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Research Article

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Temporal Pole Responds to Subtle Changes in Local Thyroid Hormone Signaling

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Abbreviations: CV, coefficient of variance; D2/DIO2, type 2 deiodinase; D3/DIO3, type 3 deiodinase; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; KMO, Kaiser-Meyer-Olkin; LT4, levothyroxine; MSigDB, Molecular Signatures Data Base; PET, positron emission tomography; RT-qPCR, real-time quantitative polymerase chain reaction; T3, triiodothyronine; T4, thyroxine; TAC, Transcriptome Analysis Console; TH, thyroid hormone; TR, thyroid hormone nuclear receptors; TRE, TH responsive element.

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Abstract

To study thyroid hormone (TH) signaling in the human brain, we analyzed published microarray data sets of the temporal pole (Brodmann area 38) of 19 deceased donors. An index of TH signaling built on the expression of 19 well known TH-responsive genes in mouse brains (T3S+) varied from 0.92 to 1.1. After Factor analysis, T3S+ correlated independently with the expression of TH transporters (MCT8, LAT2), TH receptor (TR) beta and TR coregulators (CARM1, MED1, KAT2B, SRC2, SRC3, NCOR2a). Unexpectedly, no correlation was found between T3S+ vs DIO2, DIO3, SRC1, or TRα. An unbiased systematic analysis of the entire transcriptome identified a set of 1649 genes (set #1) with strong positive correlation with T3S**+** ($r > 0.75$). Factor analysis of set #1 identified 2 sets of genes that correlated independently with T3S**+**, sets #2 (329 genes) and #3 (191 genes). When processed through the Molecular Signatures Data Base (MSigDB), both sets #2 and #3 were enriched with Gene Ontology (GO)-sets related to synaptic transmission and metabolic processes. Ranking individual human brain donors according to their T3S**+** led us to identify 1262 genes (set #4) with >1.3-fold higher expression in the top half. The analysis of the overlapped genes between sets #1 and #4 resulted in 769 genes (set #5), which have a very similar MSigDB signature as sets #2 and #3. In conclusion, gene expression in the human temporal pole can be assessed through T3S**+** and fluctuates with subtle variations in local TH signaling.

Key Words: thyroid hormones, gene expression profile, brain, hypothyroidism

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Thyroid hormone (TH) signaling is initiated by binding of the active hormone triiodothyronine (T3) to TH nuclear receptors (TR), which modifies the transcriptional activity of T3-responsive genes [1]. TH might also work through noncanonical mechanisms that require binding to TR but are independent of transcriptional regulation of T3-responsive genes [2]. The study of gene expression profiles in cells, tissues, and organs of mice and rats with hypothyroidism or thyrotoxicosis identified sets of genes that respond either positively or negatively to T3 [3, 4]. Whether these genes are directly or indirectly regulated by T3 has only been established in a few cases but, in general, genes that respond rapidly with one or more TH responsive elements (TRE) tend to be directly controlled by T3. Notwithstanding, the collective changes in gene expression triggered by TH signaling in a given tissue or organ constitute the basis for TH-dependent biological effects.

Studies in cells and animal models revealed that TH signaling in the brain depends on a triad of elements that regulate TH action: (i) transmembrane transporters, specifically, organic anion-transporting polypeptides (OATPs), monocarboxylate transporters (MCTs), and L-type amino acid transporters (LATs), that mediate passage of both T4 and T3 through the blood brain barrier and local cell membranes [5, 6]; (ii) deiodinases metabolize TH locally, specifically D2 and D3, respectively activating and inactivating TH [7]; in rats, D2-generated T3 comprises >50% of the T3 bound to TRs in the cerebral cortex [8]; (iii) TRs, mostly TRα and to a lesser extent TRβ (Fig. 1). Signaling through the triad can be initiated via circulating T3, which reaches neurons through MCT8, at the same time that circulating T4 crosses the blood-brain barrier mostly via OATPs. T4 is subsequently taken up by DIO2-expressing astrocytes, mediating T4 activation to T3; the latter acts in a paracrine fashion by entering nearby neurons via MCT8 and binding to TRs to regulate gene expression [9]. Neurons express the inactivating deiodinase D3, which converts T4 and T3 to the inactive molecules rT3 and 3,3'-T2, respectively, terminating TH signaling [10]. However, studies performed in rat brain revealed that TRs are normally almost fully occupied with T3 [8], suggesting that D3 activity in neurons does not substantially limit T3 entry or access to the neuronal nucleus. What role D3 plays in the brain remains to be determined, albeit the D3KO mouse exhibits signs of cerebral thyrotoxicosis [11]. Notwithstanding, as with all other tissues, TH signaling in the brain is likely to be unique for different areas, depending on circulating TH levels and on the exclusive blend of transporters, deiodinases, and TRs present in each area.

Less is known about TH signaling in the human brain. We know that the key genes involved in the triad that mediates TH signaling in the rodent brain (such as transporters, deiodinases, TRs, and co-regulators) are also expressed in the human brain (Allen Human Brain Atlas: http://human.brain-map.org/), and inactivation mutations of some of these genes have been reported to disrupt TH signaling. For example, male subjects with hemizygous mutations in MCT8 are afflicted with severe intellectual and motor disability, known as the Allan-Herndon-Dudley syndrome [12, 13], but data about the role of MCT10 and OATP1C1 in humans are limited [14, 15]. In adult humans, the presence of MCT8 is detected in neurons of the hypothalamus and in pituitary cells, but there are only few studies about the distribution of MCT8 protein in other areas of the well-developed human brains [16, 17]. The brains of 3 elderly individuals shows MCT8 protein almost exclusively in endothelial cells from different regions of the brain, including parietal, occipital, and limbic cortex, hippocampus, hypothalamus, amygdala, and cerebellum [18]. In addition, in human fetuses, MCT8 expression was detected in the

