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Microglia Density and Its Association With Disease Duration, Severity, and Orexin Levels in Patients With Narcolepsy Type 1

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Glossary

BMI = body mass index; MNI = Montreal Neurological Institute; MSLT = Multiple Sleep Latency Test; NSS = Narcolepsy Severity Scale; NSS-P = NSS-pediatric version; NT1 = narcolepsy type 1; PMOD = peripheral module; ROI = region of interest; SUV = standard uptake value; SUVr = SUV ratio; TSPO = 18 kDa translocator protein.

Abstract

Background and objectives:

Narcolepsy type 1 (NT1) is due to the loss of hypothalamic neurons that produce orexin (ORX), by a suspected immune-mediated process. Rare postmortem studies are available and failed to detect any inflammation in the hypothalamic region, but these brains were collected years after the first symptoms. In vivo studies close to disease onset are lacking. We aimed to explore microglia density in the hypothalamus and thalamus in NT1 compared with controls using [¹⁸F]DPA-714 PET and to study in NT1 the relationships between microglia density in the hypothalamus and in other regions of interest (ROIs) with disease duration, severity, and ORX levels.

Methods:

Patients with NT1 and controls underwent a standardized clinical evaluation and [¹⁸F]DPA-714 PET imaging using a radiolabeled ligand specific to the 18 kDa translocator protein (TSPO). TSPO genotyping determined receptor affinity. Images were processed on peripheral module interface using standard uptake value (SUV) on ROIs: hypothalamus, thalamus, frontal area, cerebellum, and the whole brain. SUV ratios (SUVr) were calculated by normalizing SUV with cerebellum uptake.

Results:

A total of 41 patients with NT1 (21 adults, 20 children, 10 with recent disease onset <1 year) and 35 controls were included, with no significant difference between groups for [¹⁸F]DPA-714 binding (SUV/SUVr) in the hypothalamus and thalamus. Unexpectedly, significantly lower SUVr in the whole brain was found in NT1 compared with controls (0.97 ± 0.06 vs 1.08 ± 0.22 , $p = 0.04$). The same finding between NT1 and controls in the whole brain was observed in those with high or mixed TSPO affinity ($p = 0.03$ and $p = 0.04$). Similar trend was observed in the frontal area in NT1 (0.96 ± 0.09 vs 1.09 ± 0.25 , $p = 0.05$). In NT1, no association was found between SUVr in different ROIs and age, disease duration, severity, or ORX levels.

Discussion:

We found no evidence of in vivo increased microglia density in NT1 compared with controls, even close to disease onset, and even unexpectedly a decrease in the whole brain of these patients. These findings do not support the presence of neuroinflammation in the destruction process of ORX neurons.

Trial registration information:

ClinicalTrials.org [NCT03754348](https://clinicaltrials.gov/ct2/show/study/NCT03754348).

Introduction

Narcolepsy type 1 (NT1) is a rare chronic sleep disorder of hypersomnolence often associated with cataplexy,¹ resulting from the loss of a small population of hypothalamic neurons that produce wake-promoting orexin/hypocretin peptides.^{2,3} Several arguments favor the involvement of an immune-mediated process and more specifically a T-cell-mediated mechanism in the pathogenesis of NT1.⁴ However, the precise mechanisms by which orexin neurons disappear from the CNS remain unclear.⁴ Interestingly, there is no evidence for microglial activation in the brain of patients with NT1. Postmortem studies failed to detect any inflammation process in the hypothalamic area,² but these brains were collected years after the first narcoleptic symptoms, and in vivo studies close to disease onset are lacking.⁵

Microglial activation is suspected to be a contributing factor in neuronal death in many neurologic disorders and is often associated with neuroinflammation.⁶ Mitochondrial 18 kDa translocator protein (TSPO) is one of the few available biomarkers of neuroinflammation for which there are clinically available PET imaging agents.⁷ TSPO is highly expressed in microglia and neuroinflammation is associated with increased levels of TSPO and binding sites for TSPO ligands.⁷⁻¹⁰ [18F] DPA-714 is a second-generation TSPO ligand for in vivo imaging PET scan and a sensitive target for imaging microglial/macrophage cell density.⁸⁻¹⁰ [18F]DPA-714 has been largely used in several neurodegenerative and neuroinflammatory disorders to quantify microglial activation such as multiple sclerosis and Alzheimer disease.¹¹⁻¹⁴

Our hypothesis was that microglial activation, in the CNS and especially in the hypothalamic and thalamic areas, would be greater in patients with NT1 than in controls and greater in patients with a short rather than a long disease duration. Our objectives were (1) to explore microglia density in the hypothalamus and thalamus in patients with NT1 compared with controls using brain PET with [18F]DPA-714 and (2) to study the relationships between microglia density in the hypothalamus and in other regions of interest (ROIs) with disease duration, severity, and CSF orexin levels in adults and children with NT1.

