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 T3 with the nuclear receptors. To understand the role of thyroid hormones on cerebral cortex development, knowledge of the genes regulated by T3 during specific stages of development is required. In our laboratory, we previously identified genes regulated by T3 in primary cerebrocortical cells in culture. By comparing these data with transcriptomics of purified cell types from the developing cortex, the cellular targets of T3 can be identified. In addition, many of the genes regulated transcriptionally by T3 have defined roles in cortex development, from which the role of T3 can be derived. This review analyzes the specific roles of T3-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 T3 during the second trimester of fetal development. The main cellular targets of T3 appear to be the Cajal-Retzius and the subplate neurons. On the other hand, T3 regulates transcriptionally genes encoding extracellular matrix proteins, involved in cell migration and the control of diverse signaling pathways.

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 T3 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
cortex development at the cellular and molecular levels as the frame to identify possible roles of T₃ on these processes. To accomplish this, I will use recent data from our laboratory on T₃-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 T₃ 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, T₄ and T₃ are present in the coelomic fluid, an ultrafiltrate of the maternal serum (Calvo et al. 2002). T₄ in the coelomic fluid is derived from the maternal pool, and T₄ concentrations in coelomic fluid and maternal serum are positively correlated. The total T₄ (TT₄) concentration (1–2 nM) is about 100 times less than the maternal concentration, although the free fraction is much higher. In the amniotic fluid, TT₄ 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 T₄ and T₃ 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 TT₄ concentration increases from around 26 nM at 12 weeks to 100–140 nM at 36 weeks, reaching maternal concentrations. Free T₄ (fT₄) increases from about 1–2 pM at 12 weeks to 25 pM at the end of gestation (Thorpe-Beeston et al. 1991, Guibourdencche et al. 2001) and TT₃ from the almost undetectable level of 0.1 nM at 12 weeks to 1 nM at 36 weeks. In contrast to T₄, T₃ levels at the end of gestation do not reach the maternal levels, most probably reflecting the immaturity of the T₄ to T₃ 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 T₃ 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), T₃, T₄, rT₃ (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⁻–NH₃⁺) (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...
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 for 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 T4 and T3, and the organic anion transporter polypeptide 1C1 (OATP1C1, encoded by the SLC01C1 gene) with much higher selectivity for T4. 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 (Partridge 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 Scl16a2−/−) mice, the accumulation of administered labeled T4, but not T3, is severely restricted (Trajkovic et al. 2007). Furthermore, T4 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 T4 in a similar way as the wild-type mice on induction of neuronal genes. The reason why T4 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 T4 to the astrocytes and conversion to T3. Proof for this explanation is that the inactivation of the Dio2 gene, highly enriched in astrocytes, in MCT8-deficient mice suppressed the effect of T4 (Morte et al. 2010). The T4 generated in the astrocytes then reaches the neurons possibly through secondary transporters (Kinne et al. 2011). Double inactivation of the Scl16a2 (MCT8) and Slc01c1 (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).

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 T4, with only 5–10% of secreted iodothyronines in the form of T3. The majority of T3 is formed from T4 in tissues by 5′-deiodination catalyzed by types 1 and 2 deiodinases (DIO1 and DIO2). T3 and T4 are inactivated by 5-deiodination catalyzed by type 3 deiodinase (DIO3) with the formation of 3,3′,5′-triiodothyronine (reverse T3, or rT3) and 3,3′-diodothyronine (T2), respectively (Bianco et al. 2002, Bianco 2011). The human brain has no DIO1 activity (Campos-Barros et al. 1996), and the only T4 to T3 converting enzyme is Dio2. As originally shown in rats by in situ hybridization (ISH), Dio2 is expressed...
predominantly in astrocytes and in the tanyocytes, 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 T₄ and T₃, which reaches the neurons from the blood directly through the BBB or indirectly from the astrocytes. Dio3 regulates critically the concentration of T₃ and dampens the effect of an excess T₃ 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 T₃, specifically through TRα1 (Barca-Mayo et al. 2011, Gil-Ibanez et al. 2014), and its placental activity may be modulated by the circulating T₃ (Bianco et al. 2002). Dio3 activity may be coupled to MCT8 transport in such a way that T₃ 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 T₃ despite high concentrations of the MCT8 protein (Grijota-Martinez et al. 2011). MCT8 transport would accumulate T₃ 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 T₃ concentrations. As shown in Fig. 1, T₃ and T₄ concentrations increase in the cerebral cortex from weeks 13 to 18 and may attain a plateau (Kester et al. 2004). The concentrations of T₄ in the cerebral cortex at 20 PMW are close to 2 pmol/g. If the brain were a homogeneous fluid, the T₃ concentration would be 2 nM, which is much higher than the TT₄ concentration in serum (Fig. 1). Years ago, we showed that T₄ was present in brain but could not be detected in other tissues where only T₃ could be detected (Bernal & Pekonen 1984). The accumulation of T₃ in the cerebral cortex during the 2nd trimester is clearly due to Dio2 activity and follows the T₄ 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 T₃ 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 T₃-binding domain. A TRα protein lacking the DNA-binding domain is attached to the plasma cell membrane and mediates the actions of T₃ on PI3K signaling (Kalyanaraman et al. 2014).
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 T3 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 T3 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 T3 agonist 3,5,3′-triiodothyroacetic acid (triac). TRβ has higher affinity for triac than for T3, 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 T3 of the rat brain TR (Perez-Castillo et al. 1985), and nearly 10-fold higher affinity for triac than for T3 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 T3. This analysis is indirect and does not result in direct proofs...
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 T₃ (Gil-Ibanez et al. 2015). By direct regulation, we mean at the level of transcription, mediated by the interaction of T₃ with the TR, and indirect regulation will be a secondary effect resulting from a primary action on another gene or genes by T₃. 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 T₃ in this process if the gene is a transcriptional target of T₃. 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 T₃ 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 T₃ 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 T₃-sensitive cellular targets of postnatal origin such as parvalbumin interneurons (Gilbert et al. 2007). With this limitation in mind, if T₃ 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 T₃. 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 T₃ 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 T₃ for 24h, and for 6h 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 T₃ 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 T₃ have a key role in defined developmental events during cerebral cortex development, and hypothesis can be formulated concerning the participation of T₃ in these events.