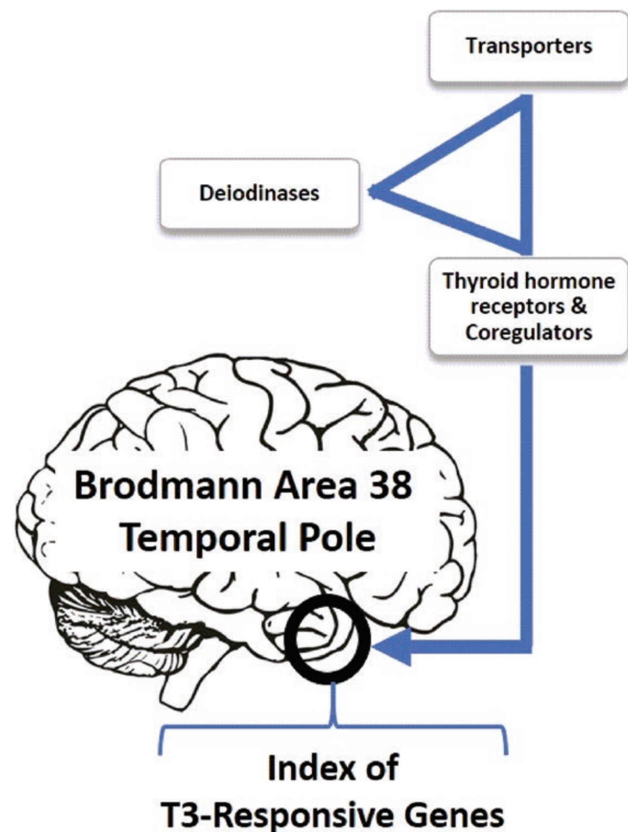

Figure 1. TH signaling in the brain depends on a triad of elements that regulate TH action: (i) transmembrane transporters, ie, OATPs, MCTs, and LATs, that mediate passage of both T4 and T3 through the blood brain barrier and local cells; (ii) deiodinases metabolize TH locally, ie, DIO2 and DIO3, respectively activating and inactivating TH; (iii) TRs, mostly TRα and to a lesser extent TRβ. In the present *in silico* studies, microarray data previously derived from 19 human Brodmann area 38 (temporal pole) samples was used to generate a T3 positive signaling index (T3S+).

blood brain barrier in all analyzed regions. Furthermore, an intense MCT8 expression was detected in other cell types, including radial glial cells in cerebral cortex and in ventricular zone [19]. Severe mental retardation (IQ < 60) is uncommon in carriers of TRβ mutations, but about 30% of affected subjects display a mild learning disability (IQ < 85), with some suggesting a relationship with attention deficit hyperactivity disorder (ADHD) [20]. At the same time, studies of patients with TRα1 mutations revealed cognitive deficits that were consistent with those seen in congenital hypothyroidism [21, 22].

The adult human brain responds to TH as illustrated by the marked changes in mood and cognition observed during the transition from hypo- to euthyroid to thyrotoxic states [23]. However, it is not clear that the human brain responds to small changes in TH signaling. For example, mood and cognition were not affected when levothyroxine (LT4)-treated hypothyroid patients were randomized to a small increase or decrease of LT4 dose [24]. On one hand, knowledge about the several elements involved in TH signaling in the rat brain [25], including the deiodinases, suggests that homeostatic mechanisms could compensate for minimal changes in TH levels, preventing changes in T3-dependent processes in the brain [26]. On the other hand, studies of T3-responsive genes in LT4-treated hypothyroid rats with normal thyrotropin (thyroid-stimulating hormone) indicate that the minimal reduction in plasma T3 leads to brain hypothyroidism [27]. The situation in the human brain is not known but could carry significant clinical implications. For example, could the relatively lower serum T3 levels observed in LT4-treated patients [28] affect TH signaling in the brain? Or explain symptoms of impaired mood and cognition seen in some of these patients [29]?

Here we used data from a previous study of the cerebral temporal pole transcriptome of 19 deceased donors that died of trauma but were relatively young and had no history of neurological or thyroid disease, to study brain TH signaling [30]. The temporal pole corresponds to Brodmann area 38 and has strong connections with the amygdala and orbital prefrontal cortex. It is considered an association cortex involved with multimodal analysis, especially in social and emotional processing [31], all known to be affected by TH signaling [23, 32]. Furthermore, studies in humans indicate that hypo- and hyperthyroidism are associated with changes in different limbic and paralimbic structures, including the temporal gyrus [33-35]. Thus, utilizing these microarray data, we created an index of TH signaling based on the expression of known T3-responsive genes in rodents (Table 1; Supplementary Table 1 [36]). This index was further used to study whether TH signaling in these donors correlates with specific patterns of gene expression in the temporal pole.

1. Materials and Methods

TH signaling index

Multiple T3-responsive genes in brain tissue were previously identified in animal and cell models by contrasting gene expression between hypothyroid and thyrotoxic states. However, even if these genes are directly regulated by T3 they may also be affected by other cellular signaling pathways. It is conceivable that by looking at the behavior of a large enough group of T3-responsive genes one would maximize the reading of T3-dependent pathways while minimizing other non-T3 related influences. To study this index of TH signaling in the human brain (**T3S+**), we looked into a data set previously published from our laboratory [30] that contains human brain (temporal pole) microarray results and asked whether these gene sets could be used to estimate TH signaling in the human brain. To answer this question, we used a previous study [4] that looked at a series of publications in mice and rats and identified a total of 4108 T3-responsive genes in the brain. For the present analysis, we used a more stringent criteria: we only include T3-responsive genes that (i) had been reported by 3 or more publications and (ii) at least one of these reports had to have been performed in cerebral cortex. These 2 criteria led us to a much smaller list of 120 genes. Next, we selected genes that were reportedly positively regulated by T3, which reduced the list to only 30 genes—unfortunately, many studies did not specify whether the gene is positively or negatively regulated by T3. We next used these 30 genes to build a preliminary T3S+ (PT3S+) index. Subsequently, we performed several correlation rounds between each one of the 30 genes *vs* the PT3S+ index, removing from the PT3S+ index any gene that returned a R (Spearman) <0.5; always starting with the ones that had smaller R values. The use of this filter was justified because we wanted to create a consistent group of T3-responsive genes, knowing that we started with 30 genes identified in rodents, not humans, and that many genes were originally observed to be responsive to T3 in developing brain or cells (Table 1). Thus, it is not surprising that some of these genes might not be good surrogates of T3 action in adult humans. This filtering eliminated 11 genes, which led us to 19 consistently T3-responsive genes (positively) that were used to build the T3S+ index (Table 1; Supplementary Table 1a and 1b [36]).

