

TITLE: 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 1,145 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 up-regulates mostly genes related to cell membrane events, such as G-protein signaling, neurotransmission, and ion transport, and down-regulates 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

Thyroid hormones

Gene regulation

Transcriptomics

Development

Subplate

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 (Bernal 2015; Berbel et al. 2014). 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 (TR α 1, TR β 1 and TR β 2) are ligand-modulated transcription factors encoded by the *THRA* and *THRB* genes (Brent 2012; Ortiga-Carvalho et al. 2014). *THRA* is the predominant 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 (Alvarez-Dolado et al. 1999; Iniguez et al. 1996; Rodriguez-Pena et al. 1993). 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 up-regulates genes involved in signaling events at the plasma membrane and down-regulates 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 supplemental Mat & Met.

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 two 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 hours 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 hours later. For the experiments involving cycloheximide (CHX, Sigma), CHX was added to the cells at a final concentration of 8 µg/ml 30 minutes before adding T3 (10 nM), and the cells harvested 6 hours 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 anti glial fibrillary acidic protein (GFAP) (Clone G-A-5, 1/500 dilution, Sigma), rabbit polyclonal anti neuronal specific nuclear protein (NeuN, 1/500 dilution, Millipore, Temecula, CA, USA), mouse monoclonal anti calbindin (D28K 1/500 dilution, Sigma), mouse monoclonal anti reelin (clone G10 1/500 dilution, Millipore), and rabbit polyclonal anti cholecystokinin (CCK-8, 1/100 dilution, Immunostar, Hudson, WI, USA). The secondary antibodies were donkey anti mouse Alexa 488 (green), goat anti mouse Alexa 488 (green), and donkey anti rabbit 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 63x/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 40x objective.

Transcriptomics

RNA-Seq was performed at the Genomics Unit of the Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid. 500 ng of total RNA were used with the TruSeq RNA Sample Preparation v2 Kit (Illumina, San Diego, CA) to construct indexed cDNA libraries. 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) (supplemental Mat & Met, 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 is 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 terms using the Bioconductor package GSeq (Young et al. 2010). GO enrichment was estimated compared to the genes expressed in the presently cultured cells. We corrected for multiple testing by Benjamini-Hochberg procedure. Significant Gene Ontology terms were represented from CellMaps (<http://cellmaps.babelomics.org/>).

Quantitative PCR assays were performed were performed using standard procedures (Supplemental Mat & Met) 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 two known T3-dependent proteins, calbindin and reelin (Supplemental fig 1), expressed by less than 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 (Supplemental 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* (Supplemental fig 1).

Transcriptomic changes induced by T3

After incubation with 10 nM T3 for 24 hours, the number of differentially expressed (DE) genes, in comparison to cells in the absence of T3 was 1,145 (false discovery rate <0.05) (Fig 1A and supplemental table 1). From these, 619 were up regulated, and 526 down regulated 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, two known T3 down-regulated genes, *Calb1* and *Aldh1a3* were present. Validation of gene expression changes was performed by qPCR in biological replicates using RNA from independent cultures. Fig 1B shows the relative expression of 24 positive genes and 6 negative genes in the T3-treated cells compared to the untreated cells. There was a good correlation between the results obtained by RNA-Seq and qPCR (Fig 1C).

Fig 1

Direct responses to T3

To proof 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 (Supplemental 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 1,145 differentially expressed genes at 24 hours were still induced or repressed 6 hours 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 hours in 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 Fig 2A, from the set of 1,145 genes regulated by T3 at 24 hours (619 positive and 526 negative), 562 responded to T3 at 6 hours (336 positive and 226 negative). From this set, 371 genes responded to T3 in the presence of CHX (254 positive and 117 negative; supplemental tables 1 and 2). As shown in supplemental 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 genes), contain proximal TR binding sites (TR binding sites located within 30Kb 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.

