Thyroid hormone regulated genes in cerebral cortex development

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Abstract

The physiological and developmental effects of thyroid hormones are mainly due to the control of gene expression after interaction of T_3 with the nuclear receptors. To understand the role of thyroid hormones on cerebral cortex development, knowledge of the genes regulated by T_3 during specific stages of development is required. In our laboratory, we previously identified genes regulated by T_3 in primary cerebrocortical cells in culture. By comparing these data with transcriptomics of purified cell types from the developing cortex, the cellular targets of T_3 can be identified. In addition, many of the genes regulated transcriptionally by T_3 have defined roles in cortex development, from which the role of T_3 can be derived. This review analyzes the specific roles of T_3-regulated genes in the different stages of cortex development within the physiological frame of the developmental changes of thyroid hormones and receptor concentrations in the human cerebral cortex during fetal development. These data indicate an increase in the sensitivity to T_3 during the second trimester of fetal development. The main cellular targets of T_3 appear to be the Cajal-Retzius and the subplate neurons. On the other hand, T_3 regulates transcriptionally genes encoding extracellular matrix proteins, involved in cell migration and the control of diverse signaling pathways.

Key Words
- brain
- development
- fetus
- gene expression
- thyroid hormone metabolism

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Introduction

The actions of thyroid hormones (TH) on brain development and function are among the more relevant of these hormones, strongly influencing neuromotor performance, cognition and mood. Multiple conditions cause impaired TH action during brain development. These include iodine deficiency, maternal and fetal hypothyroidism, maternal hypothyroxinemia, prematurity, nuclear T_3 receptor mutations (TR) and mutations of the monocarboxylate 8 transporter (MCT8) gene *SLC16A2*. These conditions may lead to various degrees of mental retardation and neurological impairment, which are particularly severe in MCT8 deficiency. Several reviews have appeared recently describing in detail the pathophysiology of these conditions (Refetoff & Dumitrescu 2007, Kurian & Jungbluth 2014, Bell *et al*. 2016, Moleti *et al*. 2016, van Gucht *et al*. 2016). In the present article, I will try to integrate recent information on genomic mechanisms of action of TH on cerebral cortex development within the physiological frame of thyroid homeostasis during fetal development. First, I will summarize the main features of TH homeostasis in the brain during human fetal stages, as the context in which cerebral maturation takes place. Then, I will use current concepts on cerebral

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cortex development at the cellular and molecular levels as the frame to identify possible roles of T_3 on these processes. To accomplish this, I will use recent data from our laboratory on T_3-regulated genes (Gil-Ibanez *et al.* 2015), many of which control key processes in cortex development. I believe that the integration of all the information will allow formulating coherent hypothesis on the general role and specific actions of TH on cortex development.

The context: fetal thyroid hormone homeostasis

Thyroid hormone concentrations in fetal fluids

The main features of thyroid hormone homeostasis in the human fetus are represented in Fig. 1. The figure represents data on TH concentrations in fetal fluids and in the developing cerebral cortex, as well as the concentrations of the nuclear T_3 receptor protein in the whole brain, during fetal development from the 6th to the 36th postmenstrual weeks (PMW). The date at which the fetal thyroid gland starts functioning is marked by a shaded vertical box between the 10th and the 12th PMW (Shepard 1967, Burrow *et al.* 1994).

During the first trimester, T_4 and T_3 are present in the coelomic fluid, an ultrafiltrate of the maternal serum (Calvo *et al.* 2002). T_4 in the coelomic fluid is derived from the maternal pool, and T_4 concentrations in coelomic fluid and maternal serum are positively correlated. The total T_4 (TT_4) concentration (1–2 nM) is about 100 times less than the maternal concentration, although the free fraction is much higher. In the amniotic fluid, TT_4 concentration is very low (0.1 nM) before the 12th week and increases to 2 nM after the 12th week and 4 nM at the end of gestation (Burrow *et al.* 1994, Calvo *et al.* 2002). The data on T_4 and T_3 concentrations in the fetal serum represented in Fig. 1 are taken from Thorpe-Beeston and coworkers (Thorpe-Beeston *et al.* 1991, Thorpe-Beeston & Nicolaides 1993) and extend from the 12th to the 36th PMW. Serum TT_4 concentration increases from around 26 nM at 12 weeks to 100–140 nM at 36 weeks, reaching maternal concentrations. Free T_4 (FT_4) increases from about 1–2 pM at 12 weeks to 25 pM at the end of gestation (Thorpe-Beeston *et al.* 1991, Guibourdenche *et al.* 2001) and TT_3 from the almost undetectable level of 0.1 nM at 12 weeks to 1 nM at 36 weeks. In contrast to T_4, T_3 levels at the end of gestation do not reach the maternal levels, most probably reflecting the immaturity of the T_4 to T_3 conversion process.

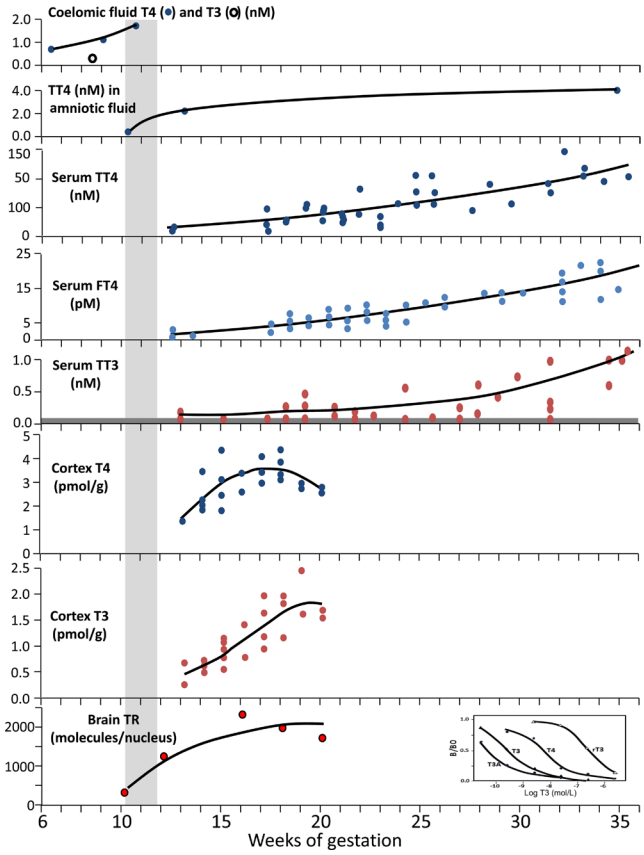

Figure 1
Thyroid hormone and TH receptor concentrations in the human fetus. This figure contains replotted data on the concentrations of TH in coelomic and amniotic fluids (Burrow *et al.* 1994, Calvo *et al.* 2002), fetal serum (Thorpe-Beeston *et al.* 1991) and cerebral cortex (Kester *et al.* 2004) as a function of fetal age expressed in postmenstrual weeks. The lower part of the figure shows the number of T_3 receptor molecules per nucleus recalculated from the original publication (Bernal & Pekonen 1984) assuming 8 pg DNA/nucleus. The inset shows the relative affinity of the human fetal receptor for triac (T3A), T_3, T_4, rT_3 (Bernal & Pekonen 1984). The shaded bar marks the date at which the fetal thyroid gland concentrates iodine and contains thyroglobulin and iodinated compounds (Shepard 1967).

