL analysis, using a commercially available detection kit (FragEL DNA Fragmentation Detection Kit, Oncogene Research Products, Boston, MA, USA), following the manufacturer's instructions. Briefly, FNC and hMSC were cultured in chamber slides both in the presence and in the absence of 1 nM T_3 for 72 h. During the last 4 h, one half of T_3 -treated and the other half of untreated

Figure 1 Detection of TR isoforms $\alpha 1$, $\beta 1$, and $\beta 2$ mRNA by RT-PCR in FNC and hMSC. C+, positive control, i.e. normal thyroid tissue. MW100 and MW50, molecular weight marker (see Materials and Methods).

chamber slides were exposed to 20 $\mu\text{g}/\text{ml}$ camptothecin. Three experiments were performed. The number of apoptotic cells/field was counted in ten $20\times$ fields for each experiment and the results were expressed as percentage of apoptotic cells/field (mean $\pm$ S.E.M.).

Results

Expression of TR in FNC and hMSC

The presence of transcripts corresponding to TR $\alpha 1$, $\beta 1$, and $\beta 2$ (i.e. the major TR isoforms with a known biological role) in

FNC and hMSC was evaluated by RT-PCR. In both the cases, detectable amounts of mRNA of these TR isoforms were found, as shown in Fig. 1.

Effect of T_3 on cell adhesion, migration, and expression of NF-M

No study has so far addressed the effect of TH in promoting the neuronal differentiation of FNC and hMSC. Therefore, the first objective of this study was to determine whether T_3

Figure 2 (A) Effect of T_3 (1 nM) on cell adhesion. (B) Amount of integrin subunits αV , $\beta 3$, and $\alpha 2b$ mRNA in FNC and hMSC, without or with T_3 exposure. C, control, i.e. absence of T_3 exposure. * $P < 0.05$ vs C.

Figure 3 Effect of T_3 (1 nM) on cell migration in FNC and hMSC. A typical experiment is shown in (A), and the quantitative analysis is represented in (B).

induces biological responses, which are expected in differentiating cells, in our particular cell models. First, the effect of TH on cell adhesion was determined. The exposure to T_3 (1 nM) for 24 h induced a significant inhibition of cell adhesion in FNC as well as in hMSC (Fig. 2A). Accordingly, the expression of integrin subunits $\alpha 2b$, $\beta 3$ and, in the case of hMSC, also αV was significantly reduced by T_3 exposure (Fig. 2B). With regard to cell migration, exposure to 1 nM T_3 determined a significant stimulatory effect in both the cell models (Fig. 3A and B). Immunocytochemistry for the neuronal marker NF-M was also performed. In untreated FNC, positivity for NF-M was rarely observed, whereas T_3 -treated cells showed a strong positivity, decorating specifically both the cell body and the neurite-like processes (Fig. 4A) (8 ± 1.1 vs 49.7 ± 6.9 cells/field, mean $\pm$ S.E.M., $P < 0.005$). Similarly, in untreated hMSC a faint positivity was observed in a few cells. Upon T_3 exposure, the intensity of the staining and the number of positive cells markedly increased (Fig. 4B) (13.2 ± 0.9 vs 41.4 ± 3.6 cells/field, mean $\pm$ S.E.M., $P < 0.05$).

Effects of T_3 on the electrophysiological properties of FNC and hMSC

Based on previous evidence indicating a positive effect of T_3 on neuron excitability (Hoffmann & Dietzel 2004), the effect of T_3 on the electrophysiological properties of FNC and hMSC was also assessed. All control FNC exhibited I_{Na} as shown in a typical experiment displayed in Fig. 5A. The voltage threshold of I_{Na} was about -50 mV. The treatment with T_3 (1 nM for 72 h) definitely increased I_{Na} amplitude (Fig. 5B) and this was consistently observed in all the experiments. The normalized I–V plot determined at the current peak related to all the cells investigated is shown in Fig. 5C. With regard to hMSC, these cells usually did not show a typical voltage-activated I_{Na} . Only a low percentage ($< 5\%$) of hMSC cultured on the glass cover slips for 3 days showed I_{Na} with a very small amplitude, but the 50% of hMSC cultured on the glass cover slips for 6 days showed I_{Na} (Fig. 5E). Again, the treatment with T_3 (1 nM for 6 days) determined an enhancement of I_{Na} (Fig. 5F) and increased the number of responsive cells to 80%. The normalized I–V plot related to all the cells investigated is shown in Fig. 5G.

