SLEEP, 2026, 49, 1–9
doi:10.1093/sleep/zsag087
Advance Access publication 31 March 2026
Original Article

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Hypothalamic volume reduction in adult patients with narcolepsy type 1

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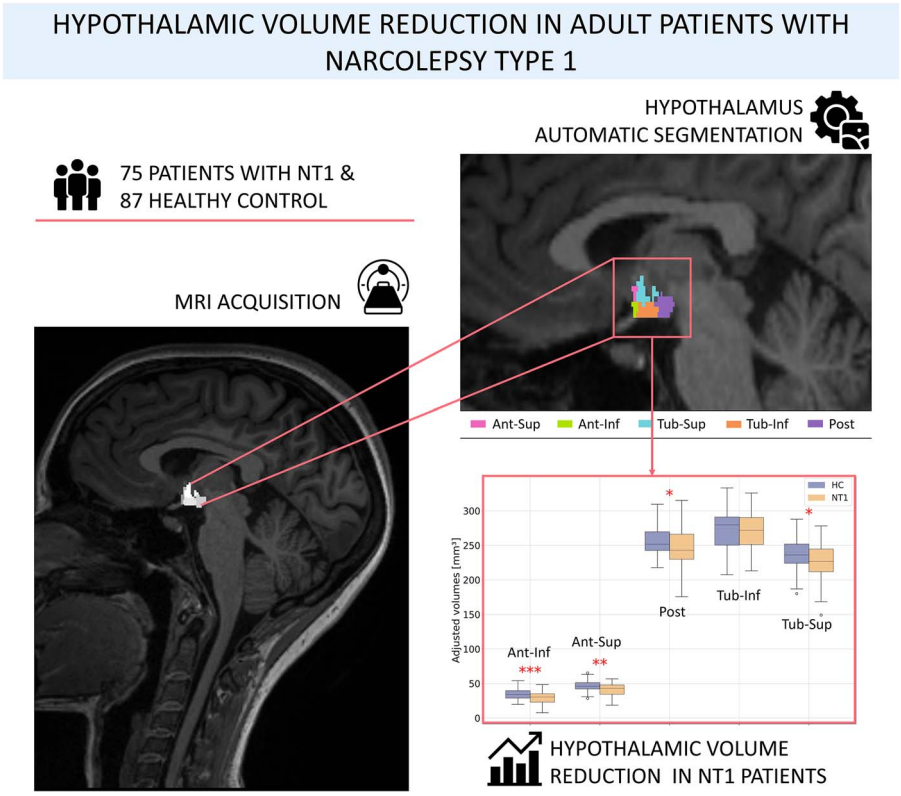
Abstract

Study Objectives: Narcolepsy-type 1 (NT1) is a neurological sleep disorder, characterized by a loss of orexin-producing cells in the posterior hypothalamus. These neurons are approximately 70 000 but have large orexin-containing projections within the hypothalamus and other brain areas. Several evidences suggest an autoimmune origin, while pathological studies in postmortem samples collected decades after onset have found variable gliosis. In this study, we studied MRI-based volumes in the hypothalamus in a large cohort of patients with NT1 versus healthy controls.
Methods: A consecutive cohort of 75 adult NT1 patients (36 females 33.1 ± 12.8 years old) and 85 age- and sex-matched healthy controls (42 females, 32.5 ± 8.2 years old) underwent neuroimaging, including T1-weighted MPRAGE MRI at 3Tesla. The hypothalamus and its subregions (anterior-inferior, anterior-superior, posterior, tuberal-inferior, and tuberal-superior) were segmented from T1 images using an automated tool. Hypothalamic volumes were compared between groups and correlated with clinical features and orexin levels in the cerebrospinal fluid.
Results: Adjusted for whole brain-volume, patients showed reductions in hypothalamic volume compared to healthy controls (p=.01). These reductions were found across most hypothalamic subregions, anterior-inferior (p<.001), anterior-superior (p=.003), posterior (p=.03), and tuberal-superior (p=.04), but not in the tuberal-inferior subregion. No significant correlations were found with clinical or laboratory variables.
Conclusions: Automated volumetric analysis of brain MRI showed hypothalamic atrophy in NT1 patients compared to healthy controls. Future pathological studies are needed to address the diagnostic and mechanistic relevance of this finding.