Fig 2

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 hours were used to construct a heatmap plot (Fig 2C) revealing different patterns of response. The most significant are illustrated in Fig 2D with individual examples. In the set of positive genes, the response to T3 at 6 hours could be lower (*Flywch2*, *Sema6c*, *Olfm4*), equal (*Klf9*, *Slc22a3*), or higher (*Bcl2l11*) than at 24 hours, defining different time-courses of response. In the presence of CHX, T3 had the same (*Flywch2*) or higher effect (*Klf9*, *Sema6c*, *Bcl2l11*) than in its absence, or no effect (*Olfm4*, *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. *Olfm4* and *Slc22a3* were secondary responses. For the negative genes, the effect at 6 h could be lower (*Ntf3*, *Dcn*) equal (*Egflam*) or higher (*Syt17*) than at 24 hours. In the presence of CHX, T3 at 6 hours had the same (*Ntf3*), or lower effect (*Syt17*) than in its absence, or no effect at all (*Egflam*, *Dcn*). These patterns can be interpreted as explained for the positive genes.

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 data set 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 2B). 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, i.e., those with more than 40-fold enrichment were regulated by T3 (Supplemental 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 2B 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 sulphate proteoglycans *Gpc6*, directly regulated, and *Sdc4*, indirectly regulated.

Table 1

To identify specific neuronal types as T3 targets we followed a similar approach. We compared our data set with a transcriptome database of cerebral cortex cell types isolated using the translating ribosome affinity purification (TRAP) 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 around 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 supplemental 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 two ways. On the one hand we included in a single set the 1,145 differentially expressed genes. In addition we separated the up-regulated and the down-regulated genes in two sets for independent analysis. All the significant GO terms (with $P_{adjust} < 0.05$) and genes included in the analysis are detailed in supplemental table 5 and supplemental figure 2. From the set combining all the differentially expressed 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.

Table 2

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 Fig 3A for the up-regulated and Fig 3B for the down-regulated genes. T3 specifically upregulates genes involved in 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 (Supplemental table 5). Among the genes up-regulated 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 differentiation are represented in both gene sets, but astrocyte differentiation is specifically induced by T3.

Fig 3

Within the Cellular Component domain, T3 induces mainly transmembrane and axonal proteins, neurofilaments, and extracellular proteins, and specifically down regulates 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 supplemental 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-hour 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 differentially expressed genes in the primary cells with previous data sets from rat and mouse cerebral cortex hypothyroidism during the fetal (Morte et al. 2010a; Dong et al. 2014) and postnatal periods (Morte et al. 2010b). From these comparisons, shown in Supplemental table 7, we can conclude that around 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 data set 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 two stages and defined a set of 1,185 genes highly expressed (fivefold or greater) in the embryonic *versus* the adult cerebral cortex and another set of 2,943 genes enriched in the adult compared to 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 two gene sets in the primary cultures. For this reason we analyzed the overlap between our gene expression data set and the over-represented embryonic and adult

transcriptomes. From the total of 14,801 genes expressed in the primary cells, approximately half of the 5X embryonic (649 genes) and of the adult gene sets (1,563 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 1,563) of the adult cortex enriched genes. Conversely, T3 positively regulated 12% (196 of 1,563) 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 1,145 genes (7.7% of expressed genes) was modified 24 hours 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 regulated genes

presented in the supplemental tables have 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 up-regulates genes involved in signaling at the plasma membrane and down-regulates genes encoding nuclear proteins associated with cell division. Cerebellar, but not cerebral hemispheres astrocytes are stimulated to proliferate by T3. But this 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 differentially expressed 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 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; Dong et al. 2014; Scobie et al. 2009; Dugas et al. 2012; Avci et al. 2012). Other transcription factors are novel transcriptional targets of T3, and mediate an array of important

actions on neural cells, including survival (*Tox3*, *Klf6*) (Dittmer et al. 2011; Salma and McDermott 2012), neuronal differentiation and specification (*Satb*, *Bhlhb5*, *Nr4a3*, *Zbtb20*, *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*, *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 F 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 data set 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 TR α 1 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 down regulated 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 up regulated 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 up regulates genes involved in signaling pathways at the cell plasma membrane, and down regulates genes involved in nuclear events associated to 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.

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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 24h (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	1,177	662	59	8.9	18	2.7
Corticostriatal Layer 5a	3,695	1,823	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

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).