Cohort of human temporal pole samples and microarray data

The present *in silico* studies were performed using microarray data reported previously [30]. In brief, experimental data were generated through the study of brain tissue samples collected from postmortem human donors at the University of Miami Brain Endowment Bank. Brain samples

Table 1. Genes Positively Regulated by T3 Used to Prepare the T3S+ Index

Gene Symbol	Function	Species	Brain Area	Main Cell Type [59]
ABCD2	Import of fatty acids into organelles	*Mus musculus/Rattus norvegicus*	Cortex [3, 60], striatum [4, 61]	Astrocytes
ATP2B2	Involved in intracellular calcium homeostasis	*Mus musculus/Rattus norvegicus*	Cortex [3, 60, 62]	Neurons
CNTNAP1	Organization of myelinated axons/ Signaling between axons and myelinating glial cells	*Mus musculus/Rattus norvegicus*	Cortex [3, 60, 63] hippocampus [63]	Neurons
DCLK1	Involved in neuronal migration, apoptosis and neurogenesis	*Mus musculus/Rattus norvegicus*	Cortex [60, 64] striatum [4]	Astrocytes, neurons
DNM3	Involved in vesicular transport	*Mus musculus/Rattus norvegicus*	Cortex [64], cerebellum [3] hippocampus [63]	Neurons, oligodendrocytes
HOMER1	Acts in regulation of group 1 metabotrophic glutamate receptor function	*Mus musculus/Rattus norvegicus*	Cortex [62, 64] Striatum [4, 61]	Microglia
ITPR1	Involved in the formation of calcium ions channel	*Mus musculus/Rattus norvegicus*	Cortex [60, 63] cerebellum [3, 65] striatum [4]	Neurons
KCNA1	Involved in the formation of potassium channels	*Mus musculus/Rattus norvegicus*	Cortex [63], cerebellum [60, 65]	Neurons
KLF9	Functions as a transcription factor regulating keratinocyte proliferation	*Mus musculus/Rattus norvegicus*	Cortex [63], cerebellum [60, 65] striatum [61]	OPC, astrocytes, oligodendrocytes
LNPEP	Acts in inactivation of peptide hormones	*Mus musculus*	Cortex [60, 64]	Astrocytes, neurons
NEFH	Biomarker of neuronal damage	*Mus musculus/Rattus norvegicus*	Cortex [60, 62, 63] cerebellum [65] hippocampus [63] striatum [4]	Neurons
NEFM	Biomarker of neuronal damage	*Mus musculus/Rattus norvegicus*	Cortex [60, 62] striatum [4]	Neurons
NMT2	Involved in regulating of signaling proteins	*Mus musculus*	Cortex [64] cerebellum [3]	Neurons
OPCML	Acts as an accessory for opioid receptor function	*Mus musculus/Rattus norvegicus*	Cortex [62, 64] cerebellum [3]	Neurons
PVALB	Involved in calcium handling	*Mus musculus/Rattus norvegicus*	Cortex [60, 63] cerebellum [3] hippocampus [63] striatum [4]	Neurons
RBBP4	Involved in chromatin metabolism regulation	*Mus musculus/Rattus norvegicus*	Cortex [64] cerebellum [3, 65]	Astrocytes, neurons, oligodendrocytes
SEMA7A	Involved in immunomodulatory and neuronal process	*Mus musculus/Rattus norvegicus*	Cortex [60, 62] cerebellum [[3] striatum [4]	Astrocytes, oligodendrocytes
SORL1	Acts in several intracellular sorting and trafficking functions/Regulation of the transformation of amyloid precursor to amyloid-β	*Mus musculus/Rattus norvegicus*	Cortex [62, 64] striatum [4]	Microglia
TBC1D30	Involved in GTPase activity/Intracellular protein transport	*Mus musculus/Rattus norvegicus*	Cortex [60, 62] striatum [4, 61]	Neurons

Abbreviations: ABCD2: ATP binding cassette subfamily D member 2; ATP2B2: ATPase, Ca++ transporting, plasma membrane 2; CNTNAP1: Contactin-associated protein 1; DCLK1: Doublecortin-like kinase 1 DNM3: Dynamin 3; HOMER1: Homer scaffold protein 1; ITPR1: Inositol 1,4,5-trisphosphate receptor type 1; KCNA1: Potassium voltage-gated channel subfamily A member 1; KLF9: Kruppel like factor 9; LNPEP: Leucyl and cystinyl aminopeptidase; NEFH: Neurofilament heavy; NEFM: Neurofilament medium; NMT2: N-myristoyltransferase 2; OPCML: Opioid binding protein/cell adhesion molecule-like PVALB: Parvalbumin; RBBP4: Retinoblastoma binding protein 4, chromatin remodeling factor; SEMA7A: Semaphorin 7A; SORL1: Sortilin related receptor 1; TBC1D30: TBC1 domain family, member 30.

from 19 male, Caucasian, middle-aged (43.6 ± 12.2 years), adult donors without known thyroid or neurologic disease were studied as described previously. The cause of death was limited to accident or sudden cardiac death without medical intervention or prolonged agonal state. Postmortem interval at specimen collection was <24 hours,

brain pH (quality measure) was >6.0. A neuroanatomist dissected homogenous samples from frozen coronal blocks based on surface and cytoarchitectural landmarks from the Brodmann area 38 (temporal cortex pole) and stored at −80 °C. Microarray analysis was performed at the Joslin Diabetes Center Genomics Core Laboratory with Genechip Human Gene 2.0 ST arrays, which utilizes a whole transcript design to assess more than 30 000 coding genes, and the Affymetrix Expression Console software [30]. Robust Multi-array Average (RMA) algorithm was used to create an expression matrix from Affymetrix data. After obtaining the expression data, a specific filter was developed by MySQL and Java software to only include unique named genes, resulting in 22 257 genes. The individual gene mRNA values of each donor were used to create the linear correlation analysis. As indicated, gene sets were analyzed using the Molecular Signatures Data Base (MSigDB) for canonical pathways enriched in the gene set. The Affymetrix data created (.CEL file) was subsequently used to analyze differentially expressed genes within the population, using Transcriptome Analysis Console (TAC, Affymetrix). These data were also processed for gene set analysis using the Gene Set Enrichment Analysis software (GSEA; Broad Institute).