TH transport to the brain

Thyroid hormones are amphipathic molecules, i.e., contain polar residues soluble in water and non-polar residues soluble in lipids. At the physiological pH, they are present in body fluids mainly in the *zwitter ionic* form (the amino acid side chain is in the form of $–COO^-$ $–NH_3^+$) (Toth *et al.* 2013) making it difficult to diffuse through the membranes, which are essentially impermeable to ions. Diffusion of TH through the cellular membranes is facilitated by membrane transporters acting on the influx and efflux to and from the cell interior, depending on the relative free hormone concentrations at either side of the membrane. Passage to the brain requires crossing

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the blood–brain barrier (BBB). The BBB is formed by the endothelial cells of brain capillaries joined together strongly by tight junctions (Abbott *et al.* 2010), severely restricting paracellular transport, and access to the brain requires crossing the luminal and abluminal membranes of the endothelial cells. The need of TH for specific transporters is now firmly established after the finding that mutations of the *SLC16A2* gene, encoding MCT8 lead to extremely severe neurological impairment and intellectual deficiency (Lopez-Espindola *et al.* 2014, Bernal *et al.* 2015). Many transporter proteins have the capacity of TH transport, but two of them are the most relevant for BBB transport: MCT8 with affinity for T_4 and T_3, and the organic anion transporter polypeptide 1C1 (OATP1C1, encoded by the *SLCO1C1* gene) with much higher selectivity for T_4. These are integral membrane proteins consisting of 12 transmembrane domains expressed in neural cells and in the BBB. They are also present in the endothelial cells of the choroid plexus. It is reasonable to assume that the major route of TH to the brain is the BBB as its exchange surface is about 5000 fold that of choroid plexus (Pardridge 1983). Transport through the choroid plexus may be more relevant at early stages of development, for example, during the formation of the cortical plate around PMW 8. At this age, the lateral ventricles are very prominent and largely occupied by the choroid plexus (O'Rahilly & Muller 2008).

A predominant role of the brain barriers on TH entry to the brain and to neural cells is supported by the effects of MCT8 deficiency in mice. In MCT8-deficient (genotype *Slc16a2*$^{-/y}$) mice, the accumulation of administered labeled T_3, but not T_4, is severely restricted (Trajkovic *et al.* 2007). Furthermore, T_3 had no effects on gene expression in the cortex and striatum when it was administered to the MCT8-deficient mice previously made hypothyroid (Ceballos *et al.* 2009). The same mice responded to the administration of T_4 in a similar way as the wild-type mice on induction of neuronal genes. The reason why T_4 is active in the absence of MCT8 is most probably due to OATP1C1 that is present in the BBB and in the astrocytes end-feet contacting the brain microvessels (Roberts *et al.* 2008). This arrangement facilitates direct access of serum T_4 to the astrocytes and conversion to T_3. Proof for this explanation is that the inactivation of the *Dio2* gene, highly enriched in astrocytes, in MCT8-deficient mice suppressed the effect of T_4 (Morte *et al.* 2010). The T_3 generated in the astrocytes then reaches the neurons possibly through secondary transporters (Kinne *et al.* 2011). Double inactivation of the *Slc16a2* (MCT8) and *Slco1c1* (OATP1C1) genes in mice induces cerebral hypothyroidism (Mayerl *et al.* 2014), but not the individual inactivation of each gene. Low concentrations of OATP1C1, as it has been shown in the monkey BBB (Ito *et al.* 2011) would make the human brain critically dependent on MCT8 for TH transport. MCT8 protein and mRNA can be detected in the human brain as early as the 7th–8th PMW (Chan *et al.* 2011).

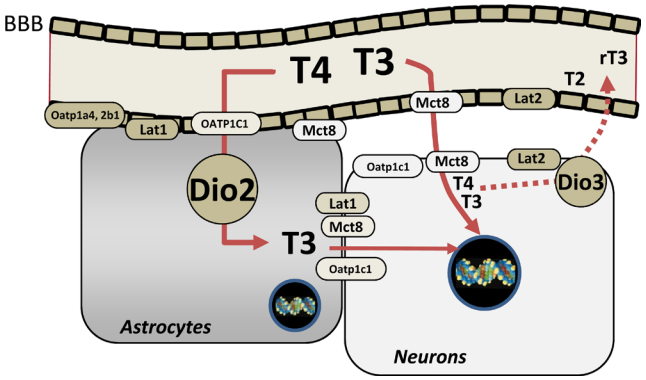

Figure 2
Model of TH transport and action in the brain. TH crosses the BBB through MCT8 and OATP1C1. MCT8 transports T_4 and T_3, whereas OATP1C1 is more specific for T_4. MCT8 and OATP1C1 are expressed in the endothelial microvascular cells and in the membrane of astrocytes and neurons. OATP1C1 is present in the astrocytic end feet in contact with the endothelial cells, so that transport of T_4 to the astrocytes is facilitated by OATP1C1. In the astrocytes, T_4 is converted to T_3. The T_3 formed has action on the astrocytes and also on neurons after crossing the cell membranes. This last step is not strictly dependent on MCT8 as it can occur in its absence. The location of other secondary transporters for TH is shown, but their specific role has not been defined. The neurons have DIO3 activity that converts T_4 and T_3 to rT_3 and T_2, respectively.

Based on the previously mentioned data, a model of TH transport and action in the rodent brain can therefore be formulated as illustrated in Fig. 2.