Methods

Participants and Design

Consecutive patients with NT1 diagnosed according to the third International Classification of Sleep Disorders criteria¹⁵ were included at the National Reference Center for Orphan Disorders, Narcolepsy and Rare Hypersomnias in Montpellier, France, from January 2019 to June 2021, with particular attention to patients with recent disease onset. They all had

excessive daytime sleepiness and typical cataplexy. They were categorized according to the recent (<1 year) or longer (\geq 1 year) onset of EDS and/or cataplexy. All patients underwent a video-polysomnography followed by Multiple Sleep Latency Test (MSLT), and completed the Narcolepsy Severity Scale (NSS),¹⁶ or its pediatric version (NSS-P).¹⁷ A lumbar puncture was performed for all patients to measure CSF orexin-A levels in duplicate, using the I125-radioimmunoassay kit from Phoenix Pharmaceuticals Inc, according to the manufacturer's recommendations. All values were back referenced to the Stanford reference samples as previously described.¹⁸ CSF orexin-A levels were considered low when <110 pg/mL and undetectable when <26 pg/mL. HLA-DQB1 typing was performed in all patients.

Two samples of controls were included, all with normal neurologic examination and no history of inflammation, neurologic, or psychiatric disorder: patients with mild sleep complaints (sleepiness or insomnia) without criteria for insomnia disorder or hypersomnolence disorder, recruited in the Sleep Unit of Montpellier, and healthy controls recruited from another study in Toulouse University Hospital (COMA-3D STUDY, NCT03482115). All participants (patients and controls) were free of inflammatory disease, and none took immunomodulatory, immunosuppressant, or anti-inflammatory medication. All were drug-free, including treatment for narcolepsy and any medications or substances that may affect sleep or alertness.

Standard Protocol Approvals, Registrations, and Participant Consents

This study was approved by the French ethics committees (Comité de Protection des Personnes [CPP], France; reference EudraCT: 2018-001584-23; CPP Number 18032). All participants provided a written informed consent to participate in the study and both parents for minors.

TSPO Genotype and Cerebral MRI

Blood samples were collected for all participants to genotype the rs6971 polymorphism which affects TSPO bindings. Genotyping analyses were performed in Tours University Hospital using PCR reaction followed by Sanger sequencing on a 3130xl Genetic Analyzer (Thermo Fisher Scientific). Data were analyzed using Sequencing analysis software. Patients were categorized as having high-affinity (C/C genotype), mixed-affinity (C/T genotype), or low-affinity (T/T) binding to TSPO sites. Low TSPO affinity binders were excluded from the analysis. Thirty-four (83%) patients with NT1, 9 (53%) controls from Montpellier, and all controls from Toulouse also underwent a brain MRI.

PET [18F]DPA-714 Imaging

PET scan was performed with a scanner acquisition (CT scan). The PET image acquisition procedure was standardized. Imaging was performed in Montpellier Hospital for 41 patients with NT1 and 17 controls and in Toulouse Hospital for 18 controls (COMA-3D Study NCT03482115) after I.V. injection of 3.5 MBq/kg of [18F]DPA-714.^{19,20} Brain PET and CT images were acquired during 1 hour after injection time.

PET and magnetic resonance (MR) images (when available) were nonlinearly transformed (16 iteration and Gaussian filter 8 mm x 8 mm x 8 mm) in the Montreal Neurological Institute (MNI) space using PET Hemifacil Smap and MR reference provided by peripheral module (PMOD) interface (version 3.5; PMOD Technologies Ltd.). Both transformed PET and MR images were coregistrated using PMOD FUSE module with rigid method. PET images were motion, attenuation, and decay corrected.

