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Global Transcriptome Analysis of Primary Cerebrocortical Cells: Identification of Genes Regulated by Triiodothyronine in Specific Cell Types

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Abstract

Thyroid hormones, thyroxine, and triiodothyronine (T3) are crucial for cerebral cortex development acting through regulation of gene expression. To define the transcriptional program under T3 regulation, we have performed RNA-Seq of T3-treated and untreated primary mouse cerebrocortical cells. The expression of 1145 genes or 7.7% of expressed genes was changed upon T3 addition, of which 371 responded to T3 in the presence of cycloheximide indicating direct transcriptional regulation. The results were compared with available transcriptomic datasets of defined cellular types. In this way, we could identify targets of T3 within genes enriched in astrocytes and neurons, in specific layers including the subplate, and in specific neurons such as prepronociceptin, cholecystokinin, or cortistatin neurons. The subplate and the prepronociceptin neurons appear as potentially major targets of T3 action. T3 upregulates mostly genes related to cell membrane events, such as G-protein signaling, neurotransmission, and ion transport and downregulates genes involved in nuclear events associated with the M phase of cell cycle, such as chromosome organization and segregation. Remarkably, the transcriptomic changes induced by T3 sustain the transition from fetal to adult patterns of gene expression. The results allow defining in molecular terms the elusive role of thyroid hormones on neocortical development.

Key words: development, gene regulation, subplate, transcriptomics, thyroid hormones

Introduction

The thyroid hormones (TH) thyroxine (T4) and 3,5,3′triiodo-l-thyronine (T3) exert important actions during vertebrate development, for example, amphibian metamorphosis (Brown and Cai 2007; Denver 2013) and are crucial for mammalian brain development (Bernal 2005, 2007). TH deficiency during maturation alters cognitive development and causes profound neurological impairment (Berbel et al. 2014; Bernal et al. 2015). An example is the dramatic phenotype caused by mutations of the specific TH transporter MCT8 (monocarboxylate transporter 8). MCT8 mutations impair T4 and T3 transport through the blood–brain barrier causing the profound intellectual deficit and neurological

impairment present in Allan–Herndon–Dudley syndrome (Bernal et al. 2015).

Knowledge of the molecular basis of TH action on brain development remains fragmentary. In rodents, TH deficiency interferes with cerebral and cerebellar cortex development in part by impairing the migration and differentiation of neurons. In the neocortex, TH deprivation impairs radial and tangential migration, and the differentiation of many neuronal types, including pyramidal cells and interneurons (Berbel et al. 1985, 1996, 2001). Most actions of TH are mediated by the interaction of the active hormone T3 with nuclear receptors (TRs). The TRs (TRα1, TRβ1, and TRβ2) are ligand-modulated transcription factors encoded by the *THRA* and *THRB* genes (Brent 2012; Ortiga-Carvalho et al. 2014). *THRA* is the predominant thyroid hormone receptor (TR) gene expressed in brain. *Thra* mutations in mice cause behavioral alterations and impair the differentiation of interneurons (Venero et al. 2005; Wallis et al. 2008). In humans, some forms of thyroid hormone resistance due to *THRA* mutations are associated with intellectual deficit (Bochukova et al. 2012).

Through the binding to the TRs, T3 modulates gene expression, and the precise identification of the gene network under control of T3 in neural cells is needed to define the role of thyroid hormones in development. Efforts in this direction have relied on in vivo and in vitro approaches. The in vivo approaches face the difficulty that the results obtained using paradigms of TH deficiency or administration are variable as a function of the brain region and the age taken for analysis making it extremely difficult to obtain a global view. For example, *Reln*, *Nrgn*, or the myelin genes are under TH regulation only during narrow windows of development, or in specific regions of the brain (Rodriguez-Pena et al. 1993; Iniguez et al. 1996; Alvarez-Dolado et al. 1999). Another difficulty is to distinguish the T3 primary transcriptional responses, from secondary and more distal responses, which may be consequences of nonspecific effects of hormonal deprivations. Transformed cell lines are useful to dissect molecular mechanisms but do not reflect the physiological situation. All these difficulties have been well summarized in a recent review (Chatonnet et al. 2015).

In this work, we have used primary mouse neocortical cells as more representative of in vivo situations than established cell lines. Primary cerebrocortical cells were used in the past to study the metabolism and action of thyroid hormones (Kolodny et al. 1985; Leonard and Larsen 1985; Lorenzo et al. 1995). Through immunofluorescence and RNA-Seq-based transcriptomic analysis, we provide evidence that the primary cells are very heterogeneous, maintaining a high phenotypic diversity in the culture. This property can be exploited by using available databases of specific cell-type transcriptomes, to dissect the actions of T3 on gene expression in discrete cellular populations. Results of transcriptomic analyses in the presence and absence of T3 show that the action of T3 depends on the expression of its nuclear receptors and that most neural cell types are transcriptionally responsive to T3. In addition, about one-third of the genes whose expression is changed by T3 are regulated by the hormone directly at the transcriptional level. T3 mostly upregulates genes involved in signaling events at the plasma membrane and downregulates genes encoding proteins involved in nuclear events related to cell division. Finally, the transcriptomic changes induced by T3 partially overlap with those taking place in the transition from the embryonic to the adult cerebral cortex. Overall, our data provide a novel and comprehensive view on the function and mechanisms of action of TH on neocortex development and open many new avenues for further investigation.

Materials and Methods

An extended description of the methodology is in Supplementary Materials and Methods.

Primary Cerebrocortical Cell Cultures

Protocols for animal handling were approved by the local institutional Animal Care Committee, according to European Union rules. Mice of a hybrid genetic background of 129/OLa^{+}129/Sv^{+} BALB/c^{+}C57BL/6 were used (Gil-Ibanez et al. 2013). Primary cerebrocortical cultures were established from the whole neocortex of 6 individual E17.5 fetuses. Each fetus originated 2 identical cell culture replicas, one for T3 treatment and another as control. The cultures were established in poly-l-ornithine-coated 12-well plates (Sigma) and incubated for 9 days, with periodic medium changes, in NB medium (Neurobasal Medium [Gibco® Life Technologies] containing 2% B27 supplement [Gibco®], supplemented with glutamine and antibiotics). B27 was removed from the NB medium 24 h before adding T3 (Sigma) at a final concentration of 10 nm. The T3 solution contained a small amount of TH-deprived serum for stabilization, and the final concentration of serum in the culture medium was 0.1%. Cells were harvested 24 h later. For the experiments involving cycloheximide (CHX, Sigma), CHX was added to the cells at a final concentration of 8 µg/mL 30 min before adding T3 (10 nm), and the cells harvested 6 h after T3 addition.