THRA expression analysis. As an additional approach to study the transcript variant 1 of the TRα gene, we performed a real-time quantitative polymerase chain reaction (RT-qPCR analysis). For this, RNA from all 19 human brain samples was extracted (RNeasy Lipid Tissue Mini Kit, Qiagen) and cDNA generated (First Strand cDNA Synthesis Kit, Roche). Genes of interest were measured by RT-qPCR (StepOnePlus Real-Time PCR Detection System, Applied Biosystems) using PowerUp SYBR Green Master Mix (Applied Biosystem) with the following conditions: 20 seconds at 95 °C, 3 seconds at 95 °C, and 30 seconds at 60 °C × 40, and 15 seconds at 95°C followed by 1 minute at 60°C and 15 seconds at 95°C. Standard curves consisting of 5 points of a serially diluted mixture of experimental and control cDNA; the amplification efficiency was 80% to 110% with a coefficient of correlation consistently >0.98. The melting curve protocol was used to verify the specificity of the amplicon generation. Cyclophilin A (CYCLOA) was used as a housekeeping internal control gene. Results were expressed as the ratio of target mRNA (THRA) to CYCLOA mRNA. The pair of primer designed are shown on Supplemental Data (Supplementary Table 2a [36]).

Homogeneity of the samples. To test for homogeneity of all brain samples, we looked at the expression levels of 10 markers of apoptosis, having found a coefficient of variance (CV) of 2.3% to 4.8%, and also that none of the markers correlated with T3S+ (P > 0.05; Supplementary Table 3a [36]). Cerebral cortex contains multiple cell types, including astrocytes, oligodendrocytes, and neurons, and we wanted to ensure that the relative representation of these cells was not markedly different among the samples. Thus, we looked at the expression of known cell-specific markers for neurons, oligodendrocytes, and astrocytes, having found a CV from 1.7% to 9.8%. More importantly, none of these genes correlated with **T3S+** (P > 0.05; Supplementary Table 3b [36]).

Statistical analysis

Nonparametric correlation (Spearman) analysis was used throughout; we qualified the correlation as strong ($r > 0.7$), moderate ($0.5 < r < 0.7$) and weak ($r < 0.5$); in the different gene set analyzes we also used the false discovery rate (FDR)-q value (adjusted P values according to FDR) to assess statistical significance. All data analyzes, including covariation, were using Microsoft Excel and GraphPad Prism 5 software. Unless otherwise indicated, data are the mean ± standard deviation (SD) and Spearman r correlation coefficient; $P < 0.05$ was used to reject the null hypothesis. We used Factor Analysis to describe variability among observed, correlated variables in terms of a potentially lower number of unobserved variables or factors. The observed variables were modelled as linear combinations of the potential factors, plus "error" terms. Factor analysis aims to find independent latent variables. We first used the Kaiser-Meyer-Olkin (KMO) analysis followed by the Bartlett test. A KMO > 0.70 was considered good and >0.85 excellent. The factor analysis used orthogonal rotation Varimax with "Kaiser" normalization; we only considered Eigenvalues > 1.0 [37].

2. Results

An index for TH signaling in the human temporal pole

The Allen Human Brain Atlas (http://human.brain-map.org/) indicates that the human temporal pole expresses key genes necessary to mediate TH signaling: (i) TH transporters at levels equivalent to the rest of the brain except for MCT8 and OATP1C that are relatively overexpressed (Z-score 1.2 to 1.5), and MCT10 that is relatively under-expressed (Z-score −0.5); (ii) TRs at levels that are below the rest of the brain (Z-score −0.7 to −1.1); (iii) deiodinases (DIO2-DIO3) at levels above the rest of the brain (Z-score 0.5-1.5).

mRNA levels for T3-responsive genes were used to create an index of TH signaling. This was done by first calculating the relative expression of each one of the 19 T3-responsive genes among the 19 donors after the level

of each individual donor was divided by the average of the group (Supplementary Tables 1a and 1b [36]). Subsequently, the relative expression level of all positively regulated genes was averaged, returning an expression index (T3S+) that varied from 0.92 to 1.1, with a mean ± SD of 1.0 ± 0.042 (±2SD = 0.92-1.08) and a CV of 4%.

Next, we wished to validate the T3S+ and asked whether it covaries with the expression of 27 genes known to play a role in TH signaling, that is, TH transporters, deiodinases, TRs, and transcriptional modulators (Set #0). In general, we found a linear relationship between some of these variables and T3S**+**, with the expected positive covariation and a moderate/strong correlation with *r* values between 0.47 and 0.82 (Table 2). Specifically, we found that the intensity of T3S+ correlated positively with the expression of LAT1, LAT2, MCT8, and MCT10 (Table 2), whereas no correlation was found with 2 OATPs (Table 2). While it is not possible to define a causal relationship between TH signaling and the group of TH transporters, the fact that the mRNA for TH transporters are only minimally affected by TH signaling in the brain [38] suggests that the expression of TH transporters could be an important factor defining the intensity of T3S+ in this tissue. There was a moderate positive correlation between DIO2 and DIO3 mRNA levels ($r = 0.52$; $P = 0.02$), but no correlation was observed between T3S+ and the expression of DIO2 or DIO3 (Table 2). The relationship between TR expression vs T3S+ was less straightforward. TRβ covaried positively with T3S+, but TRα did not (Table 2). The latter was confirmed after TRα mRNA levels were measured using RT-qPCR and primers that were specific for TRα1 (Supplementary Table 2 [36]). There was a positive correlation between T3S+ vs the coactivators CARM1, MED1, NCOA2, NCOA3, and PPARGC1A, but not vs NCOA1 or PPARGC1B (Table 2). Furthermore, the corepressor NCOR2 covaried negatively with T3S+ (Table 2). Unexpectedly, T3S+ covaried positively with NCOR1 (Table 2) and negatively with the coactivator KAT2B (Table 2).

Knowing that neurons are more responsive to T3 than other cells in the central nervous system, we filtered the genes in T3S**+** to include only genes typically expressed in neurons (n = 10) and created a **NT3S+**. We next correlated NT3S**+** with the 27 genes known to play a role in TH signaling. Remarkably, the results obtained (Supplementary Table 4a and 4b [36]) were essentially similar to when T3S+ was used in the correlations (Table 2). We also used the Microsoft Excel *"randbetween"* function to create an additional index based on genes randomly selected from the list of 22 257 genes in the microarray (**RT3S+**) that functioned as a negative control. Not surprising, RT3S**+** did not correlate with any of the 27 genes known to play a role in TH signaling (Supplementary Table 5a and 5b [36]*)*.