The hypothalamic ROI was drawn manually using spatial coordinate in MNI space with the help of MRI when available. 21 Comparison of the area under the curve in the hypothalamic ROI were made between the different coregistration (PET, PET-MRI, and PET-CT) with no significant difference found. Time activity curves were extracted from hypothalamus delimitation using “fraction” ROI integration mode proposed by PMOD. Other ROIs were analyzed: thalamus, frontal area, cerebellum, and the whole brain. Standard uptake value (SUV) was obtained by dividing the concentration in the ROI by the injected activity and by multiplying the result by the individual body weight. SUV ratio (SUV_r) was calculated dividing the SUV of interest (e.g., hypothalamus) per the cerebellum SUV,²² in line with several studies using the cerebellar gray matter as a pseudo-reference region to quantify [18F]DPA-714 binding.^{12,23} SUV and SUV_r of both control groups and narcoleptic patients were compared for the last 10 minutes of the PET acquisition (i.e., 50–60 minutes pi). Data imaging analysis was performed twice by 2 investigators blinded of the diagnosis (L.B., A.K.) to confirm the concordance of the results.

Statistical Analysis

Categorical variables were described using numbers and percentages while continuous variables were described using means and standard deviations. For the whole sample, logistic regression models were implemented to compare TSPO expression in the hypothalamus, thalamus, and other ROI between the control and NT1 groups. Nonparametric tests were used when the sample size was small (<30 in each group). The Mann-Whitney test was used to compare continuous variables between the control and NT1 groups while the χ^2 test was used to compare categorical data between the 2 groups. A correction for multiple comparisons for the SUVs and SUV_rs was applied using the false discovery rate procedure. Spearman rank order correlations were used to determine associations between continuous variables. The significance level was set at $p < 0.05$. The analyses were performed using the SAS statistical software (version 9.4; SAS, Cary, NC).

The study protocol and statistical analysis plan are available in eSAP.

Data Availability

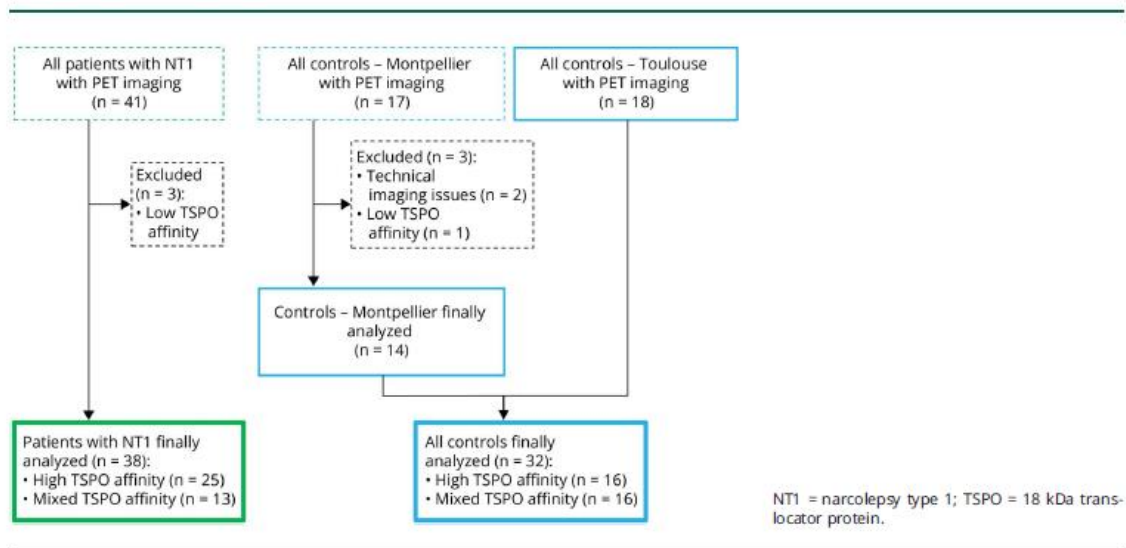
The study leaders will receive requests from researchers who provide a methodologically sound proposal as judged by the leaders for individual participant data that underlie the results reported in this article after de-identification and after the publication of study results within 3 months from initial request.

Results

Forty-one patients with NT1 (21.3 ± 9.9 years, 21 adults and 20 children (>10 years), 20 female) were included. Two samples of controls were included: 17 participants (23.34 ± 3.93 , 9 female) with mild sleep complaints, recruited in the Sleep Unit of Montpellier, and 18 healthy controls (45.94 ± 20.23 years old, 7 female) recruited from Toulouse University Hospital.