Table 2: Gene Ontology Molecular Function categories enriched in the set of genes directly regulated by T3

GO	description	adjusted_pvalue	N genes DE in GO	N 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

This table shows: the Gene Ontology (GO) term and description; the adjusted *P* value; the number (N) of genes differentially expressed (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.

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

Cortex	Number of 5X-enriched genes	Number of 5X-enriched genes expressed in the culture	Number of 5X-enriched genes regulated by T3	Percent of T3-regulated genes
Embryonic	1,185	649	Up: 22 Down: 107	Up: 3% Down: 16%
Adult	2,943	1,563	Up: 196 Down: 67	Up: 12% Down: 4%

Genes enriched at least 5X in the embryonic mouse cerebral cortex with respect to the adult cerebral cortex, and viceversa were taken from (Dillman et al. 2013).

Caption to figures

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 ($-\log_{10}P_{\text{adjust}}$). Green and blue (\log_2 of the fold change >1) dots represent genes with significant changes in expression ($P_{\text{adjust}} < 0.05$). Red dots represent non-significant changes ($P_{\text{adjust}} > 0.05$). 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 \pm 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* $r = 0.908$, $P < 0.0001$)

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

Figure 3. Selected Gene Ontology categories significantly over represented ($p < 0.05$) for the positive (**3A**) or the negative genes (**3B**). 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 up-regulated genes or in the set of the down-regulated genes are marked with a thick border.

SUPPLEMENTAL INFORMATION

Supplemental Materials and Methods: Methods for primary cerebrocortical cultures, immunofluorescence, Illumina sequencing and RNA-Seq quality controls.

Supplemental Figure 1. Characterization of primary cerebrocortical cells in culture. **A-D**, Confocal microscopy after staining the nuclei with DAPI (blue), and with antibodies against **A**: glial fibrillary acidic protein (green) NeuN (red); **B**: calbindin (green) and NeuN (red); **C**: reelin (green); **D**: cholecystokinin (green). In all cases the scale bar = 25 μ m. **E**. Expression of genes encoding proteins involved in TH transport (Slc) deiodinases (Dio), nuclear thyroid hormone receptor coregulators (Nco), and TH receptors (Thr). Data are as RNA reads per kb of transcript per million mapped reads (RPKM). **F**. Gene expression responsiveness of the primary cells 24 h after addition of 10 nM T3, as measured by qPCR. Significance of differences was calculated by the Student's t-test; ***p = 0.001.

Supplemental figure 2: Enriched GO terms in the T3-treated cultures represented using GO graphs for a better visualization of the data. Green nodes are significant functional terms associated to differentially expressed genes. The uncolored GOs are displayed to better represent the relationship between all GOs.

Supplemental table 1: List of genes regulated by T3 in primary cerebrocortical cultures. Included are three groups of data. Group 1: Effect of T3 after 24 hours of addition to the culture on gene expression with a false discovery rate (FDR) < 0.05. The columns, from left to right show: A) the Ensembl ID; B) the associated gene name; C) log2 fold change; D) logCPM (counts per million); E) LR (Likelihood ratio) ; F) P-value ; G) FDR; H) Reads Per Kilobase of transcript per Million mapped reads (RPKM) as the

mean of all untreated cultures; I) Length of mRNA . Group 2: Effect of T3 on the expression of group 1 genes after 6 hours in the absence (columns L-P) and in the presence of cycloheximide (CHX) (columns Q-U). Group 3: Lack of effect of T3 on group 1 genes after 24 hours in primary cultures derived from *Thra1^{-/-}Thrb^{-/-}* mice (columns X-AB). Gene names in red bold are directly regulated genes, i.e., FDR < 0.05 in the presence of CHX. *Directly regulated genes containing a thyroid hormone receptor binding site (Chatonnet et al. 2013).

Supplemental table 2: Genes regulated directly by T3, grouped by gene function.