Deiodinases and TH concentrations in the cortex

Thyroid follicular cell secretion consists almost entirely of T_4, with only 5–10% of secreted iodothyronines in the form of T_3. The majority of T_3 is formed from T_4 in tissues by 5′-deiodination catalyzed by types 1 and 2 deiodinases (DIO1 and DIO2). T_4 and T_3 are inactivated by 5-deiodination catalyzed by type 3 deiodinase (DIO3) with the formation of 3,3′5′-triiodothyronine (reverse T_3, or rT_3) and 3,3′-diiodothyronine (T_2), respectively (Bianco *et al.* 2002, Bianco 2011). The human brain has no DIO1 activity (Campos-Barros *et al.* 1996), and the only T_4 to T_3 converting enzyme is DIO2. As originally shown in rats by *in situ* hybridization (ISH), *Dio2* is expressed

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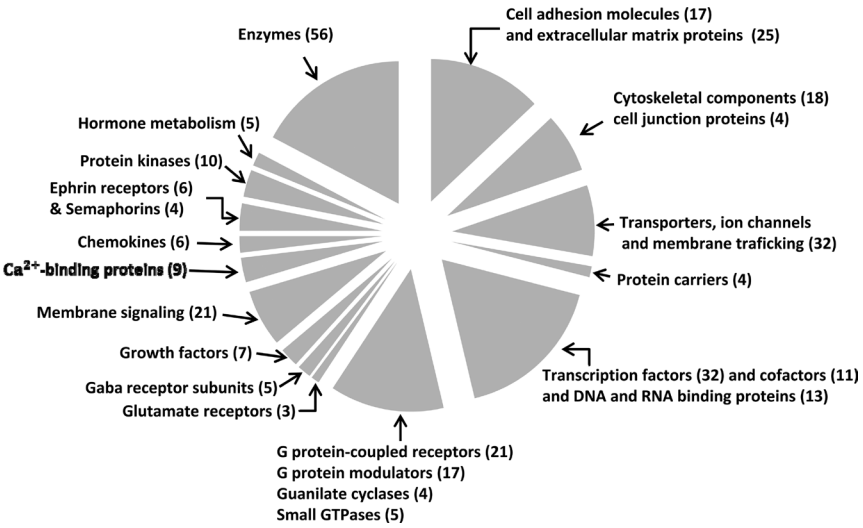

Figure 3
Genes of primary cerebrocortical cells regulated transcriptionally by T_3 classified in functional categories (Gil-Ibanez *et al.* 2015). Another group of 36 genes difficult to classify into functional categories are not included.

predominantly in astrocytes and in the tanycytes, another type of glial cells lining the lower half of the walls of the 3rd ventricle (Guadano-Ferraz *et al.* 1997, Tu *et al.* 1997). Recent transcriptomics of isolated mouse and human cortical cells have confirmed the predominant expression in astrocytes of mouse *Dio2* and of human *DIO2* (Zhang *et al.* 2014, 2016) and also confirmed early observation on *Dio2* expression in some interneurons (Guadano-Ferraz *et al.* 1999). *Dio2* mRNA is also present in cells of the oligodendroglial lineage, particularly the oligodendrocyte precursor cells (OPC) (Zhang *et al.* 2016).

Brain DIO3 is mainly a neuronal protein, attached to the plasma membrane (Baqui *et al.* 2003). DIO3 degrades T_4 and T_3, which reaches the neurons from the blood directly through the BBB or indirectly from the astrocytes. DIO3 regulates critically the concentration of T_3 and dampens the effect of an excess T_3 on gene expression (Hernandez *et al.* 2012). During early development, expression is very high in uterine structures and the placenta and restricts the passage of TH from the mother to the fetus. The *Dio3* gene is induced transcriptionally by T_3 specifically through TRα1 (Barca-Mayo *et al.* 2011, Gil-Ibanez *et al.* 2014), and its placental activity may be modulated by the circulating T_3 (Bianco *et al.* 2002). DIO3 activity may be coupled to MCT8 transport in such a way that T_3 reaching the neurons directly from the serum might be easily degraded (Stohn *et al.* 2016). This might be a possible reason why the fetal brain is apparently impermeable to T_3 despite high concentrations of the MCT8 protein (Grijota-Martinez *et al.* 2011). MCT8 transport would accumulate T_3 at the periphery of the cell where DIO3 is located, facilitating its degradation (van Mullem *et al.* 2016).

During the second trimester of human fetal development, the relative regional expression of DIO2 and DIO3 regulates local T_3 concentrations. As shown in Fig. 1, T_4 and T_3 concentrations increase in the cerebral cortex from weeks 13 to 18 and may attain a plateau (Kester *et al.* 2004). The concentrations of T_3 in the cerebral cortex at 20 PMW are close to 2 pmol/g. If the brain were a homogeneous fluid, the T_3 concentration would be 2 nM, which is much higher than the TT_3 concentration in serum (Fig. 1). Years ago, we showed that T_3 was present in brain but could not be detected in other tissues where only T_4 could be detected (Bernal & Pekonen 1984). The accumulation of T_3 in the cerebral cortex during the 2nd trimester is clearly due to DIO2 activity and follows the T_4 increase in serum and in the cortex (Kester *et al.* 2004). Interestingly, at the same ages in the cerebellum, which has high DIO3 activity, the concentration of T_3 is very low (Kester *et al.* 2004).

TH receptors

The main pathway of thyroid hormone action is at the genomic level by regulating gene expression via binding to the nuclear receptors (TRs), which function as ligand-activated transcription factors. The two TR genes, THRA and THRB, encode three proteins with full receptor function at the genomic level: TRα1, TRβ1 and TRβ2. In addition, there are several truncated proteins lacking either the DNA-binding domain or a functional T_3-binding domain. A TRα protein lacking the DNA-binding domain is attached to the plasma cell membrane and mediates the actions of T_3 on PI3K signaling (Kalyanaraman *et al.* 2014).

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The ontogeny of TRs can provide information as to the timing of CNS sensitivity to thyroid hormones at the genomic level. Using ISH techniques in rat embryos, a low signal of TRα1 mRNA is present from E11.5 onward in the neural tube and other structures (Bradley *et al*. 1992). By E15.5, there is a surge of TRα1 in the cortical plate and in the primordial hippocampus, and TRβ1 is also present in the rostral striatum (Mellstrom *et al*. 1991). At this time, nuclear T_3 binding activity becomes detectable in whole brain nuclei (Perez-Castillo *et al*. 1985). The TR is therefore present 2–3 days before onset of thyroid gland activity at E17.5, supporting the view that maternal thyroid hormones could be involved in the regulation of neural development at these stages. It is also possible that at these stages, the TR exists mainly as the aporeceptor, functioning as a developmental timer by restricting the differentiation of the neural precursors (Castelo-Branco *et al*. 2014).