Immunofluorescence

Immunofluorescence was performed on cells plated on glass coverslips fixed with absolute acetone and permeabilized with 0.5% Triton X-100. The primary antibodies were mouse monoclonal antiglial fibrillary acidic protein (GFAP) (Clone G-A-5, 1/500 dilution, Sigma), rabbit polyclonal antineuronal-specific nuclear protein (NeuN, 1/500 dilution, Millipore), mouse monoclonal anticalbindin (D28K 1/500 dilution, Sigma), mouse monoclonal antireelin (clone G10 1/500 dilution, Millipore), and rabbit polyclonal anticholecystokinin (CCK-8, 1/100 dilution, Immunostar). The secondary antibodies were donkey antimouse Alexa 488 (green), goat antimouse Alexa 488 (green), and donkey antirabbit Alexa 555 (red) used at 1/500 dilution. Nuclear staining was with 4′,6-diamidino-2-phenylindole (DAPI) (Gibco® Life Technologies). Confocal images, acquired using an inverted Zeiss LSM 710 laser scanning microscope with a plan-apochromatic objective 63×/N. A 1.3, were processed with Zen 2009 software and Adobe Photoshop. The relative abundance of different cell types as a percentage of DAPI-stained nuclei was estimated after counting 100–200 cells in sextuplicate for each culture in photographs taken using a ×40 objective.

Transcriptomics

RNA-Seq was performed at the Genomics Unit of the Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid. To construct index-tagged cDNA libraries, 500 ng of total RNA was used with the TruSeq RNA Sample Preparation v2 Kit (Illumina). Single reads of 75 base length using the TruSeq SBS Kit v5 (Illumina) were generated on the Genome Analyzer IIx following the standard RNA-sequencing protocol. Reads were further processed using the CASAVA package (Illumina).

The fasta file containing sequences of this genome was downloaded from Ensembl (http://www.ensembl.org/Mus_musculus/Info/Index). This genome was indexed from Bowtie (Langmead et al. 2009), and sequence reads were aligned using TopHat

(Trapnell et al. 2009). We quantified reads to specific genes and transcripts using the Python module HT-SEQ (Anders et al. 2015). Read and mapping data quality was determined with the application FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Qualimap (Garcia-Alcalde et al. 2012). We explored gene expression data by Principal Component Analysis and Clustering methods. Exploratory analysis was performed using the Bioconductor package NOISeq (Tarazona et al. 2011) (Supplementary Materials and Methods, RNA-Seq quality controls). RNA-Seq data were normalized using Trimmed Mean of M values (Robinson and Oshlack 2010). The paired design was analyzed from the Bioconductor package edge R (Robinson et al. 2010), fitting a Negative Binomial Generalized Linear Model. Conventional multiple testing *P*-value correction procedure proposed by Benjamini–Hochberg was used to derive adjusted *P*-values (Benjamini and Hochberg 1995). Length of genes and transcripts was estimated from only coding regions. Expression levels were estimated using the RPKM method (reads per kilobase of transcript per million mapped reads). The RNA-Seq data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE68949 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68949).

Enrichment analysis was carried out for the gene ontology (GO) terms using the Bioconductor package GOSeq (Young et al. 2010). Gene ontology enrichment was estimated compared with the genes expressed in the presently cultured cells. We corrected for multiple testing by Benjamini–Hochberg procedure. Significant GO terms were represented from CellMaps (http://cellmaps.babelomics.org/).

Quantitative PCR assays were performed using standard procedures (Supplementary Materials and Methods) on TaqMan low-density arrays (Applied Biosystems) format 48a (P/N 4342253). Data were corrected for 18S RNA and expressed relative to the values obtained for the control cells without T3.

Results

Cellular and Molecular Characterization of the Primary Cultures

The purpose of this study was to obtain a global view on the role of thyroid hormone on neocortex development, through identification of the regulated genes. This was done by transcriptomic analysis of T3 action in primary cultures of cerebrocortical cells. Primary cell cultures were established from E17.5 whole mouse cortices. Characterization of the primary cultures with specific antibodies revealed the presence of astrocytes (15%) and neurons (75%) and specific neuronal phenotypes as shown by immunofluorescence for cholecystokinin and for 2 known T3-dependent proteins, calbindin and reelin (Supplementary Fig. 1), expressed by <5–10% of the neurons. Phenotypic diversity was further confirmed by the RNA-Seq data, which showed expression of markers for different cerebral cortex layers and neuronal types.

RNA-Seq data analysis showed that 14 801 genes were expressed in the culture. From this set, we identified the genes encoding proteins involved in T3 action in the brain, such as membrane transporters, deiodinases, and nuclear receptors and coregulators (Supplementary Fig. 1). The major TH transporter expressed was *Slc16a2* (Mct8), followed by *Slco3a1* (Oatp3a1), *Slc7a5* (Lat1), *Slco1c1* (Oatp1c1), and *Slc7a8* (Lat2). Very low expression levels were found for *Slc16a10* (Mct10), a transporter expressed in microglia, and *Slco1a4* (Oatp1a4), highly expressed in endothelial cells (Bernal et al. 2015). As for the receptors, the mRNAs for the T3-binding products of the *Thra* and *Thrb* genes (*Thra1* and *Thrb*) were expressed in similar amounts, whereas the non-T3-binding splicing product of the *Thra* gene, *Thra2* mRNA, was present at a concentration 30-fold higher than *Thra1* or *Thrb*. Finally, the deiodinases *Dio2* and *Dio3* and the coregulators *Ncor1* corepressor and *Ncoa1*/*Src1* coactivator mRNAs were also present. Accordingly, the primary cells were responsive to T3, as shown by the 2–6-fold increased expression after T3 of the known T3 targets *Camk4*, *Dio3*, and *Hr* (Supplementary Fig. 1).

Transcriptomic Changes Induced by T3

After incubation with 10 nm T3 for 24 h, the number of differentially expressed (DE) genes in comparison with cells in the absence of T3 was 1145 (false discovery rate <0.05) (Fig. 1A and Supplementary Table 1). From these, 619 were upregulated, and 526 downregulated by T3. We refer to these genes as positive and negative genes, respectively. Among the positive genes with the most significant changes after T3 treatment, we identified genes well known to be regulated by T3 in vivo (Chatonnet et al. 2015), such as *Hr*, *Shh*, *Kcnj10*, *Flywch2*, *Sema3c*, *Klf9*, *Rasd2* and *Dio3*. Within the list of negative genes, 2 known T3 downregulated genes, *Calb1* and *Aldh1a3*, were present. Validation of gene expression changes was performed by qPCR in biological replicates using RNA from independent cultures. Figure 1B shows the relative expression of 24 positive genes and 6 negative genes in the T3-treated cells compared with the untreated cells. There was a good correlation between the results obtained by RNA-Seq and qPCR (Fig. 1C).