From the near 15,000 genes expressed in the CC cultures, T₃ changes the expression of 1145, with upregulation of 629 genes (positive regulation) and downregulation of 526 genes (negative regulation). Gene ontology analysis indicated that T₃ 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. T₃ 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 T₃ 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 T₃ 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.
The genes regulated directly by T₃ 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 T₃ were Cyp11a1, Hr, Shh, Dio3, Spitz, Flywhch2, Hctr1, Gpr30 and Klfl, and the repressed genes were Htr7, Aldh1a3, Rgs4, Kera, Pcdh18, Klh1f14, Ndst3, Mc4r, St8sia4 and Trhr.

It is clear that the extraordinary diversity of the T₃-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. T₃ regulates many functionally diverse genes, some of them involved in multiple regulatory cascades and having many diverse functions during development such as Shh. Additionally, T₃ 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 T₃, but with a strong synergism with glucocorticoids. Aldh1a3 is downregulated transcriptionally by T₃, and Cyp26b1 is upregulated transcriptionally. Therefore, the net effect of T₃ 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 T₃ influences RA metabolism during the early brain morphogenetic period as the T₃ receptors are still not present, and Aldh1a2 is not sensitive to T₃. During late development, T₃ 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, T₃ 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 T₃**

Data on the regulation of T₃ 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 T₃ 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 T₃, 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 T₃.

Pax6 and NeuroD have previously been related to effects of TH on neurogenesis. PXA6+ cells increase with the expression of the αvβ3 integrin, the membrane T₃ receptor mediating nongenomic actions (Stenzel et al. 2014), and diminish with maternal hypothyroidism, supporting an early effect of maternal T₃ 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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Thyroid hormones and cortex development were regulated directly or indirectly by T₃ in the primary CC cells (Gil-Ibanez et al. 2015), and thus, a primary effect of T₃ cannot be discarded. However, nongenomic effects of T₄ 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 T₃. In contrast to Aldh1a1 and Aldh1a3, the Aldh1a2 gene has not been reported to be regulated by T₃, 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

Figure 4

Scheme of cortex development and possible role of some of the genes regulated transcriptionally by T₃ 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 T₃ 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.
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.

T₃ 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 T₃ 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). T₃ 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 T₃ could be one possible candidate. T₃ 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 T₃ and contains a TR-binding site. Further examination of genes enriched in the CR cells and expressed in the cerebrocortical cultures and regulated by T₃ 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 T₃ 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, Nptn, a Ca²⁺ 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 T₃; Sulf2, a sulfatase that removes sulfate groups from heparin sulfate, and Cxcl5, a nuclear protein. Another T₃-regulated gene, Pbnnd1 a protein kinase, is enriched in CR cells at both developmental stages. Dnmt3a has recently been confirmed as a T₃-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 T₃ 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 T₃, and that immature E13 CR cells are already potentially sensitive to T₃.

**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,
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 T3. Of these, 35 genes were under direct T3 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 T3 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 T3 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 T3v 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 T3 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 T3 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 T3 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 T3. 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 Fez2, Ctip2, Tbr1 and Sox5. Of these, only Fez2 is downregulated by T3 but indirectly in CC cells. The upper layers express the transcription factors Cux1/2, Bmi1/2 and Satb2. Satb2 is a marker of a subclass of UL neurons. Interestingly, Satb2 is downregulated by T3 at the transcriptional level and contains a TR-binding site. Another subclass of UL neurons expresses Unc5d, which is also downregulated transcriptionally by T3. The regulation of these two genes induces the suspicion that T3 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 T3 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). T3 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 T3 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
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. T3 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 T3-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 T3, Adamts18 is transcriptionally downregulated. Other members of the family are indirectly upregulated by T3 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 T3. 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 T3, 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. T3 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 T3.

Many of the genes regulated transcriptionally by T3 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, T3 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 T3 and retinoic acid converge through the control exerted by T3 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 T3, as indicated by the presence of some astrocyte-enriched genes regulated transcriptionally by T3 (Supplementary Table 3 from Gil-Ibanez et al. 2015). It is unlikely that T3 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 T3. On the other hand, the CC cultures are not an appropriate system to analyze the effects of T3 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 C1qa3, were transcriptionally regulated in the cultures. A recent insight into oligodendrocyte differentiation from neural stem cells and the role of T3 has been published (Castelo-Branco et al. 2014).
Concluding remarks

Thyroid hormones have important roles during the development of the cerebral cortex. The T₃ nuclear receptor, type 2 deiodinase activity, and T₄ 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. T₃ 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 T₃-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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