Figure 4 Immunostaining for NF-M in (A) FNC and (B) hMSC in basal conditions or after exposure to T_3 . Magnification 40 $\times$.

Figure 5 Inward Na^+ currents in FNC and hMSC. Representative I_{Na} traces recorded in 20 mM TEA solution obtained from FNC in (A) control condition and (B) T_3 -treated FNC; for clarity, only current traces elicited by depolarization from -60 to $+30$ mV in increments of 10 mV are shown. (C) Normalized I–V plots determined at the current peak in control FNC ($n=16$, open circles) and under T_3 treatment ($n=17$, filled circles). (D) Normalized inactivation data in control FNC (open circles) and under T_3 (filled circles) treatment which superimposed the related Boltzmann fit (dashed lines for control and continuous lines for T_3); the related Boltzmann curves for activation are obtained from fits in C. Representative I_{Na} traces recorded from hMSC cultured for 6 days on glass cover slips (E) and in T_3 -treated hMSC (F). G. Normalized I–V plots determined at the current peak in control hMSC ($n=12$, open circles) and under T_3 treatment ($n=15$, filled circles). H. Normalized inactivation data in control hMSC (open circles) and under T_3 (filled circles) treatment which superimposed the related Boltzmann fit (dashed lines for control and continuous lines for T_3); the related Boltzmann curves for activation are obtained from fits in G. In A, B, E, and F, the voltage that elicited the maximal I_{Na} and the corresponding time to peak are reported.

The maximal current amplitude was recorded at 0 mV in control and T_3 -treated hMSC, as well as in control FNC but it was at -10 mV in FNC treated with T_3 (Fig. 5A–C and E–G). Interestingly, the time to peak decreased in T_3 -treated cells (Fig. 5A, B and E, F). Moreover, the Boltzmann parameters underwent a change because of T_3 treatment. The half voltage activation parameter, V_a , was negatively shifted to ~ 5 (FNC) and 9 (hMSC) mV in T_3 -treated cells with respect to the control (Fig. 5D and H; Table 1). The T_3 treatment determined a decrease in the k_a values, which was not significant in FNC but was significant in hMSC ($P<0.05$) (Table 1). I_{Na} inactivation in T_3 -treated FNC did not show any significant change with respect to control, whereas in hMSC a 7 mV significant shift ($P<0.05$) towards more positive potentials was observed for V_h . The T_3 treatment did not determine a significant reduction in k_h in both FNC and hMSC (Fig. 5D and H; Table 1). In conclusion, T_3 treatment determined a fast kinetics of I_{Na} and changed the V_a Boltzmann parameters for activation in both FNC and hMSC. Such changes were more evident in the less-differentiated hMSC than in FNC, in which, together with V_a , the T_3 treatment also affected k_a and the V_h Boltzmann parameters for inactivation.

All control FNC exhibited I_{Ca} (Fig. 6A). The treatment with T_3 (1 nM for 72 h) definitely increased I_{Ca} amplitude (Fig. 6B) and this was consistently observed in all the experiments. The normalized I–V plot related to all the cells investigated is shown in Fig. 6C. I_{Ca} was the result of T-type (not blocked by nifedipine or Cd^{2+}) and L-type I_{Ca} (blocked by nifedipine or Cd^{2+}). The voltage that elicited the maximal L-type I_{Ca} was shifted towards ~ 10 mV more negative potentials, and the related time to peak was reduced by ~ 100 ms in T_3 -treated cells. The related Boltzmann parameters for T-type I_{Ca} activation were not changed by T_3 treatment (Fig. 6D; Table 1). In contrast, the parameters for L-type I_{Ca} activation underwent a change as a result of T_3 treatment (Fig. 6D; Table 1); the values of V_a were shifted to ~ 7 mV towards more negative potentials ($P<0.05$) and those for k_a were significantly reduced ($P<0.05$). Again, T-type I_{Ca} inactivation in T_3 -treated FNC also did not show any significant change with respect to the control, whereas the L-type I_{Ca} showed a 4 mV significant shift ($P<0.05$) towards more negative potentials (Fig. 6D). The T_3 treatment did not determine significant changes in k_h . In conclusion, T_3 treatment caused in FNC a fast kinetics of L-type I_{Ca} and changed the V_a and V_h Boltzmann parameters for activation and inactivation respectively as observed for I_{Na} . With regard to hMSC, in both control and T_3 -treated cells, I_{Ca} was not observed, or its size was very small respect to the noise and therefore not accurately measurable (not shown).

