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**CD4<sup>+</sup> T cells affect the thyroid hormone transport at the choroid plexus  
in mice raised in enriched environment**

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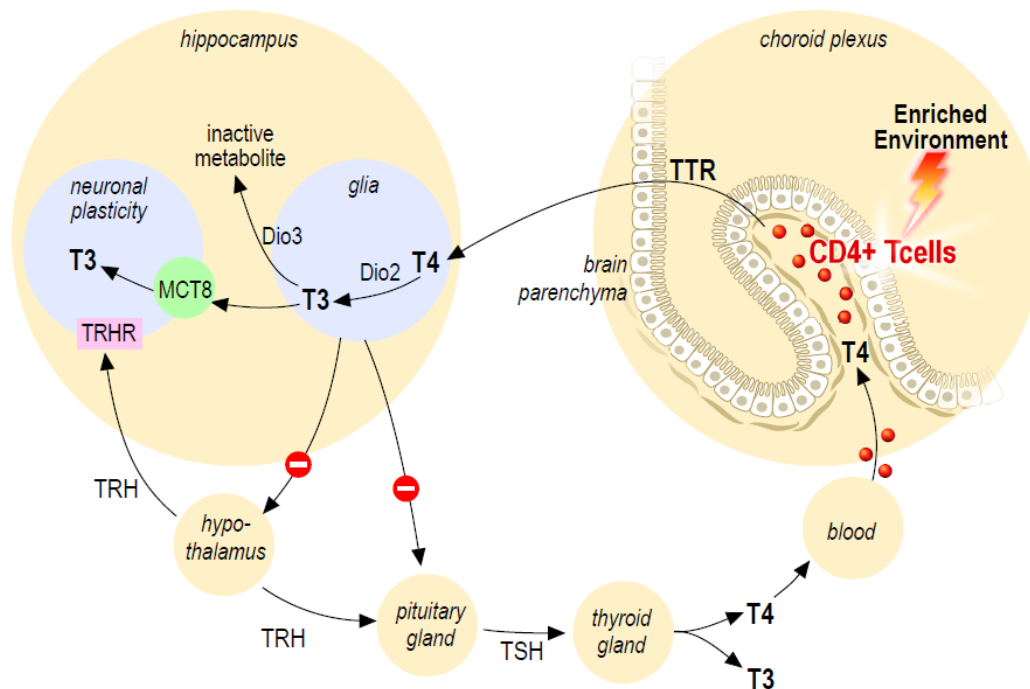
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## HIGHLIGHTS

- In the choroid plexus, CD4<sup>+</sup> T cells regulate the expression of transthyretin (TTR), the major transporter of thyroxine (T<sub>4</sub>) to the brain parenchyma.
- CD4<sup>+</sup> T cells influence EE-induced hippocampus plasticity via the thyroid hormone signaling.
- Close interactions between the immune and neuroendocrine systems are influencing the brain plasticity and function.

## GRAPHICAL ABSTRACT



## KEYWORDS

Enriched environment, CD4<sup>+</sup> T cells, hippocampus, brain plasticity, choroid plexus, thyroid system, mice

## ABSTRACT

Others and we have shown that T cells have an important role in hippocampal synaptic plasticity, including neurogenesis in the dentate gyrus (DG) and spinogenesis and glutamatergic synaptic function in the CA of the hippocampus. Hippocampus plasticity is particularly involved in the brain effects of the enriched environment (EE) and interestingly CD4<sup>+</sup> and CD8<sup>+</sup> T cells play essential and differential roles in these effects. However, the precise mechanisms by which they act on the brain remain elusive. Here, we show that CD4<sup>+</sup> T cells may influence EE-induced hippocampus plasticity via the thyroid hormone signaling by regulating in the choroid plexus the expression of transthyretin (TTR), the major transporter of thyroxine (T<sub>4</sub>) to the brain parenchyma. Our study highlights the contribution of close interactions between the immune and neuroendocrine systems in the brain plasticity and function.