Key words: resonance imaging; image segmentation; hypothalamus; narcolepsy type; sleep wake disorders

Submitted: 15 December, 2025; **Revised:** 25 February, 2026; **Accepted:** 12 March, 2026

Graphical Abstract



Statement of Significance

This study represents the largest MRI investigation to date examining hypothalamic volumetric alterations and subregional morphology in patients with narcolepsy type 1 (NT1). While previous structural MRI findings have been inconsistent, recent evidence suggests increased hypothalamic volume in NT1 cases following H1N1 vaccination. Postmortem studies have documented loss of orexin-producing neurons, proliferation of histaminergic neurons, and potential gliosis within the hypothalamus. Our findings demonstrate significant atrophy in anterior hypothalamic subregions, with relative preservation of tuberal regions. These results support and provide crucial evidence of selective hypothalamic vulnerability in NT1, assessed non-invasively in-vivo by MRI. Future research should determine whether these alterations reflect neuroinflammatory processes, gliosis, or cellular remodelling, thereby offering new insights into NT1 pathophysiology.

Introduction

Narcolepsy type 1 (NT1) is a chronic neurological disorder characterized by excessive daytime sleepiness (EDS), cataplexy- a pathognomonic symptom- hypnagogic hallucinations, sleep paralyses and disturbed nocturnal sleep [1]. NT1 typically appears in childhood or early adulthood and has a life-long course [1].

NT1 is pathophysiologically linked to the selective deficiency of orexin, a neuropeptide produced by a small population of about 70 000 cells in the hypothalamus, neurons with widespread connections to central nervous system. Orexinergic transmission is primarily involved in the regulation of the sleep–wake cycle (i.e. wakefulness promotion, rapid-eye-movement [REM] sleep suppression, non-REM sleep stabilization), as well as in other functions, notably feeding and metabolism, autonomic function, and reward [2].

Following identification of orexin receptor 2 mutations in a canine model of narcolepsy [3], a lack of orexin producing neurons has been documented in human brain samples of patients with NT1, and the deficiency of orexin peptide in the cerebrospinal fluid (CSF) became the disease specific biological marker [4–6]. Because the disease is strongly HLA class II associated, a subacute immune-mediated damage is now widely accepted as the most likely cause. A strong genetic predisposition, mostly represented by polymorphisms in the HLA-DQ gene [7] (namely HLA-DQB1*06:02), and within T cell receptor loci, also supports an autoimmune basis, as is the fact inflammatory triggers, such as flu or streptococcus infections, have been found to precede disease onset. However, the exact pathological immune mechanisms are still uncertain, as no inflammatory infiltrates have been documented in the brains of NT1 patients. A selective death of orexin producing neurons is likely, supported by several studies [5, 6, 8]. Recently, epigenetic silencing of these hypothalamic cells, has however been proposed as an alternative mechanism [9].

Beyond animal models, post-mortem studies are of fundamental importance to explore pathological processes leading to orexin loss in human NT1. Problematically, however, there is a long average interval between onset of narcolepsy (typically at young age), when the pathological process leading to orexin loss

may be still active, and subsequent death, time at which the brain is collected. Life expectancy in narcolepsy is in line with general population statistics, indeed. Alterations close to the loss of orexin neurons are thus likely missed or masked when assessed after decades.

A solution to this may be to use neuroimaging, and indeed, it has been used to investigate NT1 in-vivo closer to disease onset, with special focus on the hypothalamus as it is the core of the pathogenic process. Due to its small size (around 4 cm^3) [10] and lack of image contrast, studying the hypothalamus of patients with narcolepsy using magnetic resonance imaging (MRI) has been challenging.

The few published MRI studies that have focused with different methodologies on the hypothalamus [11–13], have shown contradictory results [14], some reporting grey matter hypothalamic reductions [12, 13], while others finding no significant differences [11], or even an increased volume [15]. Moreover, since the rarity of disease, these studies show small patients' cohort, sometimes clinically heterogenous especially for the lack of CSF orexin data.

Using a large cohort of NT1 patients and matched controls, the current study aimed at documenting volumetric changes in subregions of the hypothalamus using MRI. In contrast to most prior studies, we used a more powerful MRI and fully automated quantitative methods to assess hypothalamic volumes [16], avoiding any possible bias.

Materials and methods

Study design

Cross-sectional case–control observational study.