In the human brain, TR mRNAs are detected around 8 PMW (Iskaros *et al*. 2000). Concerning the receptor protein, there is only one study in which receptor concentrations were quantitated by ligand-binding assays (Bernal & Pekonen 1984). The results of this study showed that the receptor protein is present already by the 10th PMW and is followed by a several fold increase, indicating that this period is critical for the action of T_3 on human brain development (Fig. 1). At 10 PMW, there were around 220 mol/cell (46 fmol/mg DNA), and increased 6 and 10 times at 12 and 16 PMW, respectively. It is to be noted that the mean cell content of the TR at 10 weeks is low, similar to poorly responsive cells such as lymphocytes, but its asymmetrical distribution could result in high concentrations in specific cells. At 16 PMW, the number of molecules per cell was 2300, similar to the mean TRα1 protein per cell in the adult rat brain (Ercan-Fang *et al*. 1996). Therefore, it is likely that the sensitivity of the human fetal brain to thyroid hormone increases dramatically shortly after the end of the embryonic period (8 weeks after fertilization or 10 PMW). Semi-quantitative analyses using immunohistochemistry were in general agreement with these data (Kilby *et al*. 2000).

Mice and human differ on the distribution of TR isoforms among cellular types. Work in rodents shows that the predominant TR subtype in the brain is TRα1 at the mRNA and protein level (Strait *et al*. 1990, Mellstrom *et al*. 1991, Bradley *et al*. 1992). TRα1 and TRβ differ on the relative affinities for the T_3 agonist 3,5,3′-triiodothyroacetic acid (triac). TRβ has higher affinity for triac than for T_3, whereas TRα1 has similar affinity for both compounds (Messier & Langlois 2000). The competitive TR-binding assays for different TH analogs indicated identical affinity for triac and T_3 of the rat brain TR (Perez-Castillo *et al*. 1985), and nearly 10-fold higher affinity for triac than for T_3 of the human brain TR (Bernal & Pekonen 1984) (see inset in Fig. 1). These differences have so far been largely ignored and strongly suggest that the main TR isoform expressed during the second trimester of human fetal development is TRβ. In mice, the TRα1 protein is expressed with few exceptions in all neurons (Wallis *et al*. 2010), and transcriptomic analysis of isolated cellular types revealed that TRβ mRNA is two-fold more abundant in astrocytes and OPC than in neurons, cells of predominantly postnatal accumulation. In contrast to mice, the human TRβ mRNA is 10-fold more abundant in neurons than in astrocytes (Zhang *et al*. 2016). Therefore, it appears that TRβ is the main TR present in the human brain during the period of neuronal accumulation, which takes place during the second trimester. These observations need to be confirmed by independent studies and may be relevant for understanding the pathophysiology of TR mutation syndromes.

TH control of gene expression during cortical development

Development of the cerebral cortex is an extremely complex and dynamic process in which different neural cell types are sequentially produced from precursors, migrate to different places to form a layered structure and become integrated into functional circuits (Kaas 2006, Olson 2014, Ohtaka-Maruyama & Okado 2015, Toma & Hanashima 2015). Work in the past on the effects of hypothyroidism on cortical structure has shown that lack of TH during the perinatal period in rats leads to less-defined cortical layering, neuronal migration and differentiation defects and altered circuitry (Berbel *et al*. 2014). It was also shown that TH controls the expression of genes involved in these processes, but knowledge of the actions of TH on cortex development remains fragmentary, and we are far from having a clear picture on the specific roles of TH during the different stages of cortical development. In the past ten years, the molecular mechanisms underlying the cellular assembly during cortical development have begun to unravel, and key genes involved in different processes have been identified. One of the approaches to understand the role of TH is to identify which of these genes are regulated at the transcriptional level by T_3. This analysis is indirect and does not result in direct proofs

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that TH are indeed involved in given molecular processes, but at least working hypothesis on the role of TH can be formulated.

After this reasoning, our most recent approach has been the use of primary mouse cerebrocortical cells to identify genes regulated directly or indirectly by T_3 (Gil-Ibanez *et al*. 2015). By direct regulation, we mean at the level of transcription, mediated by the interaction of T_3 with the TR, and indirect regulation will be a secondary effect resulting from a primary action on another gene or genes by T_3. From these premises, I start the analysis with the assumption that the involvement of a gene in a given developmental process would strongly support a role of T_3 in this process if the gene is a transcriptional target of T_3. This will necessarily require the presence of functional TRs in the same cells expressing the gene under regulation, which may not be so in the early periods of embryonic development.

General actions of T_3 in primary cerebrocortical cells

Primary cerebrocortical (CC) cells derived from E14 mice and cultured in the absence of serum are composed by 80% neurons, 15% astrocytes and 5% of other minor components including oligodendrocyte precursors, microglia and endothelial cells. These cells can be used to analyze the neuronal transcriptome under T_3 regulation. Within the neuron population, different phenotypes can be identified using immunohistochemistry for specific markers (Gil-Ibanez *et al*. 2015). For example, cells expressing Reelin, Cholecystokinin or Calbindin were identified. One important limitation of the cultures is the lack of T_3-sensitive cellular targets of postnatal origin such as parvalbumin interneurons (Gilbert *et al*. 2007). With this limitation in mind, if T_3 regulates genes with more than 80–90% enrichment in a given cell type, a high probability exists that this cellular type is a direct target of T_3. Identifying cellular targets from the expression of single genes with lower enrichment is more difficult. However, groups of enriched genes provide a cell type fingerprint facilitating the identification of cellular targets from the regulatory effects on the enriched gene set. Several recent studies have been performed on the transcriptomics of purified cell types obtained from the developing mouse cerebral cortex (Cahoy *et al*. 2008, Zhang *et al*. 2014, 2016, Zeisel *et al*. 2015). These studies provide databases of gene enrichment in particular cell types, which can be compared with transcriptomic analysis of the effect of T_3 on primary CC cells. Examples of these resources are the web pages provide by Dr B Barres' lab (http://web.stanford.edu/group/barres_lab/brainseq2/brainseq2.html) and Dr S Linnarsson's lab (http://linnarssonlab.org/cortex/). In our studies (Gil-Ibanez *et al*. 2015), we performed RNA-Seq of CC cultures exposed to T_3 for 24 h, and for 6 h in the presence or absence of the protein synthesis inhibitor cycloheximide to identify the genes regulated directly at the transcriptional level. The data were compared with databases of gene expression in purified primary cell types to identify genes regulated by T_3 in specific cell types. Many of the directly regulated genes, for example, sonic hedgehog (*Shh*), are involved in the regulation of multiple pathways during development. The control by TH on the expression of this and similar wide-acting genes, which is often dependent on the cellular context and developmental time, leads to an extremely complex array of TH effects, which should be interpreted as providing 'phenotypic stability'. In other cases, some of the genes regulated transcriptionally by T_3 have a key role in defined developmental events during cerebral cortex development, and hypothesis can be formulated concerning the participation of T_3 in these events.