Supplemental table 3: T3 effect on gene expression in 5-fold enriched genes of astrocytes and neurons. The data included are: enrichment factor in untreated cells; log₂(fold change) after T3 treatment; RPKM (Reads Per Kilobase of transcript per Million mapped reads) in untreated cells as a measure of the basal expression level. Genes are ordered by enrichment in neurons or astrocytes. Gene names in red bold are directly regulated genes. *Directly regulated genes containing a thyroid hormone receptor binding site (Chatonnet et al. 2013).

Supplemental table 4: Subplate-specific genes regulated by T3. Indicated in the table are the direction of regulation, positive or negative, and the mechanism of regulation, directly at the transcriptional level, or indirectly by posttranscriptional mechanisms. The gene list was taken from Hoerder-Suabedissen et al (2013).

Supplemental table 5: Significantly enriched Gene Ontology categories (MF, Molecular Function; BP, Biological Process; CC, Cellular Component) in the T3-treated cultures 24

h. GO analysis was performed independently using all the differentially expressed genes, the positively regulated genes, and the negatively regulated genes.

Supplemental table 6: Significantly enriched Gene Ontology categories (MF, Molecular Function; BP, Biological Process; CC, Cellular Component) in the set of T3 directly regulated genes.

Supplemental table 7: Overlap between the differentially expressed genes identified in primary mouse cerebrocortical cells after T3 induction and the differentially expressed genes in cerebral cortex *in vivo* in the rat fetus (Morte et al. 2010) and the postnatal mouse (Morte et al. 2010) under conditions of thyroid hormone deprivation.