Three patients with NT1 with low TSPO affinity and 3 controls from Montpellier (1 with low TSPO affinity and 2 others with technical imaging registration issues) were excluded from all analyses (Figure 1). Controls from Toulouse were older than controls from Montpellier ($p = 0.01$), without differences for sex and body mass index (BMI). Sex and BMI were not different between patients and controls, but patients were younger ($p = 0.002$). The main characteristics of the 38 patients with NT1 finally analyzed (25 with high and 13 with mixed TSPO affinity) and the 2 control groups ($n = 32$, 16 with high and 16 with mixed TSPO affinity) are provided in Table 1. All patients with NT1 were orexin-deficient, except 1 with intermediate levels for whom the lumbar puncture was performed twice (165 pg/mL and 144 pg/mL 19 months after), but with typical cataplexy, HLA DQB1*06:02 allele, and positive MSLT results (mean sleep latency 7.2 min and 2 sleep onset REM periods).

Figure 1 Study Flowchart



PET [18F]DPA-714 Imaging Data in NT1 and Controls

Comparisons of SUV and SUVr in the 5 ROI revealed no difference between the 2 control groups (data not shown), thus allowing to group them into a single group of 32 patients (36.07 ± 19.00 years, 14 female). [18F]DPA-714 binding (SUV and SUVr) in the hypothalamus and thalamus did not differ between patients and controls (Table 2, Figure 2A). Based on their TSPO binding affinity, no SUV and SUVr differences were found in the hypothalamus and thalamus between high or mixed binder patients with NT1 and controls independently. Additional parametric tests were performed to adjust for age across the whole population and results remained unchanged (data not shown). Unexpectedly, significant lower SUVr in the whole brain was found in patients with NT1 compared with controls. The same finding

between patients with NT1 and controls in the whole brain was observed in those with high or mixed TSPO affinity, with a similar trend in the frontal area of NT1 (Table 2).

Table 1 Characteristics of Patients With NT1 and Controls With PET Imaging Analyzed

	Patients with NT1 (N = 38)	Controls from Montpellier (N = 14)	Controls from Toulouse (N = 18)
Sex, female	17 (44.74)	7 (50.00)	7 (38.89)
Age, y^a	38; 21.32 ± 10.07	14; 23.38 ± 4.21	18; 45.94 ± 20.23
Children below 18 y, yes	18 (47.37)	2 (14.29)	0 (0.00)
BMI, kg/m^{2a}	38; 24.79 ± 4.96	14; 22.58 ± 2.47	18; 23.44 ± 4.36
Obesity, yes	7 (18.42)	0 (0.00)	1 (5.56)
TSPO binding			
High	25 (65.79)	6 (42.86)	10 (55.56)
Mixed	13 (34.21)	8 (57.14)	8 (44.44)
Disease symptoms and severity			
Duration of evolution of EDS, mo	38; 45.03 ± 38.20		
<6 mo	3 (7.89)		
6 mo-1 y	3 (7.89)		
1 y-2 y	7 (18.42)		
>2 y	25 (65.79)		
Duration of evolution of cataplexy, mo^a	38; 26.22 ± 21.04		
<6 mo	8 (21.05)		
6 mo-1 y	2 (5.26)		
1 y-2 y	10 (26.32)		
>2 y	18 (47.37)		
Frequency of cataplexy, >1/d	26 (68.42)		
Sleep-related hallucinations, yes	23 (60.53)		
Sleep paralysis, yes	16 (42.11)		
Disrupted nocturnal sleep, yes	20 (52.63)		
Narcolepsy Severity Scale total score^a	20; 33.55 ± 6.58		
Narcolepsy Severity Scale-Pediatric total score^a	17; 27.59 ± 6.86		
Electrophysiologic data			
Nocturnal SOREMP, yes	29 (76.32)		
MSLT mean sleep latency, minutes^a	38; 3.97 ± 3.18		
Number of SOREMP on MSLT^a	38; 3.97 ± 1.17		
More than 2 SOREMP on MSLT, yes	38 (100)		
Biological data			
CSF orexin levels, pg/mL^a	38; 23.58 ± 27.81		
HLA DQB1*06:02 allele, yes	38 (100)		

Abbreviations: BMI = body mass index; EDS = excessive daytime sleepiness; MSLT = Multiple Sleep Latency Test; NT1 = narcolepsy type 1; SOREMP = sleep-onset REM period; TSPO = 18 kDa translocator protein.
^a Continuous variables are expressed as numbers; mean ± SD