Effect of TH on seladin-1 expression and on apoptosis

The effect of TH on the expression of *seladin-1* was assessed by real-time RT-PCR. Interestingly, T_3 (1 nM) and, yet to a lower extent, T_4 (1 nM) determined a significant increase of *seladin-1* mRNA in FNC (325 ± 7.5 fg/ μg total RNA, T_3 ; $225 \pm$

Table 1 Effect of 3,3',5-triiodothyronine (T₃) treatment on Boltzmann parameters of activation and inactivation curves for I_{Na} in fetal human neuroepithelial cells (FNC) and in 6 days cultured human mesenchymal stem cells (hMSC) and for I_{Ca} in FNC

	I _{Na}				I _{Ca} (FNC)			
	FNC		hMSC		T-type		L-type	
	Con	T ₃	Con	T ₃	Con	T ₃	Con	T ₃
V _a	−17.1±0.8	−22.0±1.2*	−8.0±0.5	−17.1±0.8 [†]	−44.2±2.2	−45.1±3.2	12.1±1.2	5.1±1.2*
k _a	7.2±0.3	6.9±0.2	8.4±0.2	7.3±0.2*	6.1±0.1	6±0.1	7.6±0.1	7.1±0.1*
V _h	−58.0±4.7	−57.2±4.8	−67.1±4.1	−60.0±3.8*	−64.0±4.3	−63.2±4.8	−27.3±2.1	−22.7±1.8*
k _h	7.8±0.6	7.5±0.8	8.5±0.6	8.0±0.6	6.4±0.5	6.5±0.6	7.1±0.5	7.6±0.5

*P<0.05 and [†]P<0.01 versus the corresponding control.

5.3 fg/μg total RNA, T₄, mean ±S.E.M.) and in hMSC (270 ± 6.4 fg/μg total RNA, T₃; 195 ± 4.8 fg/μg total RNA, T₄), when compared with the basal amount of transcript (135 ± 2.8 fg/μg total RNA in FNC; 167 ± 3.34 fg/μg total RNA in

hMSC) (Fig. 7A). Furthermore, in agreement with previous studies associating the induction of seladin-1 expression to anti-apoptotic properties (Greeve *et al.* 2001, Benvenuti *et al.* 2005, Kuehnle *et al.* 2008), we found that T₃ (1 nM) was able to significantly counteract camptothecin-induced apoptosis, as determined by TUNEL analysis, and in the case of FNC the percentage of apoptotic cells after camptothecin and T₃ treatment was even superimposable with that of control cells (Table 2).

In order to determine whether the effect of T₄ was mediated by its conversion into T₃, deiodinase (D1 and D2) expression was determined in FNC and hMSC by RT-PCR. Whereas no detectable amount of D1 transcript could be found in these cells, D2 transcript was present in both FNC and hMSC, as shown in Fig. 7B, thus suggesting that the stimulatory effect of T₄ on the expression of *seladin-1* is, at least partially, due to the intracellular conversion into T₃.

In order to clarify whether *seladin-1* may be targeted as a TH-regulated gene selectively in *in vitro* models of multipotent undifferentiated cells, the effect of TH on *seladin-1* expression was also determined in differentiated neurons. For this purpose, hMSC were differentiated into a neuronal phenotype (hMSC-n) and proven to be neurons, as described previously (Benvenuti *et al.* 2006). In hMSC-n, the expression of detectable levels of TRα1, β1, and β2 was maintained (Fig. 8A). Noteworthy, the amount of seladin-1 mRNA in hMSC-n was significantly lower (25 ± 0.5 fg/μg total RNA) compared with both FNC and hMSC, thus confirming our previous finding that seladin-1 is a predominant product of undifferentiated cells (Benvenuti *et al.* 2006)). Furthermore, neither T₃ nor T₄ was able to modify the level of transcript in hMSC-n (23.5 ± 1.2 fg/μg total RNA, T₃; 24 ± 0.8 fg/μg total RNA, T₄) (Fig. 8B).

Discussion

It is well known that TH play a fundamental role in fetal life, particularly in promoting early brain development, by affecting the expression of specific genes (König & Neto 2002, Santisteban & Bernal 2005).