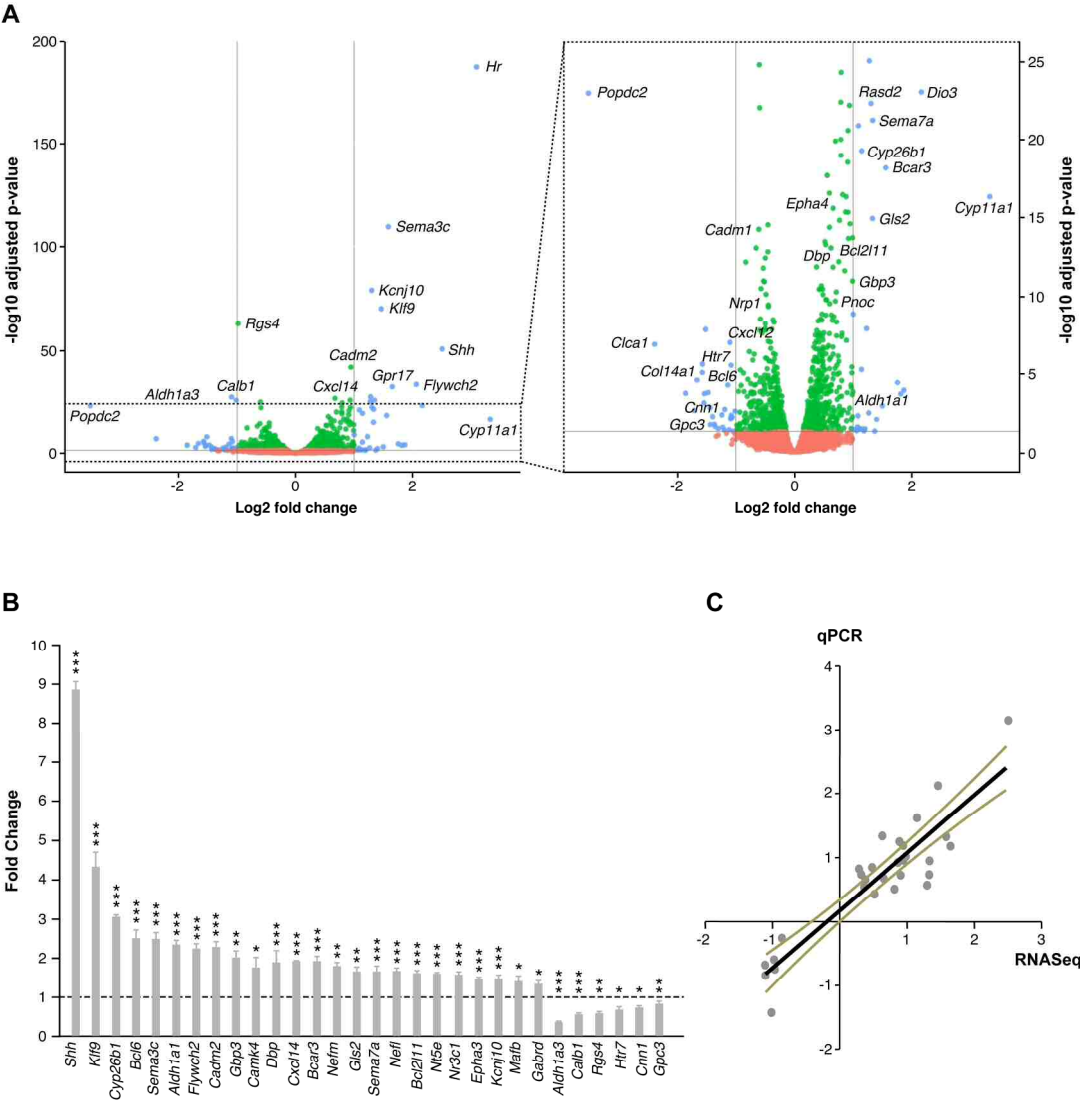


Figure 1

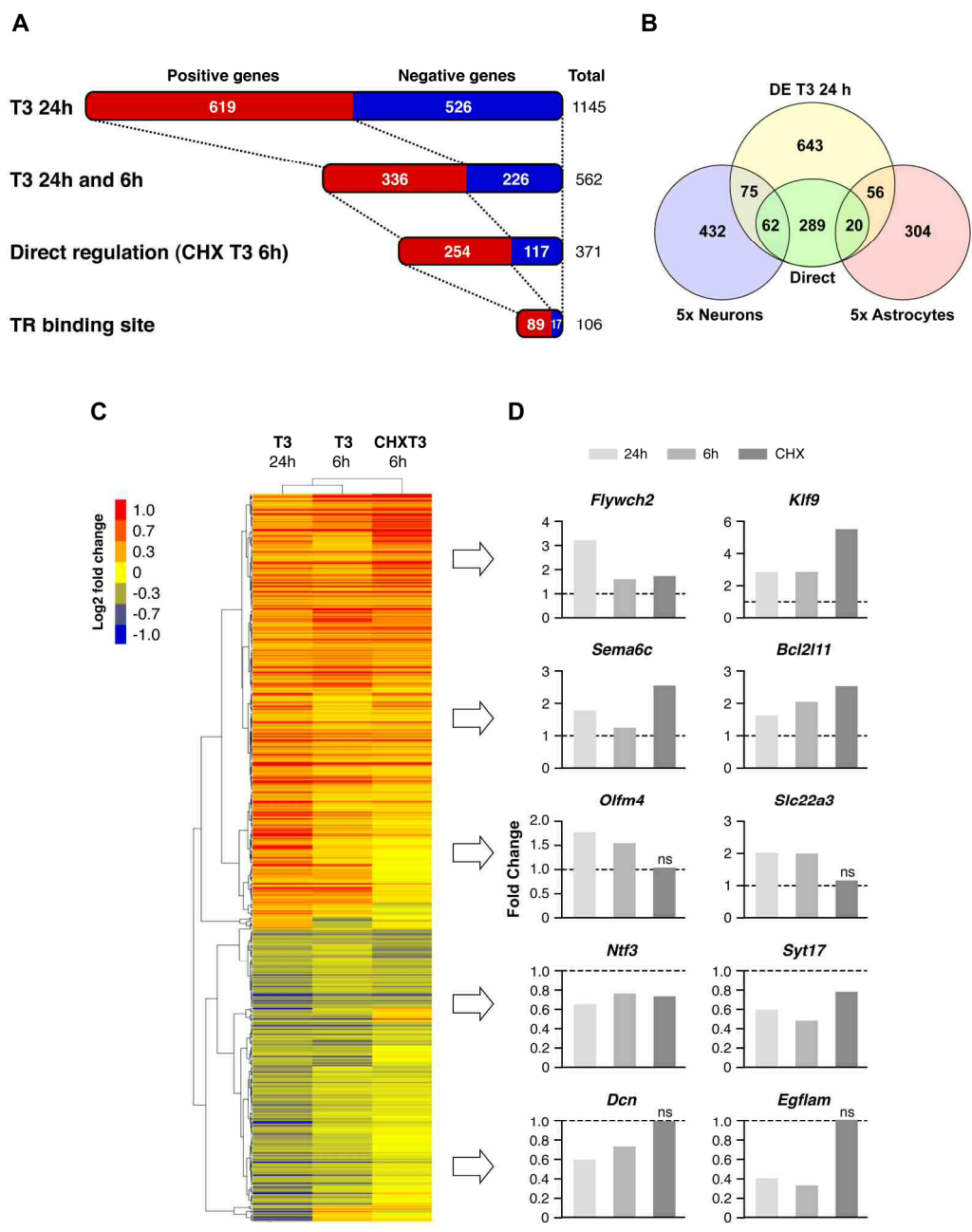