Direct Responses to T3

To prove that the T3-induced transcriptomic changes required the presence of the TRs, we performed RNA-Seq of cerebrocortical cells isolated from *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice (Gil-Ibanez et al. 2013) similarly treated or untreated with T3. No significant changes of gene expression after T3 were found (Supplementary Table 1), indicating that the transcriptomic changes induced by T3 in the wild-type cells were mediated exclusively through interaction with the nuclear receptors.

Then, we proceeded to the identification of the DE genes that were direct transcriptional responses to T3, i.e., not mediated by changes in the expression or activity of other proteins that might influence the transcription or the half-life of the target mRNAs. To this end, we used the protein translation inhibitor CHX to identify what genes within the 1145 DE genes at 24 h were still induced or repressed 6 h after T3 addition in the presence of CHX. We previously used this approach successfully to determine that *Hr*, *Klf9*, *Shh* and *Aldh1a3* were direct transcriptional responses to T3 in similar primary cultures (Gil-Ibanez et al. 2014). RNA-Seq was performed to compare cells treated with T3 for 6 h in the absence of CHX, with cells treated with T3 in the presence of CHX, and compared with the effect of T3 at 24 h.

As shown in Figure 2A, from the set of 1145 genes regulated by T3 at 24 h (619 positive and 526 negative), 562 responded to T3 at 6 h (336 positive and 226 negative). From this set, 371 genes responded to T3 in the presence of CHX (254 positive and 117 negative; Supplementary Tables 1 and 2). As shown in Supplementary Table 2, the genes regulated directly by T3 encode proteins that can be classified into diverse functional groups, especially transcription factors, G proteins, cell-adhesion and extracellular matrix proteins, and membrane transporters. We crossed our data with the chromatin immunoprecipitation data of Chatonnet et al. (2013) and found that a fraction of this set, 106 genes (89 positive and 17 negative