Figure 6 Inward Ca²⁺ currents in 6 days cultured FNC. Representative I_{Ca} traces recorded in TEA-Ca²⁺ solution obtained from FNC in (A) control condition and (B) T₃-treated FNC. For clarity, only current traces elicited by depolarization from −50 to +50 mV in increments of 10 mV are shown. The traces from −50 to −30 mV clearly show the presence of the fast activating and inactivating T-type Ca²⁺ current (indicated by an arrow in the −50 mV trace). (C) Normalized I–V plots determined at the current peak in control FNC (n=16, open circles) and under T₃ treatment (n=17, filled circles) with superimposed curves that are the best fit as a sum of two Boltzmann functions; filled triangles represent data obtained in control FNC in the presence of nifedipine with superimposed curve that is the best fit of one Boltzmann function (n=16). (D) Normalized inactivation data for T- and L-type Ca²⁺ current in control FNC (open circles for T-type and open triangles for L-type) and under T₃ treatment (filled circles for T-type and filled triangles for L-type) which superimposed the related Boltzmann fit (dashed lines for control and continuous lines for T₃); the related Boltzmann curves for activation are obtained from fits in C.

Figure 7 (A) Measurement of seladin-1 mRNA by real-time RT-PCR in FNC and hMSC, in the absence (C) or in the presence of TH (T₃ and T₄). **P* < 0.05 vs C. (B) Detection of D1 and D2 mRNA in FNC and hMSC, as assessed by RT-PCR. C+, positive control, i.e. normal thyroid tissue. MW100, molecular weight marker (see Materials and Methods).

In our study, we used two different cell models, FNC and hMSC. FNC are human neuronal precursors, which generate mature olfactory receptor neurons (Vannelli *et al.* 1995). The choice of hMSC was based on the fact that they are much more easily obtainable than neuronal stem cells, and we rationalized that they might represent an acceptable alternative to the use of neuronal stem cells in our experimental setting. In fact, *in vitro* and *in vivo* neuronal differentiation of MSC into neurons has been demonstrated (Woodbury *et al.* 2000, Sanchez-Ramos 2002, Benvenuti *et al.* 2006). In addition, transplanted bone marrow cells have been localized in mouse (Calzà *et al.* 1997, Mezey *et al.* 2000) and human brain (Mezey *et al.* 2003, Weimann *et al.* 2003), suggesting a possible differentiation into neuronal cells. Accordingly, the functional/structural parallelism between neuronal stem cells and MSC has been underlined by

Table 2 Effect of 3,3',5-triiodothyronine (T₃) (1 nM) treatment on camptothecin-induced apoptosis in fetal human neuroepithelial cells (FNC) and human mesenchymal stem cells (hMSC), as determined by TUNEL analysis. The numbers represent apoptotic/total cells (mean ± S.E.M.) in ten fields, magnification 20×

	FNC	hMSC
Control	3 ± 1.1	2.1 ± 1.1
T ₃	4 ± 0.9	2.6 ± 1.2
Camptothecin	15.9 ± 2.4*	18.1 ± 1.4*
Camptothecin + T ₃	3.6 ± 0.9†	7.5 ± 1.6*†

**P* < 0.05 versus control, †*P* < 0.05 versus camptothecin.

the proposal to use terms like ‘neuropoiesis’ to indicate the persistence of neurogenesis in the adult brain and ‘brain marrow’ to describe the brain regions that contain the cells supporting neurogenesis (Steindler *et al.* 1996, Scheffer *et al.* 1999).