Table 2. Correlation Analysis of Gene Expression of Elements Involved in T3 Signaling (Set #0) and TH Positive Signaling Index (T3S+)

Gene	T3S+	
	r	*P*
TH transporters		
SLC7A5 (LAT1)	0.55	1.38E-02
SLC7A6 (LAT2)	0.82	2.07E-05
SLC16A2 (MCT8)	0.80	4.49E-05
SLC16A10 (MCT10)	0.59	7.91E-03
SLCO1C1 (OATP1C1)	-0.34	*ns*
SLCO4A1 (OATP4A1)	-0.05	*ns*
TH receptors		
THRA (TRα)	-0.04	*ns*
THRB (TRβ)	0.81	2.54E-05
Deiodinases		
DIO2 (D2)	-0.40	*ns*
DIO3 (D3)	-0.28	*ns*
Coactivators		
CARM1	0.71	6.51E-04
CREBBP (CBP)	0.21	*ns*
EP300 (P300)	-0.12	*ns*
KAT2B	-0.76	1.80E-04
MED1 (TRAP220)	0.72	4.50E-04
NCOA1 (SRC-1)	0.38	*ns*
NCOA2 (SRC-2)	0.70	7.77E-04
NCOA3 (SRC-3)	0.65	2.37E-03
NCOA6	-0.07	*ns*
PPARGC1A (PGC-1ALFA)	0.47	4.22E-02
PPARGC1B (PGC-1BETA)	0.06	*ns*
SMARCB1 (SNF5)	0.45	*ns*
Corepressors		
HR	0.02	*ns*
NCOR1	0.56	1.31E-02
NCOR2	-0.50	2.99E-02
NR0B1 (DAX-1)	-0.45	*ns*
NR0B2 (SHP)	0.27	*ns*

Data are the Spearman *r* coefficient correlation and *P* value.

Abbreviations: CARM1: Coactivator associated arginine methyltransferase 1; CREBBP: CREB binding protein; DIO2: Type 2 iodothyronine deiodinase; DIO3: Type 3 iodothyronine deiodinase; EP300: E1A binding protein p300; HR: lysine demethylase and nuclear receptor corepressor; KAT2B: lysine acetyltransferase 2B; MED1: mediator complex subunit 1; NCOA1: nuclear receptor coactivator 1;NCOA2: nuclear receptor coactivator 2; NCOA3: nuclear receptor coactivator 3; NCOA6: nuclear receptor coactivator 6; NCOR1: nuclear receptor corepressor 1; NCOR2: nuclear receptor corepressor 2; NR0B1: nuclear receptor subfamily 0 group B member 1; NR0B2: nuclear receptor subfamily 0 group B member 2; PPARGC1A: PPARG coactivator 1 alpha; PPARGC1B: PPARG coactivator 1 beta; SLC7A5: solute carrier family 7 member 5; SLC7A6: solute carrier family 7 member 6; SLC16A2: solute carrier family 16 member 2; SLC16A10: solute carrier family 16 member 10; SLCO1C1: solute carrier organic anion transporter family member; SLCO4A11: solute carrier organic anion transporter family member 4A1; SMARCB1: SWI/SNF-related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1; THRA: thyroid hormone receptor alpha; THRB: thyroid hormone receptor beta.

We next reanalyzed the data in Table 2 using a Factor analysis to identify which of these genes or gene sets (factors) correlated independently with T3S**+**. The calculated

KMO value for this dataset was 0.973, and the Bartlett test returned a $P < 0.001$, confirming the existence of independent factors. After only ten iterations the analysis converged on 8 independent factors (Fat 1-8) with Eigenvalues > 1.0 (Table 3); combined, these 8 factors explained 90.2% of the total variability observed (Supplementary Table 6a [36]). After the Fat 1-8 scores were tested against **T3S+** through Pearson's correlation, only Fat-1 and Fat-2 reached statistical significance, which include TRβ, 6 coregulators (CARM1, KAT2B, NCOR2, SRC2-3, MED1) and 2 transporters (LAT2, MCT8) (Fig. 2; Tables 3 and 4).

Unbiased search for gene-sets and cellular pathways that correlate positively with T3S+

The fact that T3S+ correlates positively with the expression of 2 TH transporters, TRβ, and 4 coactivators, and negatively with the corepressor NCOR2 (Fig. 2) prompted us to explore the possibility that T3S+ could be used to identify T3-sensitive pathways in the human cerebral pole. To that end, we used 2 unbiased strategies: (i) correlation analysis to identify genes that correlate positively with T3S+, and (ii) TAC to contrast the transcriptome of donors with the lowest T3S+ (**LT3S+**) vs the highest **T3S+** (**HT3S+**).

Correlation analysis between individual genes and T3S+

All 22 257 named genes in the microarray were tested for Spearman correlation analysis and ranked according to "*r*". We found 1649 genes (set #1) that strongly correlated with T3S+ ($r > 0.75$; Supplementary Table 7 [36]). In contrast, when RT3S**+** was used, we only identified 6 genes that correlated strongly ($r > 0.75$) with the 22 257 genes (Supplementary Table 5c [36]). Next, we wished to validate this analysis and tested whether similar results could be obtained by correlating the 22 257 named genes with TRα mRNA or TRβ mRNA. Whereas the correlation with TRα yielded only 7 genes with $r > 0.75$ and 22 genes with $r > 0.70$ (Supplementary Table 8 [36]), TRβ correlated strongly with 1447 genes ($r > 0.75$). Remarkably, 71% of these genes (1029 genes)

Table 3. Factorial Charge of TH Signaling Related Genes (Set #0) in Each Factor

TH signaling genes	Fat 1	Fat 2	Fat 3	Fat 4	Fat 5	Fat 6	Fat 7	Fat 8
CARM1	0.899							
THRB	0.895							
KAT2B	−0.822							
NCOR2		−0.852						
NCOA3		0.838						
MED1		0.756						
SLC7A6		0.702						
NCOA2		0.691						
SLC16A2		0.540						
CREBBP			0.882					
NCOA6			0.863					
EP300			0.555					
NCOR1			0.547					
SLCO4A1			−0.479					
THRA			0.450					
DIO2				0.9				
DIO3				0.801				
SLCO1C1				0.789				
SMARCB1				-0.608				
HR					0.898			
SLC7A5					0.761			
SLC16A10						0.813		
PPARGC1A						0.789		
PPARGC1B						0.596		
NR0B1							−0.919	
NCOA1							0.688	
NR0B2								0.912

Extraction method: Principal component analysis; Rotation method: Varimax with Kaiser normalization; Rotation converged in 10 iterations.