## 1. INTRODUCTION

Living environments profoundly influence brain plasticity, particularly in the hippocampus, a brain structure that is involved in learning and memory regulation. In a previous study, we found that CD4<sup>+</sup> T cells are modified in EE relative to a standard environment (SE) leading to enhanced brain synaptic plasticity, including synaptogenesis, spontaneous excitatory synaptic transmission and synaptic plasticity.

In the present study, we focused our investigations on the role played by CD4<sup>+</sup> T cells in this reshaping of the brain in EE housing. In physiological conditions, CD4<sup>+</sup> T cells do not enter the brain parenchyma, but they are present in the blood vessels, the meninges and the choroid plexus. The choroid plexus are made of an epithelial barrier that separates the blood from the CSF contained in the ventricles. The CD4<sup>+</sup> T cells present on the choroid plexus of the lateral and third ventricles, by their strategic location close to the hippocampus, are most likely to influence the hippocampal plasticity. We hypothesized that their action could be either by secreting factors such as cytokines or chemokines that could diffuse and influence directly or indirectly (by acting on perivascular macrophages, microglia and/or astrocytes) hippocampal plasticity, or by modifying the properties of the endothelial cells of the choroid plexus by contact or through the action of these secreted factors.

We demonstrate that CD4<sup>+</sup> T cells could influence EE-induced hippocampal plasticity by a mechanism involving the thyroid hormone transportation through the epithelial barrier of the choroid plexus.

## 2. MATERIALS AND METHODS

### 2.1 EE breeding conditions

C57BL6/J female mice (Janvier Labs, France) were exposed to EE from 4 weeks after birth (i.e. at weaning) for a 1, 2, 3 or 4-week period as described in (Hosseiny et al., 2015). 82 C57BL6/J mice were used for this study. Age-matched mice (12-15) were housed in large-sized cages (9120 cm<sup>2</sup>; L x l x h: 120 x 76 x 21 cm) with nesting material, houses, running wheels, hammocks, scales, plastic toys and tunnels. Objects were changed twice a week. All mice had access to tap water and standard lab chow (diet SAFE A04, 2900 kcal/kg) *ad libitum* and were housed in a 12 hr light/12 hr dark cycle at 22-23°C with 40-60% humidity.

All animal studies were carried out in accordance with French standard ethical guidelines for laboratory animals (Agreement N° 75-178, 05/16/2000) and the European Communities Council Directive of 24 Nov 1986 (86/609/EEC), in compliance with the Institutional Animal Care and Use Committee of the University of Nice-Sophia Antipolis (permission number 010344.01) from the French “Ministère de l’Enseignement Supérieur et de la Recherche”. Formal approval to conduct the experiments described was obtained from the animal subjects review board of this institution and can be provided upon request. All efforts were made to minimize the number of animals used and their suffering.

### 2.2 T cell depletion

To achieve selective T cell sub-population depletion, each 3-week-old female mouse (C57BL6/J) was injected *ip* with 0.5 mg depleting antibody: either anti-CD4 Ab Ig (rat IgG2b, clone GK1.5, Ref BE0003-1 from BioXCell or from hybridomas culture supernatant ATCC<sup>®</sup> No. TIB-207<sup>™</sup>), or with control isotype Ab (control IgG; clone LTF-2, Ref BE0090 from BioXCell). The animals analyzed in each experiment were randomized, treated either with control or anti CD4 antibody.

Three days after the first injection, mice were placed in EE housing conditions, where they received the second and third injections (0.3 mg), 10 days apart each. The control groups received control antibody at the same time. 8 days after the last antibody injection, the mice were sacrificed, spleens were harvested and immune cells prepared to control for the absence of CD4<sup>+</sup> T cells by using flow cytometry with the anti-CD3 and anti-CD4 antibodies. No depletion was observed in control Ab-injected mice spleen, while around 98% depletion was observed with the anti-CD4 antibody (Zarif et al., 2018a).