From the near 15,000 genes expressed in the CC cultures, T_3 changes the expression of 1145, with upregulation of 629 genes (positive regulation) and downregulation of 526 genes (negative regulation). Gene ontology analysis indicated that T_3 positively regulates genes that are involved mainly in membrane processes, such as G-protein, neurotransmitter and Ephrin receptor signaling, i.e., processes related to cell differentiation, migration and communication, whereas negative regulation was associated with nuclear processes involved in mitosis and chromosome condensation. T_3 also influenced positively many genes of enhanced expression in the adult cortex, whereas it downregulated genes with increased expression in the embryonic cortex relative to the adult cortex. These data reinforce the idea that a general role of T_3 in the developing cortex is to facilitate the transition between the embryonic cortex and the adult cortex, in reminiscence of its role during metamorphosis.

From the genes sensitive to T_3 in the CC cultures, 254 positive genes and 117 negative genes were regulated directly at the transcriptional level. Many of these genes, 89 negative and 17 positive, also contained TR-binding sites as determined by TR immunoprecipitation analysis by Chatonnet and coworkers (Chatonnet *et al*. 2013). The presence of a TR-binding site further reinforces the concept of transcriptional regulation.

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The genes regulated directly by T_3 could be grouped into different functional categories (Fig. 3). The major groups were transcription factors and cofactors including DNA-modifying enzymes (56 genes), metabolic enzymes (56 genes), G-protein-coupled receptors (42 genes), extracellular matrix proteins and cell adhesion molecules (42 genes), membrane transporters and ion channels (26 genes), cytoskeletal components and cell-junction proteins (22 genes) and genes involved in membrane signaling events not classified among the previous categories (21 genes). In addition, there were glutamate and gaba receptors, ephrin receptors, semaphorins and many other genes involved in neuronal function. The induced genes more sensitive to T_3 were *Cyp11a1*, *Hr*, *Shh*, *Dio3*, *Sptssb*, *Flywch2*, *Hcrtr1*, *Gpr30* and *Klf9*, and the repressed genes were *Htr7*, *Aldh1a3*, *Rgs4*, *Kera*, *Pcdh18*, *Klhl14*, *Ndst3*, *Mc4r*, *St8sia4* and *Trhr*.

It is clear that the extraordinary diversity of the T_3-regulated transcriptome makes it very difficult to define in simple terms the biological role of TH on cerebral cortex development, apart from the generalities offered by gene ontology analysis mentioned previously. T_3 regulates many functionally diverse genes, some of them involved in multiple regulatory cascades and having many diverse functions during development such as *Shh*. Additionally, T_3 also controls the expression of genes involved in the metabolism of retinoic acid (RA) and cooperates with glucocorticoid hormones (Gil-Ibanez *et al*. 2014). RA concentrations in tissues depend on the aldehyde dehydrogenases (RALDH)-synthesizing enzymes ALDH1A1, ALDH1A2 and ALDH1A3 and degrading enzyme CYP26B1. *Aldh1a1* is upregulated indirectly by T_3, but with a strong synergism with glucocorticoids. *Aldh1a3* is downregulated transcriptionally by T_3, and *Cyp26b1* is upregulated transcriptionally. Therefore, the net effect of T_3 on RA concentrations could be to elevate or to decrease RA concentrations, depending upon the developmental pattern of the synthesizing and degrading enzymes and the local tissue concentrations of glucocorticoids. It is very unlikely that T_3 influences RA metabolism during the early brain morphogenetic period as the T_3 receptors are still not present, and *Aldh1a2* is not sensitive to T_3. During late development, T_3 may contribute to the decreased expression of *Aldh1a3* and facilitate the increased expression of *Aldh1a1* (Smith *et al*. 2001, Wagner *et al*. 2002). In this way, T_3 might modulate the actions on neuronal differentiation through control of RA concentrations in particular locations. Other functional consequences might be unrelated to RA metabolism. For example, *Aldh1a1* is expressed in ventral mesencephalic dopaminergic neurons (Liu *et al*. 2014), in which it influences dopamine metabolism and has neuroprotective effects. Lower expression of this enzyme may be relevant to Parkinson's disease (Anderson *et al*. 2011).

Sensitivity of key genes of cortex development to T_3

Data on the regulation of T_3 on gene expression in CC cells can be examined in the light of recent concepts of genetic influences on cortical development. I will refer to the human for the timing of the major events in development (de Graaf-Peters & Hadders-Algra 2006, O'Rahilly & Muller 2008) to compare with the endocrine events represented in Fig. 1. The data on gene expression during cortical development is derived mostly from studies in mice (Maeda 2015, Ohtaka-Maruyama & Okado 2015, Toma & Hanashima 2015). In the text which follows, I refer to a gene as regulated or not by T_3 from our data on the CC, if no other reference is given. These data can be examined in the Supplementary data 1 of our publication (Gil-Ibanez *et al*. 2015). Tentative roles for some of the genes regulated by T_3, on the basis of their expression during defined stages of cortical development are represented in Fig. 4.

During human development, the formation of the neural tube starts around the 5th week of gestation. The bulk of neurogenesis occurs between weeks 5 and 25, with the exception of the granular cells of the olfactory bulb, hippocampus and cerebellum, which continue to be generated postnatally. The neuroepithelial cells will give rise to neurons (excitatory or glutamatergic and inhibitory or gabaergic) and glia (astrocytes and oligodendrocytes). Early in neurogenesis, neuroepithelial cells acquire glutamatergic identity through the sequential expression of *Pax6*, *Neurog1/2* and *NeuroD*, and under the influence of FGF10, give rise to the radial glia cells (RGC) as the universal progenitor cells of the cerebral cortex (Toma & Hanashima 2015). The *Fgf10* gene is not sensitive to T_3. *Pax6* and *NeuroD* have previously been related to effects of TH on neurogenesis. PAX6+ cells increase with the expression of the αvβ3 integrin, the membrane T_4 receptor mediating nongenomic actions (Stenzel *et al*. 2014), and diminish with maternal hypothyroidism, supporting an early effect of maternal T_4 on neurogenesis (Mohan *et al*. 2012). *NeuroD* expression was altered by hypothyroidism during cerebellar development (Chantoux & Francon 2002) and by unliganded TRα1 during hippocampal neurogenesis (Kapoor *et al*. 2010). None of these genes