Figure 2

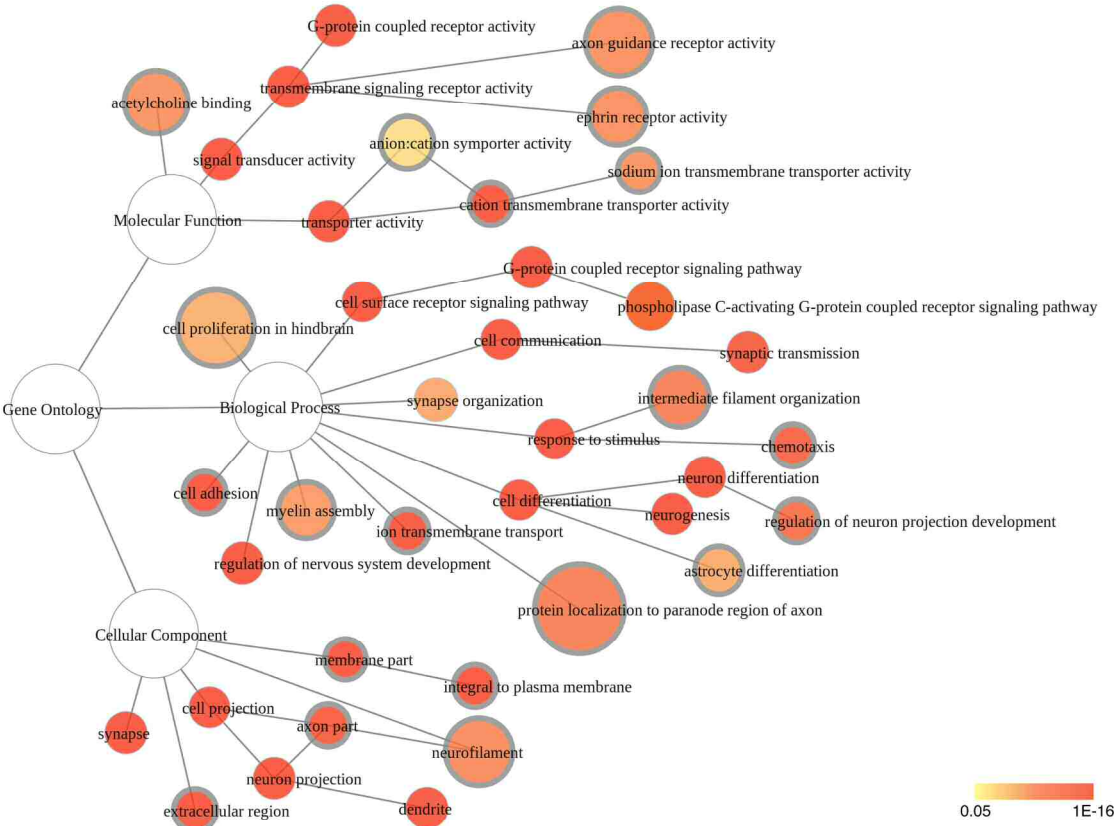


Figure 3A