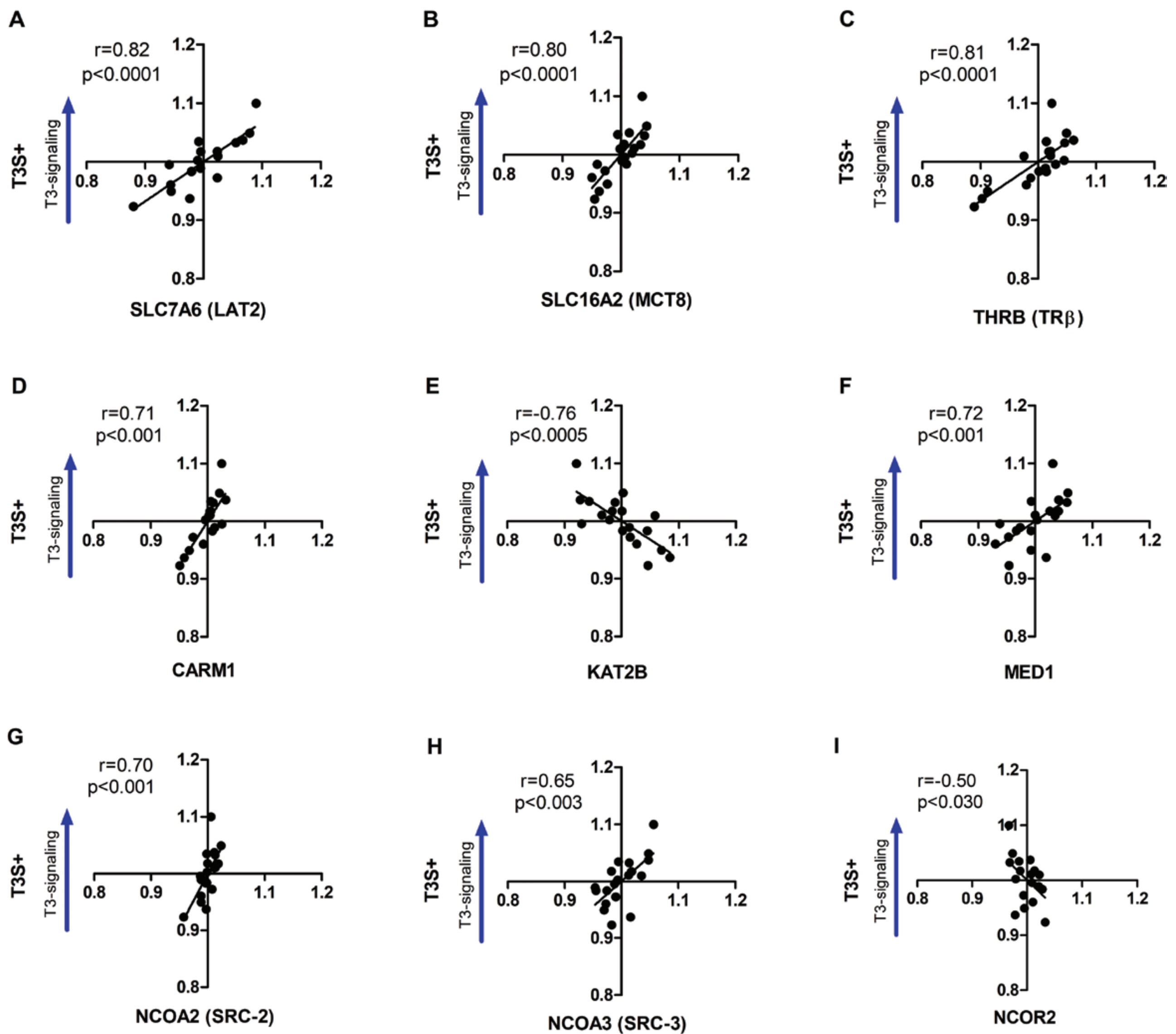

Figure 2. Correlation analysis between T3S+ and the components included at the Factors 1 and 2 that reached statistical significance in Factor Analysis. TH transporters LAT2 (A), MCT8 (B), TH receptor TRβ (C), coactivators CARM1 (D), KAT2B (E), MED1 (F), SRC-2 (G), and SRC-3 (H), and corepressor NCOR2 (I). Statistical significance is shown in each graph as well as Spearman's correlation coefficient (*r*).

overlapped with the genes in set #1 (Supplementary Table 9 [36]).

We next performed Factor analysis to identify which of these genes or gene sets (factors) correlated independently with T3S**+**. The calculated KMO value was 0.954, and the Bartlett test returned a $P < 0.001$, confirming the existence of independent factors. After 51 iterations the analysis converged on 18 independent factors (Fat 1-18) with Eigenvalues > 1.0 (Supplementary Table 10 [36]); combined, these 18 factors explained 100% of the total variability observed (Supplementary Table 6b [36]). After the Fat 1-18 scores were tested against T3S**+** through Pearson correlation, only Fat-3 and Fat-4 reached statistical significance, which included 329 (set #2) and 191 (set #3) genes, respectively (Table 4).

The physiological context of gene sets #2 and #3 was assessed through MSigDB, that searches for canonical pathways enriched in the gene sample (Supplementary Tables 11-16 [36]). These gene sets were ranked within each category according to the enrichment scores (ES). Among the top 20 GO-sets enriched for gene set #2 under "biological processes", "cellular component" and "molecular function" there were 14 to 15 sets related to synaptic transmission, mitochondria, and cellular transport,

Table 4. Pearson Correlation Between Factors Scores and T3S+

Factors	Set #0		Set #1	
	Pearson *r*	*P* value	Pearson *r*	*P* value
1	0.67	*0.002*	0.42	0.074
2	0.54	*0.016*	0.38	0.112
3	0.07	0.786	0.54	*0.017*
4	−0.23	0.35	0.59	*0.007*
5	0.15	0.549	0.04	0.872
6	0.37	0.124	0.00	0.992
7	0.10	0.683	−0.03	0.898
8	0.06	0.816	0.01	0.959
9			−0.08	0.744
10			0.12	0.616
11			−0.07	0.767
12			0.06	0.816
13			−0.02	0.948
14			0.00	0.994
15			0.05	0.831
16			0.01	0.975
17			−0.05	0.825
18			−0.01	0.956

whereas for gene set #3 the top 20 GO-sets contained 14 to 15 sets related to ionic transport and metabolic processes.