Standard protocol approvals, registrations, and patient consents

All subjects written informed consent to the study protocol, in agreement with the Convention of Helsinki. The study was approved by our local Ethical Committee (Comitato Etico Interaziendale Bologna Imola, 17 009). The collection of MRI data from healthy controls (HC-ATLAS) was approved by the Ethical Committee (77821).

Participants

The study was conducted between September 2023 and November 2024. Adult patients with NT1 were recruited among those who were referred for clinical practice to the Narcolepsy Center of the IRCCS Istituto delle Scienze Neurologiche di Bologna, Bologna, Italy, on the days with available MRI sessions (up to two exams per week, depending on clinical practice). Patients were referred either for the diagnostic hospitalization or for clinical follow-up under treatment.

Diagnoses were made according to the current criteria of the International Classification of Sleep Disorders—3rd edition Text Revision [17] (ICSD-3), following an established standardized inpatient protocol [18]. The diagnostic assessment, included a semi-structured interview on the clinical features of the disease, physical examination, a video-polygraphic test to document cataplexy, daytime and nighttime video-polysomnography, multiple sleep latency test (MSLT), blood drawn and lumbar puncture for HLA-DQB1*06:02 allele typing, and, if specific consent was given, CSF orexin measurements with radio-immuno-assay according to Stanford standards [17]. At diagnostic assessment, patients were either drug naïve or in drug withdrawal since at least two weeks.

Exclusion criteria for NT1 patients were contraindications to MRI, such as ferromagnetic implants, or claustrophobia, other neurological conditions, psychiatric or physical diseases, substance abuse, and secondary causes of narcolepsy. Additionally, patients whose MRI images exhibited poor quality due to significant motion artifacts or incidental pathological findings were also excluded.

Healthy control MRI data were obtained from the HC-ATLAS cohort. All participants underwent a clinical interview to exclude neurological, psychiatric or sleep disorders, and demographic variables (age, sex, body mass index [BMI]) were collected. Data were selected for obtaining the best age and sex-match as possible with NT1 sample.

Clinical data

In patients with NT1, the following diagnostic data were collected: age at disease onset (either EDS or cataplexy), age at diagnosis, MSLT results, HLA-DQB1*06:02 positivity, CSF-orexin level. At time of recruitment, from all patients, we collected age, disease duration, BMI, presence and frequency of cataplexy, presence of sleep-related paralysis and hallucinations, severity of daytime sleepiness assessed with the Epworth Sleepiness Scale (ESS), and ongoing treatments for NT1.

Brain MR acquisition protocol

All participants underwent high resolution 3D Magnetization Prepared Rapid Acquisition Gradient Echo (MPRAGE) MRI using a 3 T clinical scanner (Skyra-Magnetom, Siemens Healthcare), equipped with a Siemens Head/Neck 64 Coil, at the IRCCS Institute of Neurological Sciences of Bologna, Italy. The imaging protocol comprised a series of sequences designed to capture detailed structural information. The MPRAGE parameters were as follow: TE/TR = 53/2300 ms, flip angle (FA) = 9°, 1 mm^3 isotropic voxel. The acquisition time for this sequence was 5 minutes and 21 seconds. Diagnostic brain imaging including 3D T2-weighted FLAIR, susceptibility-weighted imaging (SWI) and diffusion-weighted imaging (DWI) were acquired to evaluate for the presence of structural lesions and exclude corresponding patients.

3D T1 images processing

An automated segmentation tool [16] (available from FreeSurfer v7) was used to segment the entire hypothalamus based on 3D T1-weighted data, delineating both right and left hemisphere and its five subregions: anterior-inferior, anterior-superior, posterior, tuberal-inferior and tuberal-superior regions (Figure 1). Corresponding classification of hypothalamic nuclei [19, 20] is detailed in Table 1. In the current report, subregions are named according to the classic anatomical nomenclature, not the one employed by the segmentation tool: in particular, this latter uses "tubular" instead of "tuberal" (anatomical term), as reported in its documentation (https://surfer.nmr.mgh.harvard.edu/fswiki/HypothalamicSubunits). The other subregions follow the tool's nomenclature.

The quality of the images and segmentation outputs were visually assessed by two neuroradiologists (LM, RL), with respectively 3 and more than 20 years of experience. This was done to ensure reliability and accuracy. Any data deemed unreliable or of insufficient quality (because of motion artifacts) were excluded from further analysis.