## **2.3 Gene expression profiling using Agilent system**

### **2.3.1 RNA extraction**

Total RNAs from hippocampus were isolated using the Trizol<sup>®</sup> RNA extraction kit (Invitrogen) according to the manufacturer recommendations followed by a RQ1 DNase (Promega) treatment. First-strand cDNA were synthesized from 2µg of total RNA with 200U of SuperScript III reverse transcriptase (SuperScriptIII, Invitrogen) in the appropriate buffer in the presence of 25µg/ml random primers, 0.5mM deoxyribonucleotide triphosphate mix, 5mM dithiothreitol, 40U RNAsin (Promega). The reaction was incubated 5 min at 25°C, then 50 min at 50°C then inactivated 15 min at 70 °C. Quantitative PCR was performed using the SYBRgreen method (Roche) with the LightCycler 480 sequence detector system (Roche Diagnostics). β-actin and GAPDH were used as reference genes for normalization. Primers were purchased from QIAGEN (QuantiTect primer assay, QIAGEN).

### **2.3.2 Array analysis**

mRNA expression profiling was performed with high density SurePrint G3 gene expression mouse microarrays in accordance with the protocol described by the manufacturer (Agilent).

Microarray data analyses were performed using R (<http://www.r-project.org/>). The quality control was performed using the Bioconductor package arrayQualityMetrics and custom R scripts. Additional

analyses were performed using Bioconductor package Limma. Briefly, data were normalized using the quantile method. Replicated probes were averaged after normalization and control probes removed. We used a linear modeling approach to calculate log ratios, moderated t statistics, log odds ratios of differential expression (B statistic), and P values for each comparison of interest. P values were adjusted for multiple testing using the Benjamini and Hochberg method, which controls the false discovery rate. Differentially expressed genes (Figure 4A) were selected based on a P-value under 0.01 and a log<sub>2</sub> (fold change) > 0.58 or < -0.58. Enrichment in biological themes (molecular function, upstream regulators and canonical pathways) and biological network analyses were performed using Ingenuity Pathway Analysis software (IPA™, <http://www.ingenuity.com/>).

The experimental data and microarray design have been deposited in the NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>), accession number GSE115507.

## **2.4 Cerebrospinal fluid (CSF) samples**

CSF samples were taken from the cisterna magna using the method described in (Liu and Duff, 2008). Mice were anesthetized and placed prone on the stereotaxic instrument. A sagittal incision of the skin was made inferior to the occiput. Under the dissection microscope, the subcutaneous tissue and neck muscles through the midline were bluntly separated. A microretractor was used to hold the muscles apart. Then the mouse was laid down so that the body made a 135° angle with the fixed head. In this angle, the dura and spinal medulla were visible, had a characteristic glistening and clear appearance, and circulatory pulsation of the medulla (i.e., a blood vessel) and the adjacent CSF space could be seen. The dura was then penetrated with a 6-cm-long glass capillary that had a tapered tip with an outer diameter of 0.5 mm. Following a noticeable change in resistance to the capillary insertion, the CSF flowed into the capillary. The average volume of CSF obtained was approximately 4 µl. All samples were stored at -70°C until analysis.



## **2.5 ELISAs**

Thyrotropin-Releasing Hormone (TRH) levels in hypothalamus, hippocampus and plasma were measured using a TRH ELISA kit (Wuxi Donglin SCI&TECH development CO., LTD) according to the manufacturer's guidelines. Thyroid Stimulating Hormone (TSH) levels in plasma were measured by ELISA TSH kit (ref CEA463Mu). FT4 levels in plasma were measured by ELISA (Arbor Assays K050H1).

Choroid plexus were then dissected and homogenized in lysis buffer solution (25 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, protease inhibitor cocktail). TTR measurement in mouse CSF and choroid plexus was performed with a TTR ELISA assay according to manufacturer's recommendations (Cloud-Clone Corp.). For comparison, data were normalized relative to the initial CSF volume or protein concentration, respectively for CSF and choroid plexus.

## **2.6 Data analysis**

Two-way ANOVA, followed by a post-hoc Tukey's multiple comparison tests were performed when data followed the normality Shapiro-Wilk test. Otherwise, the non-parametric ANOVA Kruskal-Wallis test was used followed by a post-hoc + Dunn's test.