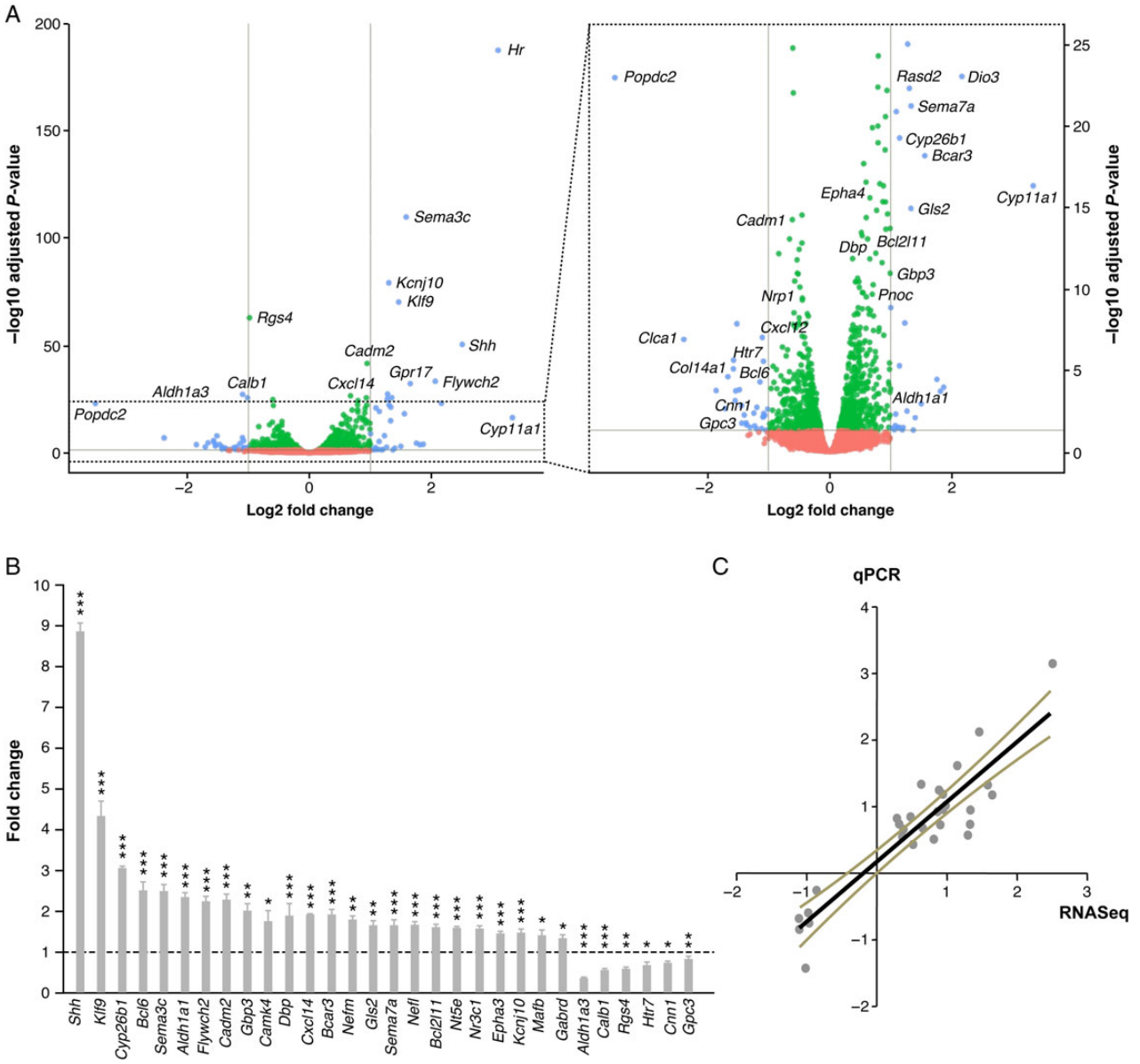

Figure 1. (*A*) Volcano plot of RNA-Seq of cells treated with T3 for 24 h and untreated cells. Scattered points represent individual genes. The X-axis is the $\log_2$ of the fold change between T3 and untreated cells. The Y-axis is the negative $\log_{10}$ of adjusted *P*-value (P_{adjust}). Given the wide values for the P_{adjust}-value, the right panel is a blow up of the region below 25 ($-\log_{10}P_{adjust}$). Green and blue ($\log_2$ of the fold change >1) dots represent genes with significant changes in expression ($P_{adjust} < 0.05$). Red dots represent nonsignificant changes ($P_{adjust} > 0.05$). The position of selected genes in the plot is illustrated with the gene symbols. (*B*) qRT-PCR validation of RNA-Seq results in a biological replicate. Results are expressed as mean ± SEM relative to the control, assigned an arbitrary value of 1.0, and represented by the horizontal dotted line. The number of individual samples in all determinations was 5. Statistical significance was calculated by the Student's *t* test. *$P < 0.05$; *$P < 0.01$; *$P < 0.001$. (*C*) Biological and technical correlation of the data of gene expression changes for 30 individual genes. In the abscissa, the data on RNA-Seq measurements of gene expression in T3-treated cultures over untreated cultures expressed as the Log_2 of fold changes. In the ordinate, the qRT-PCR data obtained for gene expression of the same genes in different cultures similarly treated. 18S RNA was used for normalization. The $\log_2$ of fold changes showed a positive correlation between the RNA-Seq and the RT–PCR (Pearson $r = 0.908$, $P < 0.0001$).

genes), contain proximal TR-binding sites (TR binding sites located within 30 Kb of a T3-regulated gene). Among the directly regulated genes were *Hr*, *Dbp*, *Klf9*, and *Gbp3* (distal binding site) considered by Chatonnet et al. (2013) as the genes with the most conclusive evidence for direct T3 regulation.

Within the negative genes, we found *Mc4r*, encoding the type 4 melanocortin receptor, also demonstrated as transcriptionally regulated by T3 through a negative T3 responsive element (Decherf et al. 2010). Individual responses of the 562 genes responding to T3 at 6 and 24 h were used to construct a heatmap plot (Fig. 2C) revealing different patterns of response. The most significant are illustrated in Figure 2D with individual examples. In the set of positive genes, the response to T3 at 6 h could be lower (*Flywch2*, *Sema6c*, and *Olfm4*), equal (*Klf9* and *Slc22a3*), or higher (*Bcl2l11*) than at 24 h, defining different time courses of response. In the presence of CHX, T3 had the same (*Flywch2*) or higher effect (*Klf9*, *Sema6c*, and *Bcl2l11*) than in its absence, or no effect (*Olfm4* and *Slc22a3*). This means that *Flywch2*, *Klf9*, *Bcl2l11*, and *Sema6c* were direct responses to T3 and that *Klf9*, *Bcl2l11*, and *Sema6c* mRNAs were in addition stabilized by CHX.

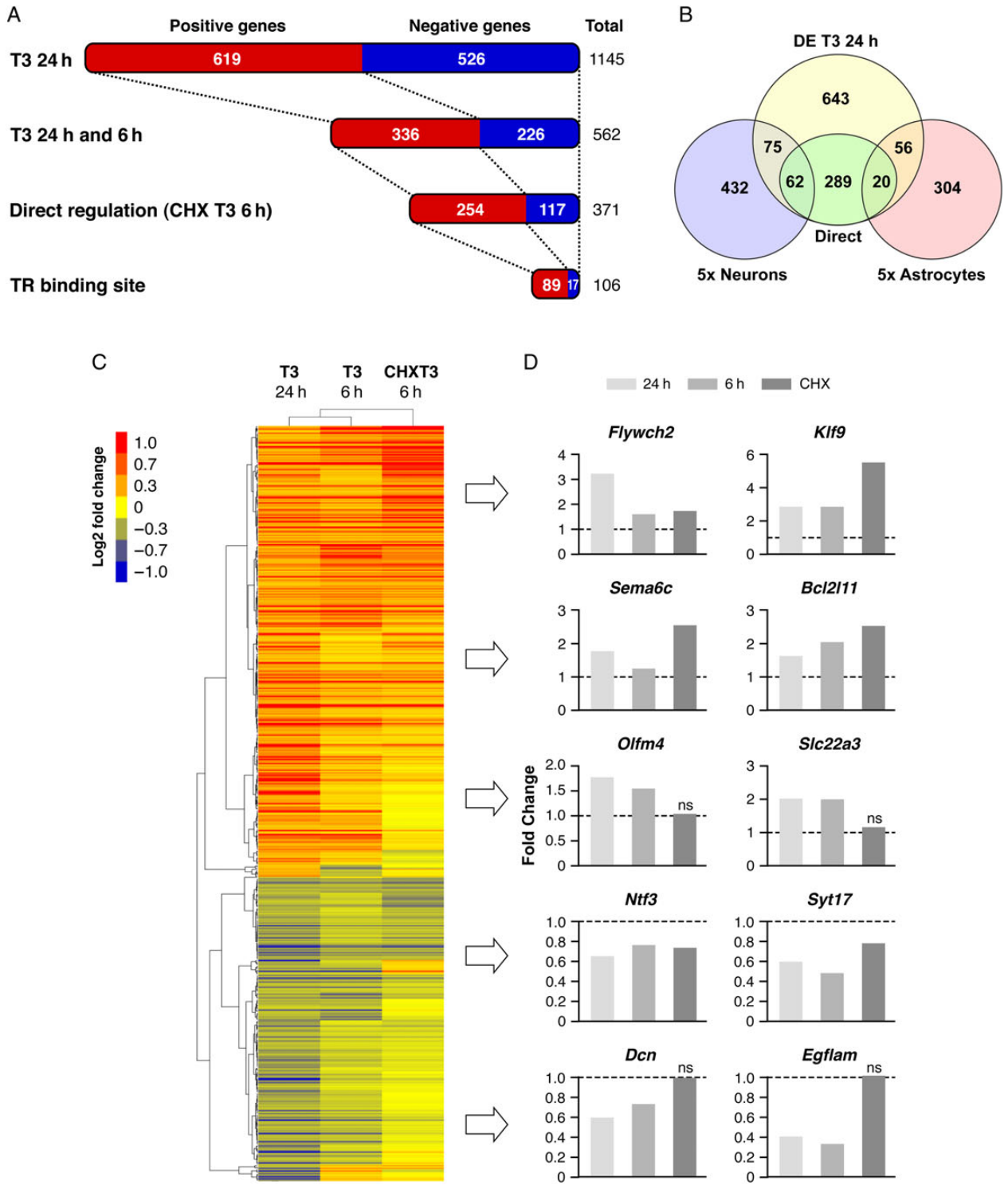

Figure 2. (*A*) From up to down: Number of upregulated (positive) and downregulated (negative) genes after addition of T3 (10 nm) to primary cerebrocortical cultures after 24. Number of genes up- or downregulated at 24 and 6 h after T3. Directly regulated genes are genes with increased or decreased expression 6 h after T3 in the presence of cycloheximide (CHX). Number of genes containing a TR binding site, as reported by Chatonnet et al. (2013). (*B*) Venn diagram representing the overlap between DE genes at 24 h, the directly regulated genes, and the 5×-enriched genes in neurons and in astrocytes expressed in the culture. (*C*) Heatmap representing changes of gene expression induced by T3 at 24, 6 and 6 h in the presence of CHX. (*D*) Examples of different gene expression patterns: Fold change after 24 h (light gray), 6 h (dark gray), and 6 h in the presence of CHX (dark). Differences were statistically significant ($P < 0.05$) except when indicated (ns = not significant).