We demonstrated that in both FNC and hMSC, which express TRα1, β1, and β2, T₃ (1 nM) was able to reduce cell adhesion. This finding was paralleled by the reduced expression of adhesion molecules, such as different α and β integrin subunits. In addition, T₃ increased cell migration, in agreement with similar findings obtained in different cell models (König & Neto 2002, Santisteban & Bernal 2005). Furthermore, an increase in both the intensity of the immunostaining and in the number of positive cells for NF-M, a typical neuronal marker, was observed in hMSC and in FNC, following T₃ exposure. Electrophysiological evaluation revealed that in these cell models, there were positive effects on Na⁺ and L-type Ca²⁺ channels upon T₃ treatment. T₃ induced a faster activation kinetics on I_{Na}, as indicated by the reduced time to peak, increased its current density, and shifted its activation and inactivation curves towards more negative and more positive potentials respectively. Such changes, which were more evident in hMSC than in FNC, indicated that T₃ was able to induce the expression of new functional ionic channels and suggested a permissive role towards the differentiation into a proper excitable neuronal phenotype. T₃ had similar effects on L-type Ca²⁺ channels expressed in FNC; it reduced L-type I_{Ca} time to peak, increased its current density and shifted its activation and inactivation curves towards more negative and more positive potentials respectively. Thus, T₃ not only increased the I_{Na} and L-type I_{Ca} but also their functionality by inducing faster kinetics and changing their voltage dependence towards a more functional state. In fact, these currents activated at less negative membrane potentials and inactivated at more positive potentials. All these observations clearly indicated that T₃ had biological effects on Na⁺ and L-type Ca²⁺ channels in FNC, which were already committed towards neuronal differentiation. These effects were more evident on Na⁺ channels in the less-differentiated hMSC, in agreement with the well-established role of TH in promoting neuronal development.

Furthermore, we determined whether TH affect the expression of *seladin-1*. We found that T₃, and to a lesser extent T₄, significantly increased the amount of seladin-1 mRNA in both the cell models. The fact that FNC as well as hMSC express type II deiodinase suggests that the effect of T₄ on seladin-1 expression may be mainly due to its intracellular transformation into T₃. Furthermore, in agreement with previous reports indicating that the up-regulation of *seladin-1* expression, such as that obtained for instance upon estrogen exposure, is paralleled by an inhibitory effect on apoptosis (Greeve *et al.* 2001, Benvenuti *et al.* 2005), we demonstrated here that T₃ also, in addition to its stimulatory role on *seladin-1* expression, was able to significantly counteract the induction of apoptosis by camptothecin. These data suggest that seladin-1 may be a mediator of the effects that TH have in the brain.

Figure 8 (A) Detection of TR isoforms in hMSC-n by RT-PCR. (B) Assessment of seladin-1 mRNA by real-time RT-PCR in hMSC-n, in the absence or in the presence of TH (T₃ and T₄). MW100, molecular weight marker (see Materials and Methods).

According to the knowledge that TH play an essential role in the CN at the time of brain development, we questioned whether their stimulatory effect on seladin-1 expression was maintained or not in differentiated neuronal cells. Thus, we differentiated hMSC toward a neuronal phenotype (hMSC-n), according to an experimental procedure validated previously by us as well as by other groups (Woodbury *et al.* 2000, Benvenuti *et al.* 2006). Admittedly, a comparison made using the same cell model at different stages of differentiation would provide striking evidence on whether or not the expression of a gene (i.e. *seladin-1*) is dependent on the degree of differentiation. In hMSC-n, the amount of expression of seladin-1 was significantly lower than that in hMSC, according to our previous findings (Benvenuti *et al.* 2006). Noteworthy, neither T₄ nor T₃ affected seladin-1 expression, thus indicating clearly that the stimulatory effect of TH appears to be selectively present in neuronal precursor cells. This finding is in keeping with observations also from *in vivo* studies in experimental animals that most of the TH-regulated genes are responsive only during a limited time interval of brain development (König & Neto 2002). The molecular reason for the absent modulation of the expression of the *seladin-1* gene by TH in hMSC-n, which express TR, remains elusive at present. However, the expression of TR does not necessarily correlate with the effects of TH. A good example is provided by the observation that in mice lacking the TR α 1, which is the predominant TR isoform in the fetal cerebellum, no structural alteration of this organ was found (Morte *et al.* 2002). On the other hand, the presence of TR does not warrant their function on gene expression, which is the result of a complex series of events, including for instance the association with co-activators or co-repressors (Zhang & Lazar 2000). Finally, the presence of membrane-regulated actions of TH has been described (Davis *et al.* 2005), and this possibility has been further highlighted by the

recent demonstration of a TH cell surface receptor on the extracellular domain of integrin α V β 3, a structural membrane protein (Bergh *et al.* 2005).