### 3. RESULTS

#### 1- CD4<sup>+</sup> depletion regulates Thyrotropin-releasing hormone (TRH) and Thyrotropin-releasing hormone Receptor (TRHR) genes expression in the hippocampus of EE mice

In order to orientate our research on the mechanisms of action of CD4<sup>+</sup> T cells on EE-induced changes in synaptic plasticity, we compared the genes expressed in hippocampus tissue from mice raised for 4 weeks in EE and treated with anti-CD4 (n=4) vs. control antibodies (n=4) by DNA microarray (Agilent, Fig 1). Interestingly, in the CD4<sup>+</sup> T cell depleted group compared to the control group, the *Trh* gene showed the greatest increase in expression ( $\log_2FC= 3.14$ ,  $p<0.13$ ) and *Trhr*, the gene coding for the TRH receptor was also modulated. In a previous study, we have shown that TRH is able to modulate synaptic plasticity (Zarif et al., 2016). The present result pointed to a role of CD4<sup>+</sup> T cells on the thyroid system.

Thyroid hormones (TH) normally exerts a feedback on the TRH system (Chiamolera and Wondisford, 2009). We hypothesized that up-regulation of TRH and TRH receptor gene expression in hippocampus tissue from CD4<sup>+</sup> T cells deprived mice could be a consequence of a lower penetration of TH in hippocampus due to a down regulation of thyroid hormone transporters (see Schema in Fig 2).

#### 2- CD4<sup>+</sup> T cell depletion affects transthyretin levels in choroid plexus and CSF of EE mice

We used RT-qPCR to investigate in choroid plexus from the lateral and third ventricles the variations in expression of transcripts encoding for transthyretin (TTR, encoded by *Ttr* gene) and the MCT8 transporter (encoded by *Slc16a2* gene), two proteins involved in the transport of TH from CSF to the brain parenchyma.

We found (Fig 3A) that choroid plexus samples from mice raised 3 weeks in EE and treated with anti-CD4 depleting antibodies, relative to control antibodies, showed a significant decrease in RNAs coding for TH transporters TTR MCT8 (*Slc16a2*).

We chose to focus on TTR protein because it is secreted in the CSF and transports the TH from the epithelium of the choroid plexus to the hippocampus parenchyma. We measured the TTR protein levels in the choroid plexus and CSF from mice raised 4 weeks in EE and depleted or not from CD4<sup>+</sup> T cells, by ELISA (Fig 3B). Interestingly, we found that anti-CD4 antibodies treatment decreased TTR levels in both choroid plexus and CSF. Therefore, in EE, CD4<sup>+</sup> T cells seem to exert a positive regulation on TTR expression in the choroid plexus, leading to higher levels of this protein in the CSF. This is in accordance with the results of Fig 1 showing an up-regulation of TRH and TRH-R gene expression in CD4<sup>+</sup> depleted mice raised in EE, which could be a consequence of a lowering of the negative feed-back exerted by TH.

### **3- CD4<sup>+</sup> T cell depletion affects hippocampal TH- regulated gene expression**

In order to estimate the local variations of TH in the hippocampus, we analyzed the expression in hippocampus of genes positively regulated by TH (*Hr*, *Dio2* and *Dio3*), which are also involved in TH metabolism and regulation. In hippocampus from mice raised in EE and treated either with control or anti-CD4 antibodies (Fig 3C), CD4<sup>+</sup> T cell depletion induced a significant increase in *Dio2* expression without affecting *Dio3* or *Hr*. Increased *Dio2* expression is a typical response of the brain to TH insufficiency, indicating an attempt to maintain brain T<sub>3</sub> levels by increasing conversion of T<sub>4</sub> to T<sub>3</sub> (Guadano-Ferraz et al., 1999; Herwig et al., 2014), suggesting that CD4<sup>+</sup> T cell depletion may have induced a local decrease of TH transport to the hippocampus.

Overall, our results suggest that CD4<sup>+</sup> T cell depletion induced perturbations of the TH transport at the level of the choroid plexus and TH signaling in the hippocampus.