TAC contrasting donors with the lowest T3S+ vs the highest T3S+

An additional approach to identify pathways that co-vary with TH signaling was to rank the group of donors according to the T3S+. This resulted in 2 subgroups of donors, one containing the lowest levels of T3S+ (LT3S+ donors # 18, 2, 17,12, 4, 9, 16, 7, 5) and the other with the highest levels of T3S+ (HT3S+ donors # 15, 8, 11, 9, 6, 11, 3, 10, 13,14) (Fig. 3; Supplementary table 1 [36]). An analysis using TAC identified 1262 genes (set #4) enriched in the HT3S+ subgroup ($P < 0.05$; fold change > 1.3; Supplementary Table 17 [36]).

Next, we compared gene sets #1 and #4 having found that 769 genes (set #5) overlapped. The physiological context of gene set #5 was assessed through MSigDB, that searches for canonical pathways enriched in the gene sample (Supplementary Tables 18-20 [36]). After gene sets were ranked within each category according to enrichment score, the top 20 gene sets under "biological processes" and "cellular components" were found to contain from 14 to 15 genes sets directly related to synaptic signaling, synaptic vesicle recycling and transport, and regulation of neurotransmitter levels and release (Supplementary Tables 18 and 19 [36]). The top 20 gene sets under "molecular function" were mostly related to transmembrane transport and cellular metabolism (Supplementary Table 20 [36]).

3. Discussion

The present studies revealed that the expression of genes in the human temporal pole fluctuates with subtle changes in local TH signaling. This was concluded based on the correlation analysis of an index of TH action (T3S+) vs expression data obtained from the human cerebral temporal pole. T3S+ correlated with the expression of 2 transporters (LAT2, MCT8), TRβ, and 6 coregulators (CARM1, KAT2B, NCOR2, SRC2-3, MED1) (Fig. 2). Furthermore, unbiased analyses of expression data identified 2 groups of hundreds of genes that correlate independently with T3S+. These sets were enriched with genes involved in cellular pathways known to be regulated by TH signaling, that is, synapsis function, cellular transport, and metabolism (Supplementary Tables 11-16, 18-20 [36]). Of course, it is not expected that these hundreds of genes are directly regulated by T3. Instead, they likely reflect fine tuning of large gene networks affected by subtle variations in TH signaling. It is likely that these changes in the transcriptome will eventually be transduced into biological processes with physiological and/or clinical relevance. A remarkable finding was that the expression of slightly more than two-thirds of the genes that correlated with T3S+ also correlated with TRβ expression, but not TRα. In fact, only a handful of genes was found to correlate with TRα. While it is conceivable that the correlation indicates that T3 stimulates the

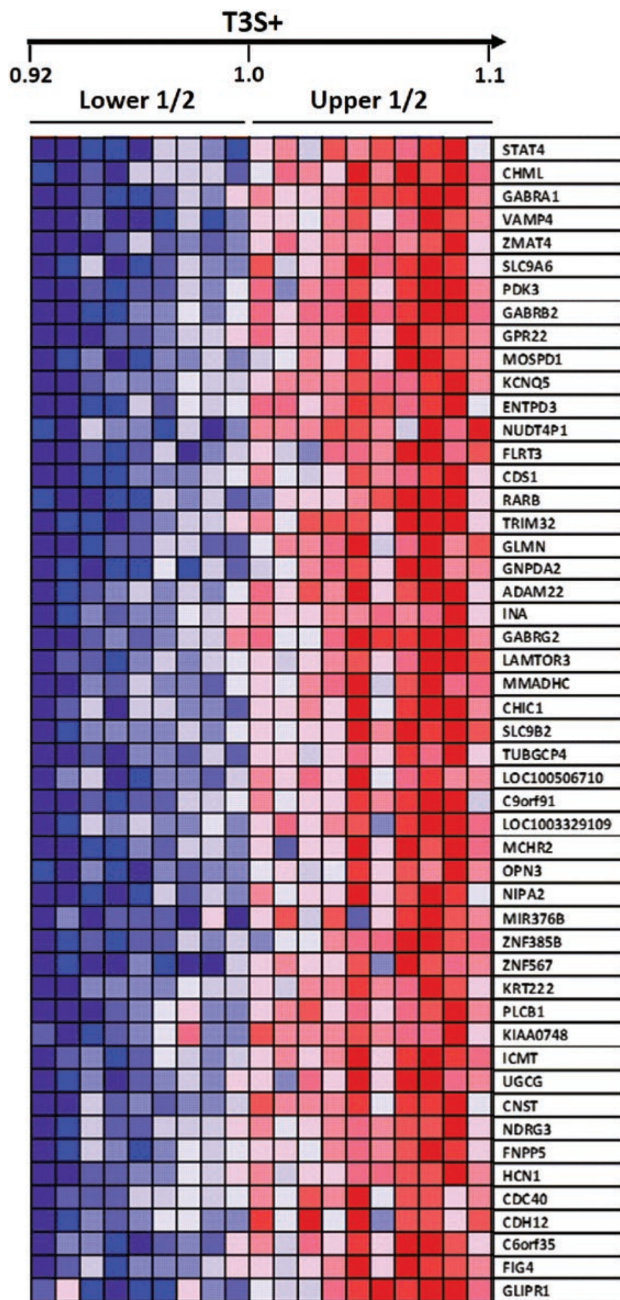

Figure 3. Heat map indicating the top 50 genes enriched in temporal pole samples when 2 subgroups of donors: lower (**LT3S+)** vs higher **(HT3S+**) were processed through GSEA.

expression of TRβ, this is unlikely given that neither TR is responsive to T3 in the brain [39]. These data need to be confirmed, including measurement of protein levels, but at face value, it indicates that TRβ might play a dominant role in TH signaling in the temporal pole.

The finding of a positive correlation between T3S+ and the expression of TH transporters suggests that TH transporters are limiting to TH signaling in the human temporal pole. This of course, is only if the transporters follow their mRNA levels. The available data indicate that the absence of functional transporters is limiting to TH signaling [12, 13]. However, it is accepted that as long as there is a minimum level of transporters available, additional transporter units are not expected to enhance TH signaling. This is because movement of T3 molecules across the membrane follows a concentration gradient, as T3 transport is not active. Thus, the present studies indicate that TH transporters might be limiting to TH signaling in some settings, even in the absence of inactivating mutations. These findings also beg the question of whether it is the availability of TH transporters that modulate TH signaling or vice versa. While we know that transporters facilitate TH signaling in the brain [12, 13], it remains unclear whether TH signaling affects the expression of TH transporters. Of note, changes in mRNA levels are small and there is conflicting evidence, with great interspecies variability [38]. However, the fact that the correlation between 2 transporters and T3S+ was moderate/strong, makes it likely that in the human temporal pole, TH transporters through which T3 can enter target cells are limiting and affect TH signaling. In contrast, T3S+ did not correlate with the expression of 2 OATPs (Table 2), transporters that preferentially facilitate the flux of T4 in/out of cells.