In a first sensitivity analysis, we compared patients and controls included only in the Montpellier site, with no difference for age (21.32 ± 10.07 years for NT1 vs 23.38 ± 4.21 years for controls) and sex (17 (45%) female among NT1 vs 7 (50%) among controls). We found a lower binding in the hypothalamus area in patients with NT1 compared with controls (SUVr: 1.42 ± 0.14 vs 1.67 ± 0.60, p = 0.04) in the frontal area (SUVr: 0.96 ± 0.09 vs 1.13 ± 0.37, p = 0.01) and in the whole brain (SUVr: 0.97 ± 0.06 vs 1.11 ± 0.32, p = 0.01). Analyses in high and mixed-TSPO binders independently did not confirm these differences, however

with a similar trend for lower binding in NT1. In a second sensitivity analysis comparing SUV_r in all ROIs between patients with high TSPO affinity and the most recent age at onset of cataplexy (<1 year) to age-matched, sex-matched, and TSPO binder-matched controls, all participants from the Montpellier site (5 patients vs 5 controls), we found no significant difference. In another sensitivity analysis, we excluded pediatric patients (<18 years) by comparing the SUV_r in all ROIs in adult patients with NT1 and adult controls. We found similar results with significant lower SUV_r in the whole brain and frontal area in patients with NT1 (in high, mixed binders and the overall NT1 population), without significant differences in the hypothalamus and thalamus (data not shown). PET [18F]DPA-714 Imaging and Characteristics of NT1

No association was found between SUV_r and age, disease duration, sex, and CSF orexin levels in the different ROI in all patients with NT1, in children with NT1, in adults with NT1, and in mixed and high TSPO affinity binders independently. No differences were found for SUV or SUV_r in all ROIs between patients with recent (<1 year, n = 10) and longer (≥1 year, n = 28) cataplexy onset or between patients with recent (<1 year, n = 6) and longer (≥1 year, n = 32) EDS onset (Figure 2, B.1 and B.2). The patient (male, age 25 years at time of PET imaging with 6 years of disease evolution) with typical cataplexy but intermediate orexin levels had average values of SUV_r in all ROIs similar to other patients with NT1. Finally, no correlation was found between SUV_r and disease severity assessed by NSS score in adults (n = 20) and by NSSP score in children (n = 18), regardless of TSPO affinity binding.

Discussion

Our study did not find differences in microglia density using the [18F]DPA-714 PET between patients with NT1 and controls in the hypothalamus and thalamus. In contrast to our hypothesis, we found lower SUV_r in the whole brain of patients with NT1, regardless of TSPO affinity binding. No association was found between SUV_r in the different brain regions and disease duration, severity, and orexin levels in NT1.

In recent years, development in molecular imaging has provided new insight into neurologic disorders involving inflammatory process, and TSPO remains the most common target used in neurologic diseases.²⁴ TSPO is highly expressed in microglia, macrophages, and astrocytes and thus emerges as a promising target of neuroinflammation.^{11,12} Several evidence indicates that microglia is involved in multiple sclerosis, the most common chronic inflammatory and neurodegenerative disease of the CNS in young adults, although the degree of this involvement and how mechanisms depending on microglia cause tissue damage remain unclear.²⁵ A recent PET study using the same [18F]-DPA-714 that we used demonstrated an increased binding in lesions and normal-appearing tissues of patients with multiple sclerosis compared with controls, which correlated with disease severity.¹⁴

Table 2 [¹⁸F]DPA-714 SUV and SUVR Data in Patients With NT1 and Controls According to Anatomic Regions