#### **4- CD4<sup>+</sup> T cell depletion affects peripheral TH levels in mice raised in EE**

TRH, by binding to its receptors (TRHR), stimulates the release of thyroid-stimulating hormone (TSH) by the pituitary, which itself induces the release of T<sub>4</sub> from the thyroid gland (Schroeder and Privalsky, 2014). We used an ELISA test to measure plasmatic free TSH and free T<sub>4</sub> (FT<sub>4</sub>) from mice raised in EE and treated with anti-CD4 or control antibodies. Interestingly, we found that CD4<sup>+</sup> T cell depletion induced a significant increase in plasmatic FT<sub>4</sub>, compared to mice treated with control antibodies raised in the same conditions (Fig 3D) and the increase in TSH was close to significance ( $p < 0.07$ ). The increase in plasmatic FT<sub>4</sub> observed in CD4<sup>+</sup> T cell depleted mice is in accordance both with the increase in *Trh* gene expression observed in this condition (Fig 1) and with the decrease in TH transportation to the brain tissue due to TTR decrease. Thus, the CD4<sup>+</sup> T cell depletion may also have repercussion in the periphery.

#### 4. DISCUSSION

In a previous study, we showed that CD4<sup>+</sup> T cells play a major role in EE-induced changes in hippocampus synaptic plasticity (Zarif Hadi et al., in press). We then hypothesized that this could be because T cells, by their strategic location at the interface between periphery and brain, could influence the transport of molecules between them. A large proportion of T cells in the brain are located in the choroid plexus. The choroid plexus is a highly vascularized structure made of endothelial and epithelial cells that produces CSF from blood. The lateral and third ventricles line the hippocampus, which makes T cells located here putative strategic contributors to influence hippocampal plasticity. We speculated that CD4<sup>+</sup> T cells found in the choroid plexus would influence (either by direct contact or indirectly by secreting factors) the transport of molecules through the choroid plexus epithelium than can then reach the CSF and the brain parenchyma (Fig 2). We first investigated by DNA microarray (Agilent) if there were variations in gene expressions in the hippocampus of EE mice whose CD4<sup>+</sup> T cells had been depleted relative to those receiving control Ab. The results of our study (Fig 1) pointed to the thyroid system, as both *Trh* and *Trhr* transcripts were up regulated in absence of CD4<sup>+</sup> T cells. TRH and TRHR expressions are under the retro-control of TH. We thus hypothesized that CD4<sup>+</sup> T cell depletion could lower the TH level in the hippocampus due to an impaired transport of TH to the brain. In support of this suggestion, we found that CD4<sup>+</sup> T cell depletion blunts the expression of several choroid plexus genes such as *Slc16a2* and *Ttr*, coding for proteins involved in TH transport.

Among the proteins involved in the transport of TH, TTR is synthesized early in development by the liver and the choroid plexus of the brain (Richardson et al., 2015) where it contributes to the transport of T<sub>4</sub> from the blood to the CSF and could have a trophic effect on neurons (Gomes 2016). In adult mice, TTR is present in the hippocampus, although it is not clear whether it is locally produced by neurons (Li et al., 2011; Stein and Johnson, 2002) or produced by the choroid plexus and then

transported to the hippocampus (Richardson et al., 2015; Sousa et al., 2004). TTR can contribute to the transport of TH from choroid plexus to the brain parenchyma as it is secreted in the CSF.

A measure of TTR protein level in CSF and choroid plexus revealed a down regulation of TTR production in CD4<sup>+</sup> T cell depleted mice, confirming our hypothesis that CD4<sup>+</sup> T cells could up regulate the TH transport to the brain.

In cerebral tissue (Fig 2), 80% of T<sub>4</sub> is locally transformed into T<sub>3</sub> through the activity of the 5'9-deiodinase type 2 (DIO 2), an enzyme that is expressed mostly by glial cells, tanycytes in the third ventricle, and astrocytes (Crantz et al., 1982). Its activity is increasing in situations of low T<sub>4</sub>, thus preserving brain T<sub>3</sub> concentrations (Schroeder & Privalsky 2016). The MCT8 transporter transports T<sub>3</sub> into brain neurons (Friesema et al., 2005) and T<sub>3</sub> is degraded by Deiodinase 3 (DIO3) in inactive metabolites (Escamez et al., 1999).