Olfm4 and *Slc22a3* were secondary responses. For the negative genes, the effect at 6 h could be lower (*Ntf3* and *Dcn*) equal (*Egflam*) or higher (*Syt17*) than at 24 h. In the presence of CHX, T3 at 6 h had the same (*Ntf3*), or lower effect (*Syt17*) than in its absence, or no effect at all (*Egflam* and *Dcn*). These patterns can be interpreted as explained for the positive genes.

Table 1 T3 regulation of cell-type and layer-enriched genes in mouse primary cerebrocortical cells

Cell type	Cell-type-enriched genes	Expressed genes in the culture	T3-regulated genes at 24 h (number)	T3-regulated genes (% of expressed genes)	Directly regulated genes (number)	Directly regulated genes (% of expressed genes)
Mixed culture		14.801	1.145	7.7	371	2.5
Neurons	719	569	137	24.2	62	10.8
Astrocytes	480	380	76	20.0	20	5.2
Corticothalamic Layer 6	594	333	43	1.9	15	4.5
Corticospinal Layer 5b	1177	662	59	8.9	18	2.7
Corticostriatal Layer 5a	3695	1823	168	9.2	54	3.0
CCK neurons	571	240	17	7.1	5	2.1
Cortistatin neurons	482	232	30	12.9	10	4.3
Pnoc neurons	114	58	14	24.1	7	12.1
Subplate	418	394	82	20.8	35	8.8

Note: Data on cell-type-enriched genes in astrocytes and neurons were from Cahoy et al. (2008). Genes enriched in Layers 6, 5b, and 5a, and the CCK, cortistatin, and pnoc neurons were from Doyle et al. (2008). The subplate data were from Hoerder-Suabedissen et al. (2013).

Transcriptomic Changes Induced by T3 in Specific Cell Types

The primary cultures contained a heterogeneous diversity of cellular phenotypes. We took advantage of this property of the culture to identify the cell types expressing genes directly responsive to T3. First, we analyzed the relative contributions of astrocytes and neurons to the transcriptomic T3 response. To this end, we compared our dataset with a transcriptome database of acutely purified astrocytes and neurons of postnatal mice (Cahoy et al. 2008) to determine to what extent genes of enriched expression in astrocytes or neurons were regulated by T3 (Fig. 2*B*). Setting a lower limit of 5-fold expression in astrocytes or in neurons to consider a gene enriched in a given cell type, a total of 137 T3-regulated genes were enriched in neurons and 76 in astrocytes (Table 1). Even some neuron- or astrocyte-specific genes, that is, those with >40-fold enrichment, were regulated by T3 (Supplementary Table 3). Of the 137 neuron-enriched genes, 62 were regulated directly by T3, and of the 76 astrocyte-enriched genes, 20 were directly regulated (Fig. 2*B* and Table 1). The results indicate that neurons and astrocytes are direct cellular targets of T3 at the genomic level. Astrocytes were proposed as mediators of many effects of TH on brain structure and function through the control of cytoskeletal proteins, growth factors, and cell-adhesion molecules (Dezonne et al. 2015). Up to 23 genes encoding extracellular matrix proteins and adhesion molecules enriched in astrocytes were regulated by T3 in our cultures. Among the genes previously proposed as T3 targets, we found the heparan sulfate proteoglycans *Gpc6*, directly regulated, and *Sdc4*, indirectly regulated.

To identify specific neuronal types as T3 targets, we followed a similar approach. We compared our dataset with a transcriptome database of cerebral cortex cell types isolated using the translating ribosome affinity purification approach (Doyle et al. 2008). This study provides the translational profiles of many cell types of the CNS and is an excellent resource to identify cell-enriched mRNAs. Table 1 shows the number of genes enriched in neurons from different cortex layers or expressing specific markers, their representation in the culture, and the number of T3 regulated genes. We found that ~50% of genes enriched in specific neurons were expressed in the culture. From these, between 7.1% (for cholecystokinin, CCK neurons) and 24% (for prepronociceptin, PNOC neurons) were regulated by T3. As for the directly regulated genes, the proportion ranged between 2.1% of the enriched genes in the CCK neurons and 12.1% in PNOC neurons, identifying this population of cells as a major cellular target of TH.

Subplate neurons have a crucial role in the maturation of cortical intrinsic and extrinsic circuits (Hoerder-Suabedissen and Molnar 2015). As shown also in Table 1, a high proportion (94%) of subplate-enriched genes (Hoerder-Suabedissen et al. 2013) was expressed in the culture, and 20% of them (82 genes) was regulated by T3. From these, 35 were regulated directly. Furthermore, 68 subplate genes have been described as being subplate-specific at any one time (Hoerder-Suabedissen et al. 2013). As shown in Supplementary Table 4, 23 of the subplate-specific genes were under T3 regulation, 8 of them in a direct fashion. Remarkably, T3 negatively regulates genes of early embryonic expression and positively regulates genes of postnatal expression.

Gene Ontology Analysis

Gene ontology enrichment analysis was performed in 2 ways. On the one hand, we included in a single set the 1145 DE genes. In addition, we separated the upregulated and the downregulated genes in 2 sets for independent analysis. All the significant GO terms (with $P_{adjust} < 0.05$) and genes included in the analysis are detailed in Supplementary Table 5 and Supplementary Figure 2. From the set combining all the DE genes, the functions represented included response to stimulus and signal transduction, especially processes related to G-protein-coupled receptor activity, regulation of nervous system development, cell communication, and axon guidance. In addition, Ca^{2+} signaling pathways were also highly represented.