	Whole population				High TSPO binders			Mixed TSPO binders		
	NT1 (N = 38)	Controls (N = 32)	M0 p value	M1 p value	NT1 (N = 25)	Controls (N = 16)	M2 p value	NT1 (N = 13)	Controls (N = 16)	M3 p value
Sex, female, n (%)	17 (44.74)	14 (43.75)	0.93	—	11 (44.00)	8 (50.00)	0.71	6 (46.15)	6 (37.50)	0.64
TSPO binding, high, n (%)	25 (65.79)	16 (50.00)	0.18	—	25 (100.00)	16 (100.00)	—	0 (0.00)	0 (0.00)	—
Age, y, mean ± SD	21.32 ± 10.07	36.07 ± 19.00	0.002	—	20.08 ± 8.80	38.38 ± 20.83	0.002	23.70 ± 12.18	33.76 ± 17.35	0.11
BMI, kg/m ²	24.79 ± 4.96	23.06 ± 3.63	0.11	—	25.04 ± 5.44	23.14 ± 3.96	0.45	24.31 ± 4.05	22.99 ± 3.39	0.31
SUV, mean ± SD										
Hypothalamus	1.100 ± 0.261	1.070 ± 0.309	0.85	0.37	1.151 ± 0.265	1.264 ± 0.302	0.45	1.003 ± 0.231	0.876 ± 0.160	0.21
Thalamus	0.987 ± 0.262	0.985 ± 0.325	0.98	0.29	1.049 ± 0.264	1.206 ± 0.313	0.20	0.867 ± 0.220	0.764 ± 0.126	0.21
Frontal	0.741 ± 0.173	0.769 ± 0.250	0.85	0.43	0.777 ± 0.181	0.918 ± 0.26	0.20	0.672 ± 0.139	0.620 ± 0.118	0.21
Cerebellum	0.779 ± 0.198	0.720 ± 0.225	0.43	0.12	0.818 ± 0.209	0.856 ± 0.237	0.45	0.704 ± 0.154	0.584 ± 0.097	0.09
Full brain	0.753 ± 0.183	0.766 ± 0.236	0.88	0.37	0.790 ± 0.193	0.916 ± 0.237	0.20	0.681 ± 0.143	0.617 ± 0.107	0.21
SUVR, mean ± SD										
Hypothalamus	1.421 ± 0.142	1.534 ± 0.426	0.43	0.16	1.417 ± 0.147	1.567 ± 0.596	0.45	1.429 ± 0.139	1.501 ± 0.137	0.27
Thalamus	1.194 ± 0.291	1.311 ± 0.487	0.43	0.43	1.224 ± 0.264	1.387 ± 0.599	0.20	1.138 ± 0.342	1.235 ± 0.346	0.21
Frontal	0.958 ± 0.091	1.087 ± 0.250	0.005	0.05	0.957 ± 0.082	1.114 ± 0.347	0.05	0.961 ± 0.110	1.061 ± 0.086	0.04
Full brain	0.970 ± 0.064	1.083 ± 0.215	0.002	0.04	0.969 ± 0.059	1.109 ± 0.299	0.03	0.971 ± 0.074	1.057 ± 0.068	0.04

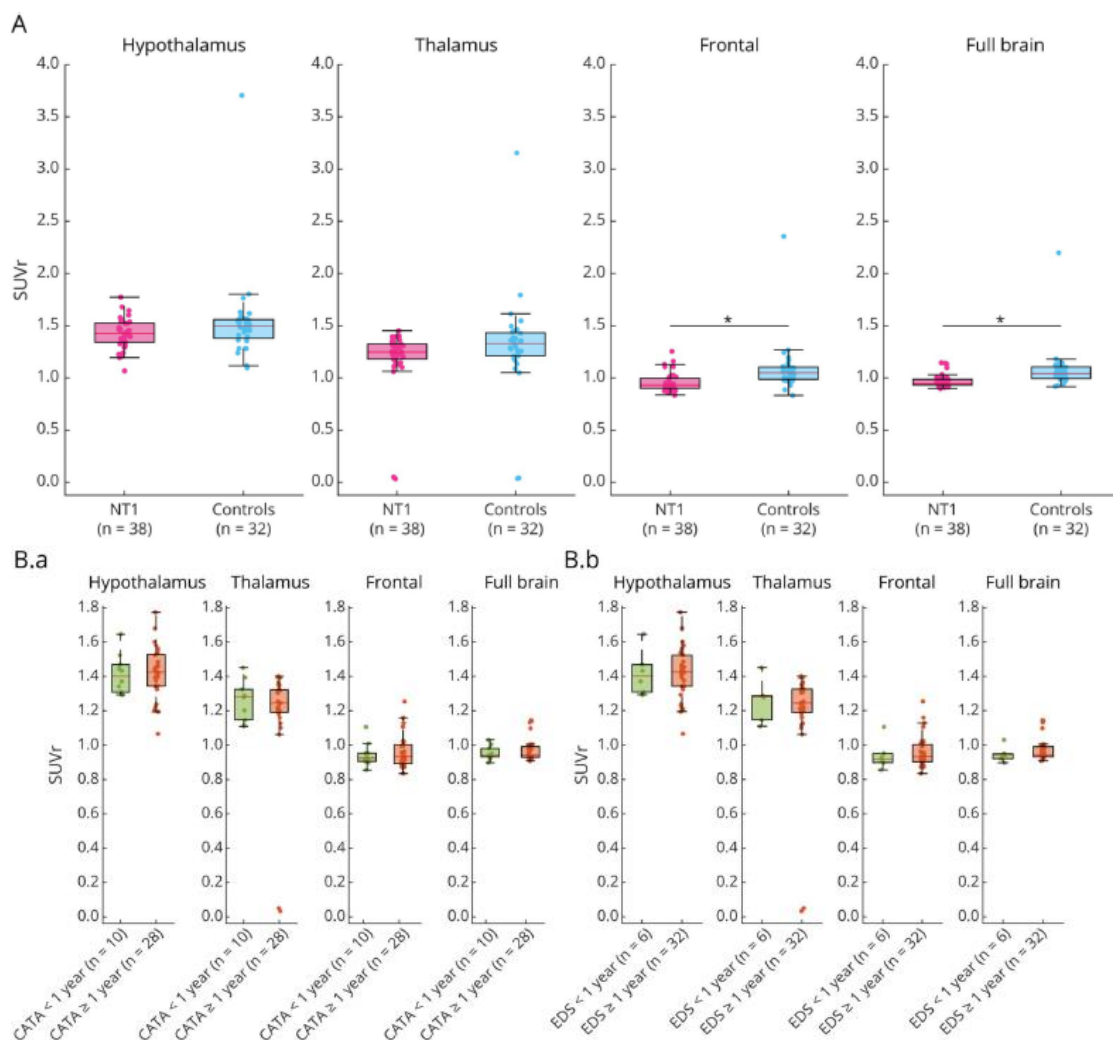
Abbreviations: BMI = body mass index; FDR = false discovery rate; NT1 = narcolepsy type 1; SUV = standard uptake value; SUVR = SUV ratio; TSPO = 18 kDa translocator protein.
M0: Crude association, with FDR correction for SUV and SUVR data; M1: Model adjusted for age, and with FDR correction for SUV and SUVR data; M2 and 3: Crude association (nonparametric test), with FDR correction for SUV and SUVR data.