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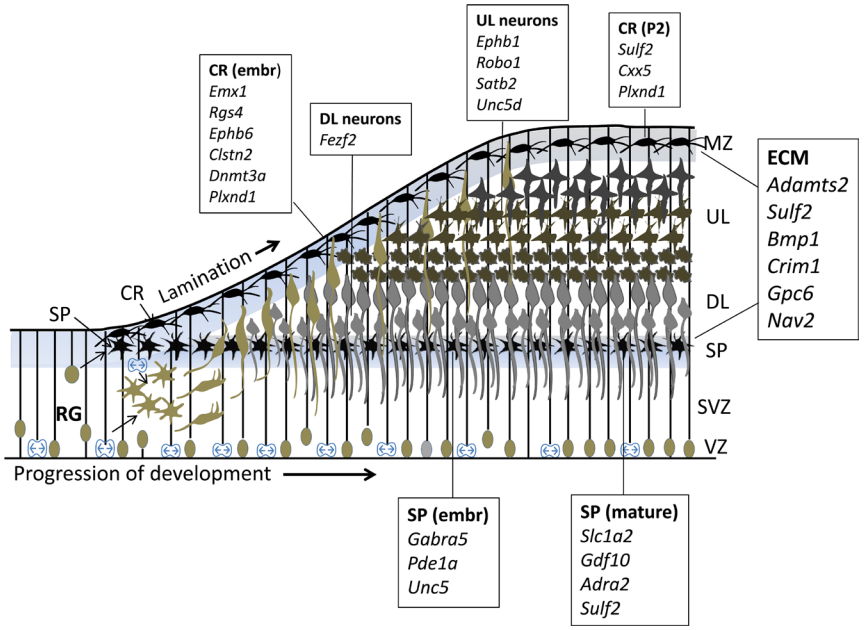

Figure 4

Scheme of cortex development and possible role of some of the genes regulated transcriptionally by T_3 in the cerebrocortical cultures. Different stages of cortex development are represented: proliferation of precursors, multipolar to bipolar transition, appearance of the Cajal-Retzius cells (CR) and the subplate cells (SP) with the formation of the preplate, arrival of the first migrating neurons with the splitting of the preplate and formation of the cortical plate, and the inside out migration process to form first the deep layers (DL) 5 and 6, and then the upper layers (UL) 2 to 4. Genes regulated by T_3 have been selected on the basis of specific expression at a certain developmental stage in the embryonic or P2 CR cells, the SP cells at two stages of development, specific markers genes for the DL or the UL neurons, and the extracellular matrix (EM). The extracellular matrix is represented by a shade on the marginal zone and the subplate, which stain more strongly by glycosaminoglycans, the major component of brain EM. Adapted, under the terms of the CCBY license, from Ohtaka-Maruyama C & Okado H (2015) Molecular pathways underlying projection neuron production and migration during cerebral cortical development, *Frontiers in Neuroscience*, volume **9**, article 447.

were regulated directly or indirectly by T_3 in the primary CC cells (Gil-Ibanez *et al.* 2015), and thus, a primary effect of T_3 is unlikely. However, nongenomic effects of T_4 cannot be discarded.

The RGC extends a basal process in contact with the pial surface, and an apical process in contact with the ventricular surface. The basal process serves as a guide for migrating neurons. The RGC undergoes symmetrical division forming two identical progenitor cells or divide asymmetrically to generate one progenitor and one neuron. The transition between symmetrical and asymmetrical modes of cell division is regulated by RA produced by the meninges by the ALDH1A2 enzyme (Siegenthaler *et al.* 2009) under the influence of the transcription factor FOXC1. RA reaches the RGC through the basal process. *Foxc1* expression is insensitive to T_3. In contrast to *Aldh1a1* and *Aldh1a3*, the *Aldh1a2* gene has not been reported to be regulated by T_3, and these processes most likely occur independently of TH.

The different types of neurons are generated sequentially by tightly regulated mechanisms involving the loss and acquisition of competence, expression of specific transcription factors and epigenetic modifications. Different waves of cell subtypes are generated and integrated into the developing cortex in the following order: first the non-projection neurons Cajal-Retzius (CR) and subplate (SP) cells, then the projection neurons of cortex layers 6–2 and finally the glial cells.

Asymmetrical division of the RGC produces the first neurons of the cerebral cortex, the CR and SP cells. When these cells are formed, they accumulate in a transient structure, the preplate or primordial plexiform layer, immediately above the ventricular zone (VZ) and underneath the meninges. CR cells and SP cells, and the appearance of the preplate, occur around the 5th–7th week of gestation in humans. In mice, the peak of CR cell formation occurs between E10 and E11 (Takiguchi-Hayashi *et al.* 2004). These processes most probably occur in the complete absence of TH. The first founder cortical cells, also cortical progenitor pool, generated by asymmetric division of the RGC split the preplate into a marginal zone or future layer 1 containing the CR cells, and the subplate. This process is known as preplate splitting and occurs at 13.5 in mice and in the 7th–8th

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weeks in humans. The subsequently arriving neurons accumulate between the marginal zone and the SP and form the cortical plate (CP).

Cortical founder neuroblasts can divide again symmetrically in the VZ, increasing the tangential surface of the cortex and consequently the number of cortical columns, whereas asymmetrical divisions will originate migrating neurons increasing the number of cells per column (Rakic 2009). In developing hypothyroid rats, the tangential surface and the thickness of the barrel cortex are decreased, indicating reduced symmetrical and asymmetrical divisions (Berbel *et al.* 2001).

Neurons migrating along the RGC processes pass existing neurons and displace the older neurons back, in a process known as 'inside-out' migration (Sidman & Rakic 1973). The extracellular matrix protein Reelin (RLN), produced by the CR cells, plays a fundamental role in this process. It halts the migration of arriving neurons impeding their progression to the marginal zone (future layer 1) (Rice & Curran 2001). In this way, the deep layers 6 and 5 (DL) of the cortex are the first to form, between E10.5 and E14.5 in mice, and the upper layers 4–2 (UL) between E14.5 and E16.5. The SP acts as a gateway for neurons entering the CP, accommodating the large pool of arriving neurons and guiding the thalamic afferents to establish synaptic contacts.

T_3 has important influences on gene expression of CR and SP cells and on matrix and extracellular proteins involved in migration.