Table 1. Classification of hypothalamic nuclei subregions

Hypothalamic subregions	Hypothalamic nuclei
Anterior–superior	Paraventricular nucleus, preoptic area
Anterior–inferior	Suprachiasmatic nucleus, supraoptic nucleus
Posterior	TMN, mamillary body (including medial and lateral mamillary nuclei), lateral hypothalamus
Tuberal-superior	Lateral hypothalamus, dorsomedial nucleus, paraventricular nucleus
Tuberal-inferior	TMN, infundibular (or arcuate) nucleus, ventromedial nucleus, supraoptic nucleus, lateral tuberal nucleus

Classification of hypothalamic nuclei according to Bocchetta et al. [19] and Makris et al. [20].

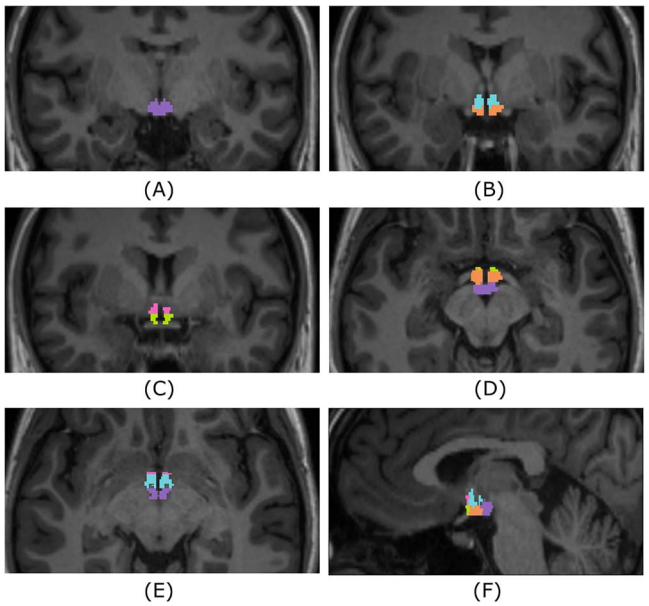


Figure 1. Example of hypothalamus automatic segmentation. Coronal (A, B, C), axial (D, E), sagittal (F) views. Different colours represent hypothalamus subunits: Posterior (purple), tuberal-inferior (orange), tuberal-superior (blue), anterior-inferior (green), and anterior-superior (pink). Views of 3D T1- weighted sequence of a healthy control (F, 25 yo).

Statistical analysis

Volumetric hypothalamic data were adjusted for the effects of sex, age, and total intracranial volume (TIV) using a residual method. Specifically, a multivariate linear regression model was first fitted between each variable of interest (region volumes) and the three confounding variables within the control group. Following this, estimated parameters were used to correct raw values for every subject. The Shapiro–Wilk test was then applied to residuals of each subject to assess normality. In case of two-group comparisons, analysis of variance or Mann–Whitney U tests were used to compare groups, depending on normality of the dependent variable under investigation. To assess statistical correlation between morphometric and clinical variables, partial Spearman correlation tests on raw values was calculated, controlling for sex, age, and TIV. In addition, CSF orexin concentrations were categorized into tertiles and quartiles, and partial correlations were re-computed within these strata to assess potential threshold effects.

The Bonferroni method was applied to correct for multiple comparisons and correlations were corrected using the false discovery rate procedure. Statistical significance was set at adjusted-$p<.05$. p-values were further classified as significant ($p<.05$), highly significant ($p<.01$), and extremely significant ($p<.001$). The statistical analysis was conducted using an in-house script developed in Python 3.11. Data are presented as mean ± standard deviations for continuous variables, and as frequency (%) for categorial variables.

Results

Study sample

Eighty-two patients with NT1and 87 age and sex matched HC underwent brain MRI. Seven patients were excluded from the study due to MRI motion artifacts, leaving 75 patients. Clinical features and ongoing treatment of patients, and comparison of demographics with HC, are presented in Table 2. All patients exhibited definite cataplexy (either video-documented at the time of the diagnosis or reported by the patient with typical features), CSF-orexin was already dosed at time of the study in 56 patients and levels were below 110 pg/mL in all subjects.