Some of the enriched GO terms were specifically represented in one of the sets of positively or negatively regulated genes. For better visualization of the data, the most representative terms for Molecular Function, Biological Processes, and Cellular Component domains are summarized in Figure 3A for the upregulated and Figure 3B for the downregulated genes. T3 specifically upregulates genes involved in the transmission of the nerve impulse, processes involving ion transport (i.e., anion–cation symporter activity, sodium ion transmembrane transport), ephrin receptor activity, cell adhesion, and chemotaxis (Supplementary Table 5). Among the genes upregulated by T3 are also genes involved in myelin assembly and in protein localization at the paranodal region. The genes negatively regulated by T3 are specifically enriched in cell division, particularly in chromosome segregation and organization during the M Phase of cell cycle. Regulation of chemokine-mediated signaling pathway is also highly represented. Genes involved in neurogenesis and in neuron

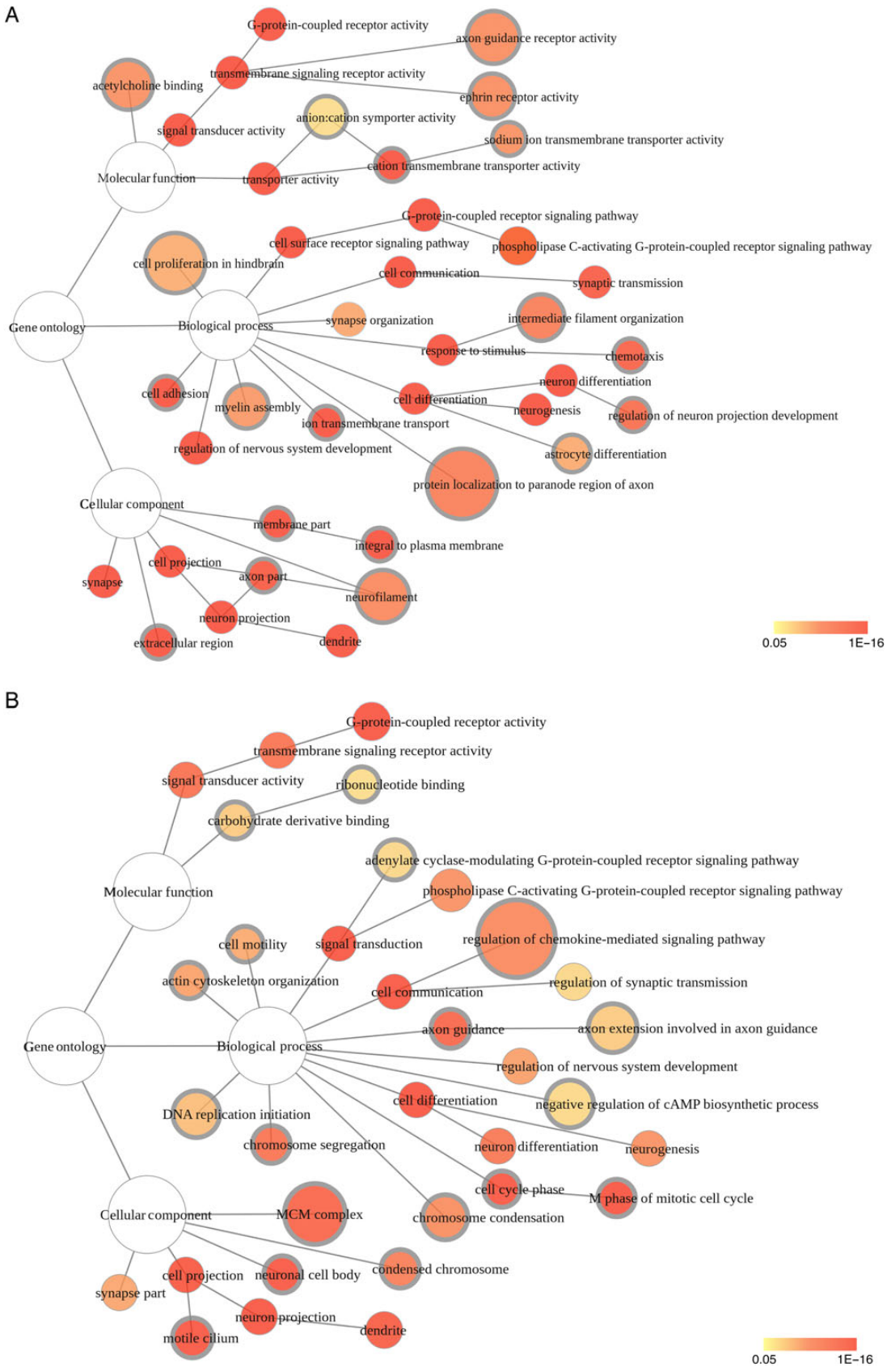

Figure 3. Selected GO categories significantly over-represented ($P < 0.05$) for the positive (*A*) or the negative genes (*B*). The color gradient, from yellow to red, represents the degree of significance of the categories by hypergeometric test with Benjamini–Hochberg FDR correction. The circle size represents the level of enrichment of the categories. Specific GO terms significantly represented either in the set of upregulated genes or in the set of the downregulated genes are marked with a thick border.

Table 2 Gene ontology molecular function categories enriched in the set of genes directly regulated by T3

GO	Description	Adjusted_pvalue	Number of genes DE in GO	Number of genes in GO	Perc_enrichement
GO:0004872	Receptor activity	1.16E – 09	50	532	0.09398
GO:0004888	Transmembrane signaling receptor activity	4.68E – 09	41	380	0.10789
GO:0038023	Signaling receptor activity	5.82E – 09	44	444	0.09910
GO:0004871	Signal transducer activity	2.10E – 08	52	638	0.08150
GO:0060089	Molecular transducer activity	2.10E – 08	52	638	0.08150
GO:0004930	G-protein-coupled receptor activity	0.00051	21	203	0.10345
GO:0005102	Receptor binding	0.00136	43	794	0.05416
GO:0005003	Ephrin receptor activity	0.00615	5	13	0.38462
GO:0005515	Protein binding	0.00778	167	4940	0.03381
GO:0005488	Binding	0.00841	254	8275	0.03069
GO:0004714	Transmembrane receptor protein tyrosine kinase activity	0.02102	8	48	0.16667
GO:0042562	Hormone binding	0.02818	7	49	0.14286
GO:0005125	Cytokine activity	0.03492	7	60	0.11667
GO:0005005	Transmembrane-ephrin receptor activity	0.04750	3	5	0.60000
GO:0005230	Extracellular ligand-gated ion channel activity	0.04895	7	46	0.15217

Note: This table shows: the GO term and description, the adjusted *P*-value, the number (*N*) of genes DE contained in each of the GO definitions, the total number of genes present in the GO definitions, and the percent enrichment of DE within each definition.

differentiation are represented in both gene sets, but astrocyte differentiation is specifically induced by T3.