In control EE mouse choroid plexus, we observed a peak of expression of *ttr* (and *SLC16a2*) at 3W that was abolished in choroid plexus from CD4<sup>+</sup> T cell depleted mice. In agreement, we found after 4 weeks of EE housing a decrease in TTR protein level in both choroid plexus and CSF from CD4<sup>+</sup> T cell depleted mice compared to control mice.

A TTR downregulation in CD4<sup>+</sup> T cell depleted animals would lead to a lower T<sub>4</sub> transportation towards the brain parenchyma inducing a lower feedback control of TH on TRH and TSH, and thus an increase in their production, inducing the plasmatic elevations of both TSH and T<sub>4</sub> levels that we observed in our experiments. Of note, the changes in plasmatic TSH and FT<sub>4</sub> we observed in CD4<sup>+</sup> T cell depleted mice raised in EE were low in comparison with those found for instance in hyperthyroidism, which are then multiplied by 10-20 (Rakov et al., 2017), and this makes very unlikely a direct influence of CD4<sup>+</sup> T cells on the thyroid or pituitary gland function.

Similarly, a down-regulation of TTR expression in choroid plexus should lead to a decreased level of TH in the brain parenchyma. We did not measure the TH levels directly in hippocampus for technical reasons. However, in CD4<sup>+</sup> T cell depleted animals, we observed a *Dio2* transcript expression

increase with no change in *Dio3*, which could constitute a mechanism of compensation to restore a sufficient level of T<sub>3</sub> in EE. Indeed, increased *Dio2* expression is a typical response of the brain to TH insufficiency, indicating an attempt to maintain brain T<sub>3</sub> levels by increasing conversion of T<sub>4</sub> to T<sub>3</sub> (Guadano-Ferraz et al., 1999; Herwig et al., 2014).

TH act on neurons and promote brain plasticity, for instance neurogenesis (Gilbert et al., 2017) and LTP (Bernal, 2000; Calza et al., 1997). In addition, TH exert feedback regulation of TRH production, thus maintaining a balance between TRH and TH (Ortiga-Carvalho et al., 2016). We have shown that TRH can block LTP by interacting with NMDA receptors (Zarif et al., 2016). CD4<sup>+</sup> T cell depletion, by impairing the TTR production, could affect the TH-TRH balance, which could directly influence neurons (neurogenesis, LTP). Similarly, TRHR1 is expressed in granule cells of the DG where it influences neurogenesis (Bernal, 2007) and we have recently shown that TRH affects synapse activity (Zarif et al., 2016). Finally, TTR itself has recently been shown to promote neurite outgrowth and to have neuroprotective properties (Sousa et al., 2007b) by interacting with different receptors such as megalin and IGF1R (Gomes et al., 2016) and could therefore act directly to promote plasticity. However, this hypothesis is difficult to test as it is challenging to quantify TTR levels in isolated hippocampus tissue due to recurrent contamination by adherent choroid plexus cells (Sousa et al., 2007a).

Other indirect pathways could also explain this phenomenon. For instance, TH could also stimulate ApoE in astrocytes, which modulates lipid synthesis and is permissive for the increase in synaptic plasticity (Kim et al., 2014; Roman et al., 2015). TH could also modify the microglial profile and thus regulate their capacity to regulate synaptic contacts. Furthermore, other putative factors, apart from the thyroid system, could also mediate the effects of CD4<sup>+</sup> T cells on brain plasticity. Indeed, choroid plexus epithelial cells have been shown to produce other neurotrophic factors (Thouvenot et al., 2006). Finally, some factors produced by CD4<sup>+</sup> T cells in EE such as adiponectin, which has been shown to be produced by these cells in under certain conditions (Crawford et al., 2010; Danturiti et al.,

2017) could favor brain plasticity (Nicolas et al., 2015; Zhang et al., 2016). Overall, our results suggest that the thyroid system could be involved in the way CD4<sup>+</sup> T cells influence EE-induced hippocampal plasticity. However, the situation is complex because EE promotes neurogenesis and hippocampal plasticity while decreasing at 4 weeks the amount of TTR present in choroid plexus. This suggests that EE triggers other mechanisms promoting brain plasticity, independently of the TTR transporter and TH signaling (Zarif et al., 2018b).