Cajal-Retzius cells

Cajal-Retzius cells are under T_3 control in rodents (Garcia-Fernandez *et al.* 1997, Alvarez-Dolado *et al.* 1999). These cells are a minor population of cerebral cortex neurons located in layer 1. They secrete the extracellular matrix protein REELIN, which acts as a barrier for the newly arriving neurons from the ventricular layer during the formation of the cortical layers. The REELIN-DAB1 pathway is under transient control by thyroid hormones in rodents (Alvarez-Dolado *et al.* 1999). It is possible that hypothyroidism also affects the human brain similarly, because a fetus with mutated MCT8 transporter showed lack of neurofilament staining of the CR cells (Lopez-Espindola *et al.* 2014). T_3 does not have a direct transcriptional control on the *Rln* gene, and therefore, the effects of hypothyroidism are probably exerted on genes having an effect on CR generation, migration or differentiation. *Emx1* a transcription factor gene upregulated transcriptionally by T_3 could be one possible candidate. T_3 also regulates, but indirectly, two genes involved in the migration of the CR cells, the chemokine *Cxcl12* expressed in the meninges and its receptor *Cxcr7* expressed in the CR cells. A related chemokine, *Cxcl14*, is regulated transcriptionally by T_3 and contains a TR-binding site. Further examination of genes enriched in the CR cells and expressed in the cerebrocortical cultures and regulated by T_3 at the transcriptional level gave surprising information. Previous studies have identified genes enriched in CR cells at two stages of mouse development, E13 and P2 (Yamazaki *et al.* 2004). Interestingly, when the genes regulated transcriptionally by T_3 in the primary CC cultures are compared with these data sets, 5 genes enriched in E13 CR cells were found: *Rgs4*, a G-protein modulator, *Npnt*, a Ca^{2+} and integrin-binding ECM protein, *Ephb6*, an ephrin receptor, *Clstn2*, a cell adhesion molecule, and *Dnmt3a*, a DNA methyl transferase; 2 genes enriched in P2 CR cells are also transcriptionally regulated by T_3: *Sulf2*, a sulfatase that removes sulfate groups from heparin sulfate, and *Cxx5*, a nuclear protein. Another T_3-regulated gene, *Plxnd1* a protein kinase, is enriched in CR cells at both developmental stages. *Dnmt3a* has recently been confirmed as a T_3-regulated gene in neuroblastoma cells and in the postnatal mouse brain (Kyono *et al.* 2016). P2 CR cells also expressed the universal transcriptional target of T_3 *Klf9*. Our data indicate that specific cells such as the CR cells, with an important role in cortex development, expressed genes under transcriptional regulation by T_3, and that immature E13 CR cells are already potentially sensitive to T_3.

Subplate neurons

The SP is a transient structure of the developing cerebral cortex formed when the PP is split, by the arriving neurons of layer 6, between the upper marginal zone or future layer 1 and the SP (Hoerder-Suabedissen & Molnar 2015). In the process of neurogenesis, the newly arriving neurons from the ventricular zone cross the SP and arrive at the CP, which will then mature to form the cortex layers. SP neurons are located between the white matter and cortex layer 6. PP splitting occurs in human at PMW 7–8 (E13.5 in mice), reaches a maximum thickness at about 29 weeks of gestation and regresses by around PMW 31–38. In mice, SP neurons are generated at around E12 and the SP persists postnatally. The SP plays a pivotal role in axonal routing from and to the cortex and also influences the tangential migration of interneurons. Fibers from the thalamus,

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the basal forebrain and the contralateral and ipsilateral hemispheres destined to the cortex, first arrive at the SP and establish transient synaptic contacts, before heading for the final destination. The SP neurons, therefore, have a crucial role for the maturation of cortical intrinsic and extrinsic circuits.

Recent gene expression studies were aimed at identifying genes specific from the SP neurons to understand the molecular basis of SP function. Four hundred sixteen genes were identified as SP enriched (Hoerder-Suabedissen & Molnar 2013, Hoerder-Suabedissen *et al*. 2013). Most of these genes (394) are expressed in CC cultures and 82 of them were regulated by T_3. Of these, 35 genes were under direct T_3 regulation (Gil-Ibanez *et al*. 2015). These data point to an important regulatory effect of thyroid hormone in SP development and function. Furthermore, of the genes expressed in the SP 68 were identified as being SP specific at any one time of development. From this set of genes, 23 were under T_3 regulation, and 8 of them directly at the transcriptional level: *Gabra5*, the γ5 GABA A receptor subunit, which also increased in expression in P21 hypothyroid mice (Morte *et al*. 2010); *Pde1a* encodes a phosphodiesterase; *Unc5c* encodes a netrin receptor, involved in axon extension and cell migration, and its expression is upregulated by T_3 *in vivo* (Dong *et al*. 2014); *Slc1a2*, a glutamate and aspartate transporter involved in glutamate clearance at the synapses; *Alcam*, a cell adhesion molecule; *Gdf10*, a member of the BMP and TGF family; *Adra2a*, the α2 adrenergic receptor; and *Sulf2*, also expressed in CR cells as mentioned previously. Some of these genes were expressed in the embryonic SP (*Gabra5*, *Pde1a* and *Unc5c*) and were downregulated by T_3, and others were expressed at more mature stages (*Slc1a2*, *Gdf10*, *Adra2a* and *Sulf2*) and were upregulated. This is in agreement with the general trend in T_3 regulation of gene expression during neural development with downregulation of embryonic genes and upregulation of adult genes (Dillman *et al*. 2013, Gil-Ibanez *et al*. 2015).

It is known that hypothyroidism interferes with the formation of cortical maps by altering the proper development of cortical circuitry (Lucio *et al*. 1997). Direct actions of T_3 on the SP may underlie the effects of hypothyroidism on these processes (Navarro *et al*. 2014). It also could be of particular relevance for the etiology of autism (Berbel *et al*. 2014). Subplate-specific or enriched genes regulated by T_3 such as *Cdh18*, *Gabra5*, *Prss12*, *Sema5a* and *Cdh10*, have been linked to autism (Hoerder-Suabedissen *et al*. 2013) and *Slc1a2* to schizophrenia (Hoerder-Suabedissen *et al*. 2013).

Layer projection neurons and the formation of cortical layers

The switch of RGC progenitor cells from producing CR cells to projection neurons involves repression by two transcription factors, FOXG1 and LHX2. These transcription factors are expressed in the CC cultures and are not regulated by T_3. As indicated previously, the first layer projection neurons destined to form layer 6 split the PP and the cells start accumulating in the CP. Sequential rounds of RGC asymmetric division originate the rest of the layer projection neurons, and form layers 5 to layer 2 through the inside-out migration process. The peak of neuronal migration in humans occurs between the 12th and 20th weeks, coinciding with the increase in the TR (Fig. 1) and is completed by the 30th week. The DL neurons of layers 5 and 6 contain corticofugal projection neurons, the upper layers 2–3 contain ipsilateral and contralateral corticocortical projection neurons and layer 4 receives subcortical afferents especially from the thalamus. The DL neurons express the transcription factors *Fezf2*, *Ctip2*, *Tbr1* and *Sox5*. Of these, only *Fezf2* is downregulated by T_3 but indirectly in CC cells. The upper layers express the transcription factors *Cux1/2*, *Brn1/2* and *Satb2*. *Satb2* is a marker of a subclass of UL neurons. Interestingly, *Satb2* is downregulated by T_3 at the transcriptional level and contains a TR-binding site. Another subclass of UL neurons expresses *Unc5d*, which is also downregulated transcriptionally by T_3. The regulation of these two genes induces the suspicion that T_3 might be involved in the production or more likely in the migration or timing of integration of the UL neurons in the developing cortex. In this context, *Robo1*, a gene involved in the radial dispersion of UL neurons, is regulated by T_3 but indirectly. In addition to the radial migration of neurons along the RGC processes to form the cortical layers, lateral dispersion contributes to the formation of the cortical columns. EPHRIN A (*Efna*) signaling through the EPHRIN A receptor (*EphA*) is involved in this process (Ohtaka-Maruyama & Okado 2015, Toma & Hanashima 2015). T_3 regulates transcriptionally many of the components of EPHRIN signaling components in primary CC cells, and at least one of them, *Ephb1*, is involved in lateral neuron dispersion and the formation of cortical columns.