Within the Cellular Component domain, T3 induces mainly transmembrane and axonal proteins, neurofilaments, and extracellular proteins and specifically downregulates nuclear proteins related to the condensed chromosome and the MCM complex, and genes encoding proteins of the motile cilium.

It was also of interest to perform GO analysis of the genes directly responsive to T3 at 6 h in the presence of CHX. The full data are given in Supplementary Table 6, and a summary with the most enriched GO terms within the Molecular Function domain is in Table 2. In general, the terms enriched were similar to those obtained when the 24-h gene set was analyzed, with the top definitions represented by transmembrane signal transduction processes, especially those involving G proteins. Molecular function terms involving ephrin signaling were also significantly represented.

Comparison with In Vivo Studies

To extrapolate the identified cellular T3 target genes to in vivo regulation, we compared the DE genes in the primary cells with previous datasets from rat and mouse cerebral cortex hypothyroidism during the fetal (Morte, Diez et al. 2010; Dong et al. 2015) and postnatal periods (Morte, Ceballos et al. 2010). From these comparisons, shown in Supplementary Table 7, we can conclude that ~20% of the brain genes altered in expression by fetal and postnatal hypothyroidism in vivo are real cellular targets of T3 and not altered as a distal effect of hypothyroidism. The overlapping list of 329 T3-regulated genes represents a valuable dataset to break through the genomic targets of TH involved in brain development.

T3 Favors an Adult versus Embryonic Profile of Gene Expression

The transition between the embryonic and adult brain involves substantial changes in the expression of genes related to developmental processes. Dillman et al. (2013) performed comparisons of transcriptome profiles between these 2 stages and defined a set of 1185 genes highly expressed (5-fold or greater) in the embryonic versus the adult cerebral cortex and another set of 2943 genes enriched in the adult compared with the embryonic cortex. Given the importance of TH in brain maturation, it was of interest to analyze whether T3 was involved in the relative expression of these 2 gene sets in the primary cultures. For this reason, we analyzed the overlap between our gene expression dataset and the over-represented embryonic and adult transcriptomes. From the total of 14 801 genes expressed in the primary cells, approximately half of the 5× embryonic (649 genes) and of the adult gene sets (1563 genes) were present (Table 3). Sixteen percent (107 of 649) of the genes enriched in the embryonic cortex were negatively regulated by T3, in contrast to only 4% (67 of 1563) of the adult cortex-enriched genes. Conversely, T3 positively regulated 12% (196 of 1563) of the adult cortex genes versus 3% (22 of 649) of the embryonic genes.

Discussion

Efforts to identify the gene network regulated by T3 during brain development have given only fragmentary information, highly depending on the region of the brain, developmental stage, and compounding factors of in vivo studies. We here have employed RNA-Seq to analyze the transcriptomic changes induced by T3 in mouse primary cerebrocortical cells in culture. These cells are a very heterogeneous mix of cells with an extraordinary diversity of cell phenotypes, as confirmed by immunofluorescence and by the outcome of the transcriptomic analysis. We believe that these cultures allow obtaining a global view of T3 action on the different cell types of the neocortex. The T3-dependent patterns of expression may then be extrapolated to in vivo development to define the "potentiality" of T3 action on neocortex development, at least at the cellular level (Gil-Ibanez et al. 2014).

Not surprisingly T3 had a large effect on the transcriptome of the primary cells. The expression of 1145 genes (7.7% of expressed genes) was modified 24 h after exposure to T3. The response to T3 was mediated through the TRs, as no effect was found in cells from TR null mice. Furthermore, 32% of these genes were directly regulated by the hormone at the transcriptional level. The full list of

Table 3 Regulation by T3 of genes enriched in the embryonic and adult cerebral cortex

Cortex	Number of 5×-enriched genes	Number of 5×-enriched genes expressed in the culture	Number of 5×-enriched genes regulated by T3		Percent of T3-regulated genes	
Embryonic	1185	649	Up:	22	Up:	3
			Down:	107	Down:	16
Adult	2943	1563	Up:	196	Up:	12
			Down:	67	Down:	4

Note: Genes enriched at least 5× in the embryonic mouse cerebral cortex with respect to the adult cerebral cortex, and vice versa were taken from Dillman et al. (2013).

regulated genes presented in the supplemental tables has a great and powerful heuristic value to identify the molecular basis of TH action on developmental processes known to be affected by these hormones and to discover new processes so far unsuspected as being under the influence of thyroid hormones (Berbel et al. 2014). Some of the T3-regulated pathways profoundly influence developmental processes, for example the sonic hedgehog pathway. Among the genes with a more robust transcriptional induction by T3 was *Shh*, a gene previously known to be regulated by T3 in the mouse brain in vivo and in cortical cells (Desouza et al. 2011). Regulation of this gene by T3 likely influences many biochemical processes in the developing and adult brain (Ho and Scott 2002; Alvarez-Buylla and Ihrie 2014). A challenge for future work is the clarification of the specific role of T3 on developmental processes acting through *Shh* expression, beyond the local regulation of deiodinase activity (Dentice 2011).

The GO analysis led to the surprising conclusion that T3 preferentially upregulates genes involved in signaling at the plasma membrane and downregulates genes encoding nuclear proteins associated with cell division. Cerebellar but not cerebral hemispheres astrocytes are stimulated to proliferate by T3. But this is shown as a delayed and indirect effect due to stimulation of secretion of astrocytic growth factors (Trentin et al. 1995). Most actions of T3 are related to migration or differentiation. A crucial process of cortical development is the migration of postmitotic neurons from the proliferative zones to form the cortex layers (Rakic 1990). Among the most relevant actions of T3 on the developing neocortex is the control of cell migration. Transient maternal hypothyroxinemia at onset of corticogenesis alters radial and tangential migration of neurons (Lavado-Autric et al. 2003; Auso et al. 2004; Cuevas et al. 2005). Given the age at which the cultures were established, the effects of T3 on genes involved in migration will likely affect the neuronal migration to Layers 2/3, especially radial distribution of callosal projecting neurons and subsequently their connectional pattern (Lucio et al. 1997). In agreement with these in vivo effects of thyroid hormones, up to 17 cell-adhesion molecules and 25 extracellular matrix proteins were regulated directly by T3 in the primary cultures. Many of the DE genes are involved in the tangential migration of GABAergic interneurons from the medial ganglionic eminence. These include genes encoding transcription factors implicated in interneuron development (Mafb, Etv1, Npas1); receptors regulating migration such as ErbB4, and Cxcr7 and its ligand Cxcl12, chemorepulsive molecules (Slit2, Slit3, Robo1, Robo2, EphrinA5, and EphA4), as well as Sema3a and its receptor Nrp1 that are important to maintain the interneuron migrating route. Many of the encoding genes are under direct transcriptional control by T3, such as *Mafb* and *Robo2*, *Etv1*, *Slit2*, *Sema3a*, and *Nrp1*.