NT1 group hypothalamus volumetric comparative analysis

Table 3 summarizes uncorrected volumes of the entire hypothalamus and its subregions in patients with NT1 versus HC. After adjusting for age, sex, and TIV, patients with NT1 exhibited significant atrophy of the entire hypothalamus compared to HC ($p=.009$). More specifically, significant reductions in volume were found in the anterior inferior ($p=.0008$), anterior superior ($p=.003$), posterior ($p=.03$), and tuberal superior regions ($p=.04$). No statistically significant volume alteration was found in tuberal-inferior subregion (Figure 2). Detailed statistical results, including test statistics, effect size, and confidence intervals, are provided in Supplementary Table 2.

Correlation with clinical data

No significant correlations (Supplementary Table 3) were observed between corrected hypothalamic volumes (whole hypothalamus and its subunits) and demographic, clinical, or laboratory data, including disease duration and CSF orexin levels (scatterplots of CSF orexin levels versus hypothalamic volumes are shown in Supplementary Figure 1). Moreover, no significant correlations were found between hypothalamic atrophy and medical therapy, neither with respect to the presence or absence of treatment nor to the specific drug employed. In additional sensitivity analyses, CSF orexin concentrations were categorized into tertiles and quartiles to account for potential threshold effects. Adjusted analyses across these strata confirmed the absence of significant associations between hypothalamic volumes and orexin levels (Supplementary Table 4).

Discussion

In this study we investigated alterations in the volume of the hypothalamus and its subunit components in 75 adult patients with sporadic NT1 compared with 87 HC. This large study was performed using an automated analysis tool applied to high

Table 2. Clinical and demographic features of patients with narcolepsy type 1 (NT1) and healthy controls (HC)

	NT1 (n = 75) n (%) or mean±SD	HC (n = 87) n (%) or mean±SD	*p*-value
Sex male (n)	39 (52.0%)	45 (51.7%)	0.866
Age at MRI (years)	33.1 ± 12.9	32.5±8.2	0.409
Age at diagnosis (years)	26.2 ± 12	-	
Disease Duration (years)	13.8 ± 9.7	-	
Follow up (years)	7 ± 7.9	-	
BMI (kg/m2)	27.4 ± 6.1	-	
EDS (n)	75 (100%)	-	
EDS onset (years)	18.6 ± 10.7	-	
Cataplexy (n)	72 (96.0%)	-	
Cataplexy onset (years)	20.7 ± 11.5	-	
Cataplexy frequency (n)		-	
absent	3 (4.3%)		
<1/year	2 (2.9%)		
1/year-1/month	7 (10.0%)		
1/month-1/week	15 (21.4%)		
1/week-1/day	17 (24.3%)		
>1/day	26 (37.1%)		
Sleep paralysis (n)	43 (58.9%)	-	
Hypnagogic hallucinations (n)	46 (63.0%)	-	
Disrupted nocturnal sleep (n)	51 (72.9%)	-	
ESS (score)	14.7 ± 3.9	-	
HLA-DQB1*0602 (n)	69 (95.8%)	-	
CSF-Orexin (pg/mL)	31.9 ± 32.6	-	
MSLT mean sleep latency (min)	3.1 ± 2.5	-	
MSLT SOREMPs (n)	3.8 ± 1.3	-	
Stable Treatment at MRI (n)			
None/started <1 year	16 (21.3%)		
Wake promoting agents(modafinil or solriamfetol)	28 (37.3%)		
Sodium Oxybate	28 (37.3%)		
Pitolisant	18 (24.0%)		
Antidepressants	10 (13.3%)		

Clinical features of patients with narcolepsy type 1 (NT1) and comparison of demographic data with healthy controls (HC). Detailed statistical results are provided in Supplementary Table 1. Legend: BMI, body mass index; EDS, excessive daytime sleepiness; CSF-orexin, cerebrospinal fluid orexin-1 level; MSLT, multiple sleep latency test; SOREMP, sleep onset rapid eye movement period.

Table 3. Hypothalamus and its subregions volumes

Hypothalamic regions	NT1 patients (n = 75) volumes	Healthy controls (n = 87) volumes
Whole hypothalamus	807.1 ± 76.1	847.6 ± 67.2
Anterior–superior	41.1 ± 9.3	46.4 ± 8.2
Anterior–inferior	28.9 ± 8.9	34.8 ± 7.1
Posterior	245.3 ± 29.8	256.2 ± 21.4
Tuberal-superior	221.6 ± 28.7	237.8 ± 25.9
Tuberal-inferior	270.0 ± 31.1	272.4 ± 28.3

Hypothalamus and its subregions volumes in patients with confirmed type 1 narcolepsy (NT1) and healthy controls (HC). Results are reported as uncorrected mean volumes (mm^3) and standard deviations of the entire hypothalamus and its subregions [16].

resolution T1-weighted MR images using high-field 3 T scanner. Compared to a sample of 87 HC, we found an overall volumetric reduction in the hypothalamus of patients with NT1. Further, individual subunits of the hypothalamus were also reduced, with stronger significant effects anteriorly. In this analysis, only the tuberal-inferior segment was not significantly affected, although a trend toward volumetric reduction was observed (Figure 2).