The biological role played by the TH-mediated stimulation of seladin-1 expression in neuronal precursors remains to be elucidated. For this purpose, additional studies, designed for instance to make seladin-1 expression in these cells silent, will be performed, in order to determine which of the TH-mediated function/s is/are directly due to this protein. However, in consideration of the fact that one of the best characterized properties of seladin-1 so far is its anti-apoptotic activity (Greeve *et al.* 2001, Kuehnle *et al.* 2008), which has been further confirmed also by our group in FNC cells (Benvenuti *et al.* 2005, Luciani *et al.*, submitted), it might be hypothesized that one of the functions associated with the increased seladin-1 levels in the developing brain may be to protect neuronal precursor cells from death. From this point of view, seladin-1 might therefore be regarded as a factor, which helps in maintaining a pool of young and self-renewing multipotent cells, to then be made available to other TH-regulated genes, which specifically direct the differentiation toward a neuronal phenotype.

In summary, our study showed for the first time that *seladin-1* is a novel TH-regulated gene in neuronal precursors and suggested that it might be an additional mediator of the effects of TH in the developing brain.

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References

- Allan WC, Haddow JE, Palomaki GE, Williams JR, Mitchell ML, Hermos RJ, Faix JD & Klein RZ 2000 Maternal thyroid deficiency and pregnancy complications: implications for population screening. *Journal of Medical Screening* **7** 127–130.
- Benvenuti S, Luciani P, Vannelli GB, Gelmini S, Franceschi E, Serio M & Peri A 2005 Estrogen and SERMs exert neuroprotective effects and stimulate the expression of seladin-1, a recently discovered anti-apoptotic gene, in human neuroblast long-term cell cultures. *Journal of Clinical Endocrinology and Metabolism* **90** 1775–1782.
- Benvenuti S, Saccardi R, Luciani P, Urbani S, Deledda C, Cellai I, Francini F, Squecco R, Rosati F, Danza G *et al.* 2006 Neuronal differentiation of human mesenchymal stem cells: changes in the expression of the Alzheimer's disease-related gene seladin-1. *Experimental Cell Research* **312** 2592–2604.
- Bergh JJ, Lin HY, Lansing L, Mohamed SN, Davis FB, Mousa S & Davis PJ 2005 Integrin α V β 3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. *Endocrinology* **146** 2864–2871.
- Calzà L, Giardino L & Aloe L 1997 Thyroid hormone regulates NGF content and p75LNGFR expression in the basal forebrain of adult rats. *Experimental Neurology* **143** 196–206.
- Davis PJ, Davis FB & Cody V 2005 Membrane receptors mediating thyroid hormone action. *Trends in Endocrinology and Metabolism* **16** 429–435.
- Gamble JR & Vadas MA 1988 A new assay for the measurement of the attachment of neutrophils and other cell types to endothelial cells. *Journal of Immunological Methods* **109** 175–184.
- Gittoes NJ, McCabe CJ, Verhaeg J, Sheppard MC & Franklyn JA 1997 Thyroid hormone and estrogen receptor expression in normal pituitary and nonfunctioning tumors of the anterior pituitary. *Journal of Clinical Endocrinology and Metabolism* **82** 1960–1967.
- Greeve I, Hermans-Borgmeyer I, Brellinger C, Kasper D, Gomez-Isla T, Behl C, Levkau B & Nitsch RM 2001 The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress. *Journal of Neuroscience* **20** 7345–7352.
- Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J, O'Heir CE, Mitchell ML, Hermos RJ, Waisbren SE *et al.* 1999 Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *New England Journal of Medicine* **341** 549–555.
- Hoffmann G & Dietzel ID 2004 Thyroid hormone regulates excitability in central neurons from postnatal rats. *Neuroscience* **125** 369–379.
- Kester MHA, Martinez de Mena R, Orregom MJ, Marinkovic D, Howatson A, Visser T, Hume R & Morreale de Escobar G 2004 Iodothyronine levels in the human developing brain: major regulatory role of iodothyronine deiodinases in different areas. *Journal of Clinical Endocrinology and Metabolism* **89** 3117–3128.
- König S & Neto VM 2002 Thyroid hormone actions on neural cells. *Cellular and Molecular Neurobiology* **22** 517–544.