In conclusion, we propose, for the first time, a possible pathway by which CD4<sup>+</sup> T cells could influence the hippocampal plasticity promoted by enriched environment. This pathway involves the control of gene expression, including *Ttr*, in choroid plexus epithelial cells. In turn, this may enable the permissive effects of the thyroid system on the plastic changes elicited by enriched environment.



## Figure Legends

**Figure 1: Agilent analysis of genes expressed in hippocampus of mice raised in EE and treated with control (n= 4) vs. CD4 antibodies (n=4)**

**A-** MA-plot for the comparison between hippocampus from mice raised in EE treated with control vs. anti-CD4 antibodies. X-axis: average gene expression levels. Y-axis: log<sub>2</sub> Fold-Change between control and CD4 antibodies. Red dots: p value < 0.01

**B-** Heatmap of the 42 most differentially expressed genes when comparing hippocampus from mice raised in EE treated with control vs anti-CD4 antibodies. Expression levels are gene centered, so that all genes have an average expression level equal to zero. Hierarchical clustering of the samples and genes used Euclidean distance and complete linkage. logFC > 0.58 or <-0.58, p<0.01.

**C-** Gene expression levels of Trh and Trhr genes.

Symbol	baseMean	log2FoldChange	pvalue
Trh	9.60	3.15	7.1 e-4
Trhr	10.04	0.77	1.2 e-3

**D-** Top canonical pathways significantly enriched when comparing genes expressed in hippocampus from mice enriched environment treated with control vs anti CD4 antibodies with an IPA analysis.

**Figure 2: Schema illustrating the permissive effect of CD4<sup>+</sup> T on enriched environment-induced hippocampus plasticity through thyroid system regulation.**

Enriched environment modifies the phenotype of CD4<sup>+</sup> T cells, including those located at the choroid plexus. These CD4<sup>+</sup> T cells regulate transthyretin (TTR) and Claudin 2 and 11 expressions at the choroid plexus blood / CSF epithelial barrier, influencing the T<sub>4</sub> transport from the blood to the CSF and the brain parenchyma. T<sub>4</sub> is then converted to T<sub>3</sub> in astrocytes and tanycytes by Dio2 and which acts on hippocampal neurons together with TRH to regulate neurogenesis and synaptic plasticity.

CD4<sup>+</sup> T cell depletion, by down regulating TTR expression induces a decrease in T<sub>4</sub> in the hippocampus, as reflected by the expression of genes such as Dio2, Dio3 and Hr, and a decrease in the T<sub>3</sub> negative feedback on TRH, TSH and T<sub>4</sub>, resulting in an increase in FT<sub>4</sub> levels in the blood.

**Figure 3: CD4<sup>+</sup> T cell depletion affects the thyroid system in brain and periphery**

**A-** Transcript levels of TH transporters *Ttr* (left) and *Slc16a2* (right) in choroid plexus measured using RT-qPCR. Choroid plexus from the lateral and third ventricles were micro-dissected and gene levels were determined by quantitative PCR from the brains of mice from different housing durations (2w, 3w and 4w) in SE or EE conditions. Bars represent the mean expression levels  $\pm$  SEM of the  $2^{-\Delta\Delta C_t}$ . For each gene, we first performed a Kruskal-Wallis test on the  $\Delta C_t$  values followed by an exact two-sample Fisher-Pitman permutation test comparing SE to EE at 3W and 4W, and comparing each group (SE or EE) 3W to 2W and 4W to 2W. The p values were corrected using the FDR method of Benjamini-Hochberg for 8 comparisons per gene. \*:  $p < 0.05$ , N=6 for each group.

**B-** Relative TTR protein levels in CSF (left) and choroid plexus (right) measured by ELISA. Each dot represents one mouse.

**C-** Transcript levels of the TH-regulated genes *Hr*, *Dio 2* and *Dio3* in dissected hippocampus from mice of the 4 different groups measured using RT-qPCR. N=6 per group.

**D-** Plasmatic levels of TSH (left) and FT<sub>4</sub> (right) measured by ELISA.

Each dot represents one mouse. Histogram represents the mean  $\pm$  sem.

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