The inside–out pattern of radial migration during corticogenesis is critically controlled by the extracellular matrix protein reelin produced by the Cajal–Retzius neurons (Rice and Curran 2001). In rats, hypothyroidism alters the expression of *Reln* transiently during the perinatal period (Alvarez-Dolado et al. 1999). Here, we find that *Reln* was not a primary, but a secondary response to T3. Transcription factors regulating the *Reln* promoter, *Sp1*, *Pax6*, and *Tbr1* (Grayson et al. 2006), were not regulated by T3 in our study. However, the transcription factor *Emx1*, important for the production of Cajal–Retzius cells and subplate neurons (Shinozaki et al. 2002), was regulated directly by T3. Therefore, T3 might control the expression of the *Reln* gene by facilitating the generation or differentiation of the Cajal–Retzius cells.

Related to the above-mentioned discussion, we found that a large fraction of subplate-enriched and specific genes were regulated by T3, and many of them directly at the transcriptional level. We believe that this is an important finding, given the role of the subplate in the organization of intracortical and thalamocortical circuitry, and in tangential migration of interneurons (Hoerder-Suabedissen and Molnar 2015). A direct action of T3 on subplate maturation may underlie the effects of hypothyroidism on disruption of cortical circuitry and the formation of cortical maps and could be of relevance in the etiology of autism (Berbel et al. 2014). Actually the subplate-specific genes *Cdh18*, *Gabra5*, and *Prss12* and the subplate-enriched genes *Sema5a* and *Cdh10*, all regulated by T3, have been linked to autism (Hoerder-Suabedissen et al. 2013). *Slc1a2*, which contains a TR-binding site (Chatonnet et al. 2013), has been linked to schizophrenia (Hoerder-Suabedissen et al. 2013).

About two-thirds of the DE genes were not direct transcriptional responses to T3. Regulation of these genes might be secondary to a primary effect of T3 on the expression of transcription factors or components of signaling cascades. Among the DE genes, we found 101 transcription factors or transcriptional cofactors and coregulators, of which 48 were directly regulated by T3. Within this category, it is worth mentioning *Klf9*, a transcription factor already known to be under TH control in many cell types (Hoopfer et al. 2002; Martel et al. 2002; Denver and Williamson 2009; Scobie et al. 2009; Avci et al. 2012; Dugas et al. 2012; Dong et al. 2015). Other transcription factors are novel transcriptional targets of T3 and mediate an array of important actions on neural cells, including survival (*Tox3* and *Klf6*) (Dittmer et al. 2011; Salma and McDermott 2012), neuronal differentiation and specification (*Satb, Bhlhb5*, *Nr4a3*, *Zbtb20* and *Emx1*) (Britanova et al. 2008; Joshi et al. 2008; Cocas et al. 2009; Xie et al. 2010; Eells et al. 2012), progenitor cell division (*Mycn* and *Emx1*), and maintenance (*Zhx2*). *Emx1* is important for Cajal–Retzius cells and the subplate as mentioned above, and *Mycn* is involved in differentiation of radial glial precursors (Zinin et al. 2014). The circadian oscillator Dbp is known as a T3-dependent transcription factor in several animal models (Chatonnet et al. 2015). In this study, we also obtained *Dbp* as a DE, directly regulated gene. Interestingly, another transcription factor, Nfil3, directly regulated by T3, cooperates with Dbp in determining the length of the circadian oscillator (Yamajuku et al. 2011).

The primary culture dataset allows drawing conclusions on T3 action in specific cellular types by comparing the data with transcriptomic databases of different cell types (Cahoy et al. 2008; Doyle et al. 2008). We found that T3 exerts direct transcriptional

regulation in astrocytes and in neurons. In astrocytes, we identify direct targets among genes involved in neuron migration and differentiation, such as the heparan sulfate proteoglycan gene *Gpc6*. Remarkably, all the neuronal types analyzed are direct transcriptional targets of T3, in agreement with TRα1 expression in practically all neurons (Wallis et al. 2010). The relatively high number of DE genes enriched in different cell types indicates a prominent role of TH in the maintenance of most cellular phenotypes. The Pnoc neurons (Doyle et al. 2008) appear as unique T3 target cells given the relatively high number of directly regulated genes. Other known cell targets of T3, the oligodendrocytes and the Parvalbumin-expressing interneurons, were not present in the culture, given their postnatal appearance during development.

The partial overlap between the gene network involved in the transition from the embryonic to adult brain and the T3-regulated genes suggests a formulation for the biological role of thyroid hormones during development. The embryonic brain is enriched in genes included in the GO categories of cell division, M phase of cell cycle, and chromosome segregation and organization, whereas the mature nervous system is enriched in genes involved in neurotransmission and ion transport (Dillman et al. 2013). The GO categories more highly represented in the set of genes downregulated by T3 in the primary cells highly overlap with the enriched functions in the embryonic brain. Conversely, the categories more represented in the set of upregulated genes overlap with the enriched functions of the mature brain. Therefore, regulation of gene expression during development of the nervous system by TH facilitates the transition from the embryonic to the mature brain. The same conclusion can be drawn from the effects of T3 on the subplate-specific genes.

In summary, T3 regulates many signaling pathways during cerebral cortex development through the control of the expression of many different classes of genes. A large fraction of these genes are direct transcriptional targets of T3, involved in cerebral cortex development. Remarkably T3 preferentially upregulates genes involved in signaling pathways at the cell plasma membrane and downregulates genes involved in nuclear events associated with cell division. By doing so, T3 is a critical factor in processes of neuron migration, axon elongation, and synaptogenesis and promotes the transition from the embryonic to adult pattern of gene expression. In this work, we identify many genes involved in T3 action and differentiate between primary and secondary transcriptional targets of T3. The databases accompanying this paper will be a most valuable resource to address the involvement of T3 in specific signaling pathways and cellular fates and provide a starting point to understand the role of T3 in neural disorders.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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