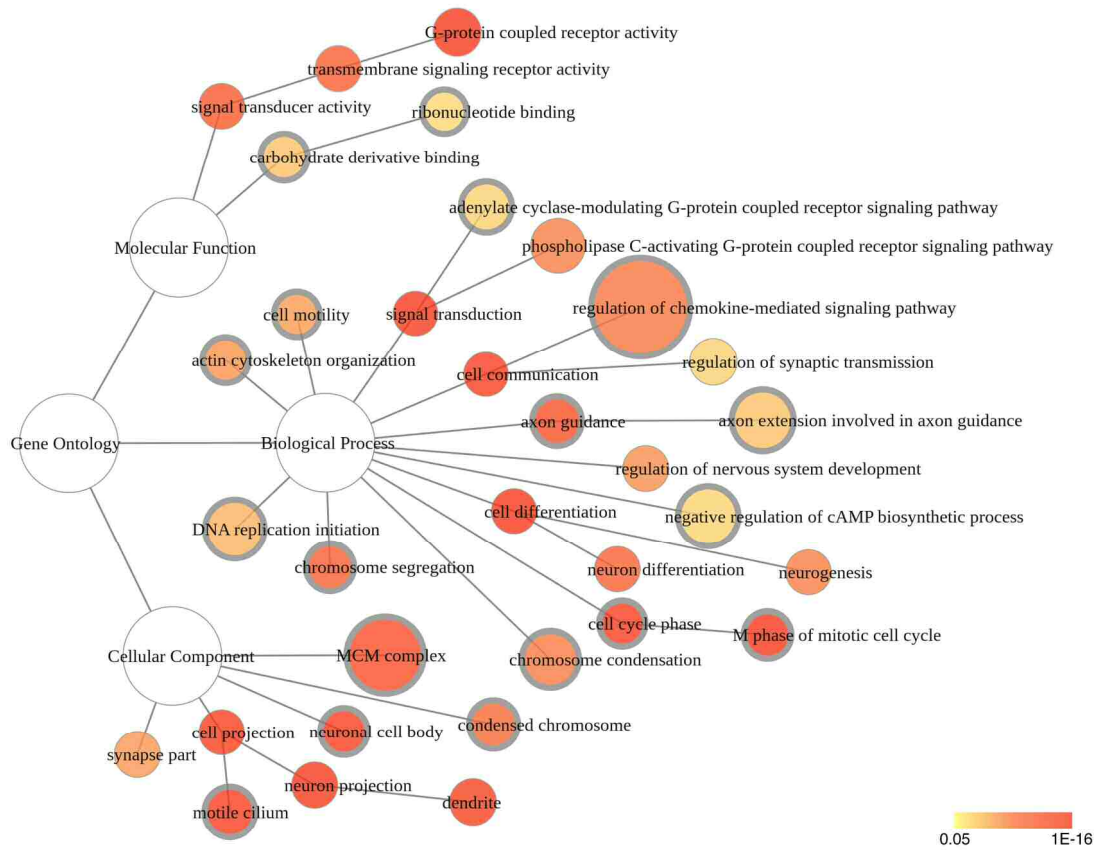


Figure 3B

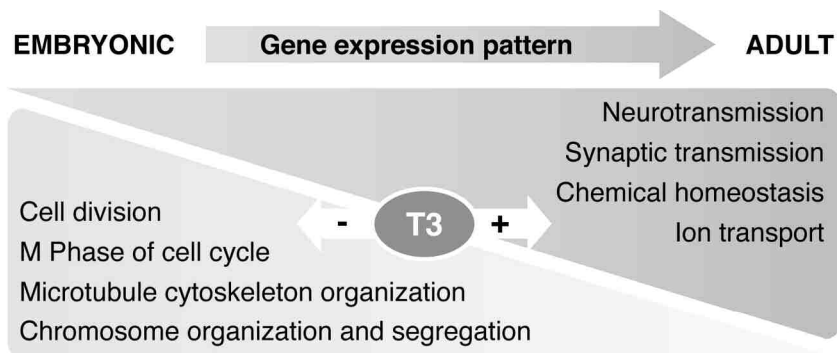


Figure 4