Actions of T_3 on genes encoding extracellular matrix (ECM) proteins

The ECM (Mouw *et al*. 2014) constitutes about 20% of the brain parenchyma and fills the extracellular space. It

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forms a lattice-like structure composed of a heterogeneous group of molecules such as neurotrophic factors, adhesion molecules, laminin, fibronectin, collagen, hyaluronan proteins, proteoglycans and other components. The ECM proteins are involved in many processes during development and in the adult brain, such as neurogenesis, neuronal and glial migration, axon outgrowth and guidance, synaptic plasticity and recovery from injury. T_3 has a direct transcriptional control of at least 25 genes encoding ECM proteins, of which 7 contain TR-binding sites. These 7 genes are *Adamts2*, *Lingo3*, *Mfap3l*, *Bmp1*, *Megf10*, *Nav2* and *Crim1*, to which we will limit the discussion below.

ADAMTS2 is a member of a large family of proteinases (disintegrin and metalloproteinase with thrombospondin motifs) involved in proteolysis of proteoglycans (PG) (Gottschall & Howell 2015). The PGs are formed by a protein moiety bound to the cell surface containing lateral chains of glycosaminoglycans (Maeda 2015), the major components of the brain ECM. Glycosaminoglycans are enriched in the marginal zone and in the subplate during development, and their sulfation state is regulated by the activity of sulfatases, one of them, *Sulf2* has been mentioned previously as a T_3-regulated gene in CR and SP cells. The chondroitin sulfate-bearing proteoglycans comprise the hialectans or lecticans, the glypicans, the syndecans and others. Although *Adamts2* is transcriptionally upregulated by T_3, *Adamts18* is transcriptionally downregulated. Other members of the family are indirectly upregulated by T_3 such as *Adamts17* or downregulated such as *Adamts1* and *Adamts19*. The action of thyroid hormone is very selective as other members of the family, specifically *Adamts* 4, 5, 8, 9, 15 and 20, which degrade hialectan/lectican proteoglycans, the major class of PG present in the CNS are not regulated by T_3. *In vivo*, hypothyroidism decreases the expression of *Adamts2* and increases *Adamts18* in agreement with the regulation in the cultured cells (Morte *et al*. 2010; and Supplementary Table 7 of Gil-Ibanez *et al*. 2015). Another metalloproteinase transcriptionally upregulated by T_3, *Bmp1*, is a bone morphogenetic protein that cleaves procollagens. *Crim1* regulates the processing of BMPs preproteins into mature proteins and delivery to cell surface.

The glypicans (GPC) are a class of PGs bound to the cell surface by a glycosylphosphatidylinositol anchor. There are six Gpc genes in the mammalian genome, *Gpc1* through *Gpc6*, and all of them are expressed in the CC cultures. They regulate the activity of several signaling pathways, including the SHH pathway. T_3 upregulates the expression of *Gpc6* directly at the transcriptional level, whereas *Gpc3* is downregulated but indirectly. *In vivo*, hypothyroidism increases *Gpc3* expression in the mouse cerebral cortex (Morte *et al*. 2010). Other *Gpc* genes are unaffected by T_3.

Many of the genes regulated transcriptionally by T_3 encoding adhesion proteins and proteins of the extracellular matrix are involved in axon outgrowth, axon pathfinding and axon guidance. In addition to the genes mentioned previously, T_3 controls the expression of *Nav2* (Neuron navigator 2) in CC cells (Gil-Ibanez *et al*. 2015), and *NAV2* in the human skeletal muscle is downregulated in hypothyroidism (Visser *et al*. 2009). *Nav2* is the mammalian ortholog of Caenorhabditis elegans unc-53 required for axonal elongation of mechanosensory neurons (Luo *et al*. 2006). *Nav2* is also under control of retinoic acid in neuroblastoma cells (Luo *et al*. 2006). *Nav2* regulation is potentially a crucial regulatory crossroad where signaling pathways regulated by T_3 and retinoic acid converge through the control exerted by T_3 on retinoic acid synthesizing and degrading enzymes, as explained elsewhere in this review.

A note on glial cells

The termination of neurogenesis in the cerebral cortex involves a switch from the UL neurons to generation of astrocytes, although progenitor cells able to generate neurons and glia are already present at E10 in mice. Mature astrocytes are transcriptional targets of T_3, as indicated by the presence of some astrocyte-enriched genes regulated transcriptionally by T_3 (Supplementary Table 3 from Gil-Ibanez *et al*. 2015). It is unlikely that T_3 regulation takes place in the transition from the UL neurons to astrocyte generation, because genes involved in this transition (Toma & Hanashima 2015), *Ring1b*, *Ezh2* and *Neurog1*, and the DNA methyltransferase *Dnmt1*, expressed in the CC cultures are not regulated by T_3. On the other hand, the CC cultures are not an appropriate system to analyze the effects of T_3 on oligodendrocyte maturation, a process that takes place postnatally. One percent of the genes expressed in the CC cultures are highly enriched in cells of the oligodendroglia lineage, and among them, the oligodendroglia-specific genes *Enpp2*, *Lgi3* and *C1ql3*, were transcriptionally regulated in the cultures. A recent insight into oligodendrocyte differentiation from neural stem cells and the role of T_3 has been published (Castelo-Branco *et al*. 2014).

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Concluding remarks

Thyroid hormones have important roles during the development of the cerebral cortex. The T_3 nuclear receptor, type 2 deiodinase activity, and T_3 concentrations increase in the human fetal brain and the developing cortex from the 10th week of gestation, with maximum levels attained around the 18th–20th postmenstrual weeks. In this period, and continuing throughout gestation, important developmental events take place leading to the expansion of the neuronal population, and migration of neurons to form the cortex layers. T_3 regulates, at the transcriptional level, genes involved in many of these processes. In this review, we have identified many of these genes and provide a first approach to understanding the molecular basis of thyroid hormone action on cerebral cortex development. Many of the T_3-regulated genes are expressed in the Cajal-Retzius cells or the subplate or encode proteins of the extracellular matrix. These three are critical regulators of cortical development. Alterations caused by thyroid hormone imbalance during corticogenesis may lead to irreversible damage and may have implications in neurological and mental diseases.

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