It is unexpected that the intensity of T3S+ also did not correlate with the expression of deiodinases (Table 2). Glia-specific D2KO animals exhibit a phenotype of brain hypothyroidism [40] and the global D3KO mouse exhibit a phenotype of brain thyrotoxicosis [41, 42]. At face value, this suggests that within this narrow range of TH signaling in the human brain, deiodinases play a more homeostatic role preserving TH signaling, rather than defining it. The lack of correlation could also be explained by other factors that affect deiodinase synthesis. For example, all deiodinases are selenoproteins, which require cotranslational incorporation of selencysteine into the nascent polypeptide chain [43]. Thus, mRNA levels in this case might not be the only limiting factor for the expression of the active deiodinases. In addition, there are specific mechanisms for D2 and D3 regulation. D2 is mostly regulated at posttranslational level via ubiquitination [44, 45]; D3 is not known to be ubiquitinated but has been shown to suffer posttranslation changes in subcellular localization, as it relocates to the nuclear membrane and reduces TH signaling [46].

TH coactivator and corepressors are not regulated by TH signaling in the postnatal developing cerebellum [47]. We are not aware of similar studies performed in other brain areas. Thus, the positive correlation between T3S+ and the expression of CARM1, SRC2-3, and MED1 is reassuring that these molecules positively affect TH signaling in the temporal pole (Table 2). Likewise, the negative correlation between NCOR2 and T3S+ suggests that the expression of this corepressor dampens TH

signaling in the temporal pole. However, it was unexpected to see that neither TRα or SRC1 mRNA levels correlated with T3S+, and that KAT2B correlated negatively with T3S+. Both TRs are expressed in brain and mediate TH signaling, but TRα1 protein account for about 70% to 80% of total receptor content [48]. Likewise, at least in the rat brain, SRC1 is expressed ubiquitously [49]. The significance of these findings is unknown, but it could suggest that neither TRα nor SRC1 is unlikely to constitute a limiting factor for TH signaling in the temporal pole. KAT2B is an acetyl transferase previously shown to interact with TR and coactivate T3-induced gene expression [50]. However, KAT2B also coregulates other important pathways in the central nervous system, including the Hedgehog-Gli pathway [51], which inhibits TH signaling [52, 53], possibly explaining the inverse relationship with T3S+.

The analyses of the gene sets that correlated with **T3S+** revealed important cellular functions in the human brain that fluctuate with TH signaling, for example, to synaptic signaling, synaptic vesicle recycling and transport, and regulation of neurotransmitter levels and release (Supplementary Tables 18-20 [36]), all carrying a high energetic cost. Thus, it is not surprising that TH signaling accelerates metabolism and energy expenditure in the human brain. In fact, this is a concept that has evolved over time. In the 1950s, while measuring cerebral blood flow and O_2 consumption, investigators failed to detect differences between hyperthyroid patients and normal subjects [54, 55]. The idea of brain refractoriness to TH signaling persisted for decades until more accurate measurements could be performed. The utilization of 2-[$^{(14)}$C]-deoxyglucose in mice revealed that TH signaling accelerates glucose utilization via TRα and not TRβ. While glucose utilization remained almost identical in 19 brain regions of the homozygous TRβ-PV mutant mice and their wild-type siblings, it was markedly reduced in all brain areas of the heterozygous TRα-1PV mice [56]. In humans, this was elegantly demonstrated with the use of positron emission tomography (PET) with [$^{(18)}$F]- deoxyglucose to compare the relative regional cerebral glucose metabolism between untreated hypothyroid patients and healthy volunteers [57]. Hypothyroid patients exhibited lower regional activity than control subjects in the amygdala, hippocampus, and areas of the anterior cingulate cortex, and right posterior cingulate cortex. These differences were abolished with TH replacement therapy, restoring metabolic activity in brain areas that are integral to the regulation of affect and cognition [57]. In contrast, [$^{(18)}$F]- deoxyglucose PET studies in hyperthyroid patients showed a decreased glucose metabolism in the limbic system (uncus and inferior temporal gyrus), but increased anxiety yielded a positive correlation with glucose metabolism in the bilateral sensory association cortex [58].

The present studies have several limitations. Although the donors were not known to have a history of neurologic or thyroid disease, we did not have serum thyroid function tests documenting biochemical euthyroidism at or before the time of death. Also, we did not measure brain tissue T3 content, but the samples were frozen, not perfused, thus were not appropriate for this measurement. In these *in silico* analyses, we did not measure protein levels of the TH transporters, deiodinases, or TRs. These 19 donors were relatively homogenous in demographic characteristics: all male, Caucasian, and adults. Thus, whether these results extrapolate to more diverse populations, or other brain regions, is unknown. Another limitation is the difficulty in demonstrating the homogeneity of the samples. We used the expression of genes typically expressed in different cell types of the central nervous system (Supplementary Table 3 [36]) but it is conceivable that these genes might very well be responsive to T3 as well. We know these samples were dissected by a neuroanatomist but small variations among samples might exist. Even considering all of these limitations, the strengths of the study include the relatively large number of high-quality, anatomically homogenous samples from healthy, young adult, donors that expired from traumatic accidents. The **T3S+** was validated against a panel of 27 genes known to be involved in TH signaling by Factor analysis, high degree of correlation with NT3S+ and TRβ expression. A randomly generated index RT3S+ exhibited no significant correlations.

In conclusion, the transcriptome of the human temporal pole can be assessed through an index of TH signaling T3S+ and was found to correlate with subtle variations in TH signaling. The expression of several hundred genes related to synaptic signaling and cellular metabolism as well as other multiple cellular processes, correlated positively with T3S+, within a narrow ~10% window. It is fascinating to see that TH signaling in the temporal pole exhibits subtle fluctuation that is likely to have significant physiological/pathophysiological consequences. At this time, we do not know the source of this fluctuation, which could due to normal physiological variations around an optimal level, or just reflective of individual-specific variations in the brain setup of TH signaling parameters, or both.

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Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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