However, the frequent assumption that increased TSPO expression in the human brain reflects proinflammatory activation of microglia came from rodent studies.²⁶ TSPO expression did not increase in activated human microglia in vitro.^{26,27} TSPO signal is associated with microglia/ macrophages in active lesions; however, its expression predominantly reflects cell density rather than activation of these cells.²⁶⁻²⁹ Analyses of lesions in multiple sclerosis reveal that TSPO is not restricted to proinflammatory microglia/ macrophages, but it is also present in reparative microglia.²⁷ Accordingly, microglial activation may have different roles, with diversity of microglial responses and complexity of the balance between proinflammatory and neuroprotective microglial activation.³⁰ Until now, no PET tracer can distinguish between different microglial subtypes or capture the transition between these different states.³¹ All these findings have implications for the interpretation of TSPO PET signal in neurologic disorders.

To our knowledge, our study is the first to explore TSPO expression in NT1. Our findings did not confirm the hypothesis of increased TSPO expression in the hypothalamus of NT1, but in contrast we found a lower binding in the whole brain of patients with NT1, regardless of TSPO affinity binding. These findings do not support the presence of neuro-inflammation in the process of destruction of orexin neurons. Postmortem studies also failed to detect hypothalamic inflammation process (microglial or T-cell infiltration) in NT1, but with some controversy regarding the presence of gliosis.^{2,3} However, as these brains were collected several decades after the first narcoleptic symptoms, an inflammatory process at disease onset cannot be ruled out. A single postmortem study of a patient with concomitant Ma2-antibody encephalitis, 4 months after the onset of symptoms of narcolepsy, revealed extensive CD8 T-cell infiltration and gliosis of the hypothalamus.³² In our current in vivo study, we selected several patients with NT1 with recent onset (<1 year of evolution of sleepiness or cataplexy) or even very recent onset (<6 months of evolution), but we did not find significant differences

in TSPO expression between these patients and those with a longer course or compared with matched controls. One of the initial objectives and perspectives of this study was the identification of patients who may be responders to immunomodulators, with neuroinflammation in the hypothalamus (i.e., high TSPO expression) shortly after disease onset when the potential neuronal destruction process is not too advanced and thus potentially reversible.^{5,33} Although the main hypothesis still favors the involvement of a T-cell-mediated mechanism in NT1, evidence for hypothalamic inflammation or T-cell infiltration is lacking in NT1.⁴ Recent data also suggest the possibility that epigenetic silencing of the orexin gene rather than the loss of these neurons causes the NT1 phenotype.³⁴ Finally, given that the glymphatic system could play a role in neuroinflammation, future research could also study the functioning of astrocytes and the glymphatic system in patients with NT1.³⁵

Figure 2 Boxplots Representation of Global and Regional [¹⁸F]DPA-714 SUVR Data



(A) In the whole sample of patients with NT1 (n = 38) vs controls (n = 32). *p < 0.05. (B) In the group of patients with NT1, according to the duration of evolution of CATA (recent onset, <1 year) (B.1) and duration of evolution of EDS (recent onset, <1 year) (B.2). Boxplots represents interquartile range (IQR) with median, Q1 = 25th Percentile and Q3 = 75th Percentile. CATA = cataplexy; EDS = excessive daytime sleepiness; NT1 = narcolepsy type 1; SUV = standard uptake value; SUVR = SUVR ratio.