- Kuehnle K, Cramer A, Kálin RE, Luciani P, Benvenuti S, Peri A, Ratti F, Rodolfo M, Kulic L, Heppner FL *et al.* 2008 Prosurvival effect of DHCR24/Seladin-1 in acute and chronic responses to oxidative stress. *Molecular and Cellular Biology* **28** 539–550.
- Lavado-Autric R, Auso E, Garcia-Velasco JV, del Carmen Arufe M, Escobar del Rey F, Berbel P & Morreale de Escobar G 2003 Early maternal hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny. *Journal of Clinical Investigation* **111** 1073–1082.
- Lie DC, Song H, Colamarino SA, Ming GL & Gage FH 2004 Neurogenesis in the adult brain: new strategies for central nervous system diseases. *Annual Review of Pharmacology and Toxicology* **44** 399–421.
- Luciani P, Ferruzzi P, Arnaldi G, Crescioli C, Benvenuti S, Valeri A, Greeve I, Serio M, Mannelli M & Peri A 2004 Expression of the novel adrenocorticotropin-responsive gene selective Alzheimer's disease indicator-1 in the normal adrenal cortex and in adrenocortical adenomas and carcinomas. *Journal of Clinical Endocrinology and Metabolism* **89** 1332–1339.
- Mezey E, Chandross KJ & Harta G 2000 Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science* **290** 1779–1782.
- Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD & Crain B 2003 Transplanted bone marrow generates new neurons in human brain. *PNAS* **100** 1364–1369.
- Morreale de Escobar G 2001 The role of thyroid hormone in fetal neuro development. *Journal of Pediatric Endocrinology and Metabolism* **14** 1453–1462.
- Morreale de Escobar G, Obregón MJ & Escobar del Rey F 2004 Role of thyroid hormone during early brain development. *European Journal of Endocrinology* **151** U25–U37.
- Morte B, Manzano J, Scanlan T, Vennström B & Bernal J 2002 Deletion of the thyroid hormone receptor α 1 prevents the structural alterations of the cerebellum induced by hypothyroidism. *PNAS* **99** 3985–3989.
- Patel AJ, Hayashi M & Hunt A 1987 Selective persistent reduction in choline acetyltransferase activity in basal forebrain of the rat after thyroid deficiency during early life. *Brain Research* **422** 182–185.
- Pop VJ, Brouwers EP, Vader HL, Vulsma T, van Baar AL & de Vijlder JJ 2003 Maternal hypothyroxinemia during early pregnancy and subsequent child development: a 3-year follow-up study. *Clinical Endocrinology* **59** 282–288.
- Redmond GP 2002 Hypothyroidism and women's health. *International Journal of Fertility and Women's Medicine* **47** 123–127.
- Sanchez-Ramos JR 2002 Neural cells derived from adult bone marrow and umbilical cord blood. *Journal of Neuroscience Research* **69** 880–893.
- Santisteban P & Bernal J 2005 Thyroid development and effect on the nervous system. *Reviews in Endocrine and Metabolic Disorders* **6** 217–228.
- Scheffer B, Horn M, Blumcke I, Laywell ED, Coomes D, Kukekov VG & Steindler DA 1999 Marrow-mindedness: a perspective on neurogenesis. *Trends in Neuroscience* **22** 348–357.
- Selkoe DJ 2001 Alzheimer's disease: genes, proteins, and therapy. *Physiological Reviews* **81** 741–776.
- de Souza Meyer EL, Dora JM, Wagner MS & Maia AL 2005 Decreased type 1 iodothyronine deiodinase expression might be an early and discrete event in thyroid cell dedifferentiation towards papillary carcinoma. *Clinical Endocrinology* **62** 672–678.
- Steindler DA, Kadrie T, Fillmore H & Thomas LB 1996 The subependymal zone: 'brain marrow'. *Progress in Brain Research* **108** 349–363.
- Surks MI, Ortiz E, Daniels GH, Sawin CT, Col NF, Cobin RH, Franklyn JA, Hershman JM, Burman KD, Denke MA *et al.* 2004 Subclinical thyroid disease: scientific review and guidelines for diagnosis and management. *Journal of the American Medical Association* **291** 228–238.
- Vannelli GB, Ensoli F, Zonefrati R, Kubota Y, Arcangeli A, Camici G, Barni T, Thiele CJ & Balboni GC 1995 Neuroblast long-term cell cultures from human fetal olfactory epithelium respond to odors. *Journal of Neuroscience* **15** 4282–4294.
- Weimann JM, Charlton CA, Brazelton TR, Hackman RC & Blau HM 2003 Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. *PNAS* **100** 2088–2093.
- Woodbury D, Schwarz EJ, Prockop DJ & Black IB 2000 Adult rat and human bone marrow stromal cells differentiate into neurons. *Journal of Neuroscience Research* **61** 364–370.
- Zhang J & Lazar MA 2000 The mechanism of action of thyroid hormone. *Annual Review of Physiology* **62** 439–466.

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