Our study has several limitations. The first is the small size of hypothalamus area in the human brain. We used brain MRI to confirm the location of the hypothalamus, and the technique of ROI delineation was then implemented in the images analyses process for the other participants, when MRI was not available. However, the PET scanner used in this study has a clinical resolution measured at 8 mm across the entire field of view (measured with activity lines in a water-filled phantom). Thus partial volume effects lead to an underestimation of SUV in anatomic structures with 1 dimension smaller than 16 mm, which may affect hypothalamic measurements. In addition to the hypothalamus, we also analyzed other larger regions of interest, including the area of the thalamus encompassing the hypothalamus. Indeed, the suspected brain inflammatory process can infiltrate other CNS structures, even with less intensity, as shown in a patient with concomitant NT1 and Ma2-antibody encephalitis and in immune-mediated experimental models of NT1.^{30,34} A second limitation is the potential heterogeneity of the patients included, with different stages of evolution and disease duration. The natural history of orexin neurons loss in NT1 remains largely unknown, and the gradient and slope of the destruction could vary between patients, some developing a narcoleptic phenotype (e.g., sleepiness and cataplexy) within few weeks, others in several years.^{1,36,37} A third limitation is the relatively small number of healthy controls, but for ethical reasons it is problematic to expose healthy subjects to PET radiations. The composition of the control groups is also questionable. Children (<16 years old) could not be included for ethical reasons. The adult controls from the Montpellier Sleep Unit were not healthy controls but presented mild sleep complaints (sleepiness or insomnia). However, the latter complaints were not clinically significant, and therefore, the patients remained without criteria for insomnia disorder or hypersomnolence disorder. The age range was wide; however, this is unlikely to have changed our overall results, as we performed several sensitivity analyzes to account for this, adjusting for age, removing children, and finally with a paired analysis between cases and controls, with unchanged results. Finally, we may also lack statistical power when categorizing the participants according to their TSPO binding affinity, age groups, and in subgroups with the most recent disease onset.

Our study also has several strengths. NT1 is a rare disease, and the inclusion of patients with short disease duration (e.g., cataplexy onset within the last 6 months for 8 patients) was a real challenge.³⁸ Patients with NT1 were well characterized, all with CSF orexin measurement. All participants (patients and controls) were genotyped for rs6971 polymorphism which affects TSPO bindings. Participants with the low-affinity binding status were excluded, and we analyzed the results as function of high affinity and mixed-affinity together and independently. Images were processed twice on PMOD, by 2 investigators blinded to the diagnosis, with results confirming the concordance of the findings. The robustness of our findings was also confirmed by several statistical approaches: correction for multiple comparison, parametric tests, and taking into account age (by adjusting for age, removing children, and analyzing children and adults separately), with age being a potential confounding factor. We added a second population of healthy controls from another center (with no difference between the 2 control groups) to increase the control group size. At last, we confirmed our findings in several sensitivity analyses and especially within subgroups of patients with the shortest duration of evolution of the symptoms and high TSPO affinity.

In conclusion, contrary to our hypothesis, we reported no *in vivo* increase in microglial density measured by brain PET with [¹⁸F]DPA-714 in the hypothalamus and thalamus in patients with NT1 compared with controls, but unexpectedly, we found a decrease in the whole brain of these patients. These findings do not support the presence of neuroinflammation in the process of destruction of orexin neurons. Further *in vivo* studies are

needed to search for new biomarkers to reliably identify patients with recent-onset NT1, with possible brain inflammation or evidence of T cells associated with orexin neurons damage.

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Alexandre Da Costa, MSc	ToNIC, Toulouse NeuroImaging Center, UMR 1214, INSERM, Université Paul-Sabatier, Toulouse, France	Data imaging analysis (confirmation), manuscript revision
Michel Lecendreux, MD	Pediatric Sleep Centre, Hospital Robert-Debré, Paris, France	Manuscript revision, inclusion of patients
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