Previous neuroimaging studies analysing the hypothalamus of narcoleptic patients utilized two different approaches: voxel-based morphometry (VBM) [11–13, 21–25] that quantified the whole hypothalamus but not its subunits (eight studies), and, for two recent studies [15, 26], the same automated method [16]. A summary overview of these studies is presented in Table 4. VBM studies showed conflicting results: four found grey matter hypothalamic volume reduction [12, 13, 21, 22] in a total of 94 NT1 patients while the other four did not find any volume differences in a total of 55 NT1 patients [11, 23–25]. Further, seven of eight of these VBM studies were conducted using 1.5 T MRI scanners. In contrast with VBM or segmentation methods such as older versions of FreeSurfer (before v.7.2) and FSL, our study employed an automated segmentation tool that allows the delineation of hypothalamic subunits [16]. Traditional VBM method, while effective for whole-brain assessments, are limited by partial volume effects and spatial resolution constraints, reducing precision in small size structures like the hypothalamus. Similarly, the older versions of FreeSurfer and FSL, despite their strengths in cortical and larger subcortical segmentation, provide a sub-optimal anatomical sensibility for hypothalamic subregions [27]. In one study [26] on hypothalamic segmentation in sporadic narcolepsy, the authors found no significant volumetric alterations in the whole hypothalamus and its subunits between patients and HC. However, we pinpoint that this study included a small size group of 15 patients, with mixed diagnoses, nine with NT1 and six with NT2, without evidence of orexin deficiency.

Using the same MRI analytical approach in a cohort of 54 patients who developed narcolepsy following *Pandemrix*®

Table 4. Previous MRI studies on hypothalamic alterations in patients with narcolepsy

	Patients' group	Patients (Male: Female)/HC; Narcolepsy type Age range (mean), in years	Disease duration, range (mean), in years	MRI Scanner	Analysis method	Hypothalamus volume changes
1.	*Draganski et al* [13], *2002*	29 (NA)/29 Narcolepsy type not reported (39.7)	NR	1.5 T	VBM	Grey matter decreasing in bilateral hypothalami
2.	*Kaufmann et al* [23], *2002*	12(6:6)/32 All NT1 22-65 (36.99 ± 15.8)	(12)	1.5 T	VBM	No alterations
3.	*Overeem et al* [24], *2003*	15(NR)/16 All NT1 21-70 (44.7)	2-50 (19.2)	1.5 T	VBM	No alterations
4.	*Brenneis et al* [11], *2005*	12(8:4)/12 11 with cataplexy, 1 without cataplexy, no orexin available. 22-72 (35.8 ± 13.2)	3-55 (15)	1,5 T	VBM	No alterations
5.	*Buskova et al* [22], *2006*	19(19:9)/16 All NT1 (43.4 ± 13.8)	4-40 (11.9)	1.5 T	VBM	Decreasing
6.	*Kim et al* [12], *2009*	17(13:4)/17 All NT1 17-35 (24.6 ± 4.9)	(8.8)	3 T	VBM	Decrease of right hypothalamus
7.	*Joo et al* [21], *2009*	29(15:14)/29 All NT1 (31.2)	NR	1.5 T	VBM	Reduction of grey matter concentration in bilateral hypothalami
8.	*Scherfler et al* [25], *2012*	16(12:4)/12 All NT1 40-70 (56.8)	6-59 (30.6)	1.5 T	VBM	No alterations
9.	*Kim H et al* [26], *2022*	15(7:8)/19 9 NT1, 6 NT2 (33.2 ± 15.5)	NR	3 T	Automated tool [16]	No alterations
10.	*Juvodden et al* [15], *2023*	54(15:39)/114 All post-H1N1 vaccine NT1 10-33 (21.8)	4-7 (5.8)	3 T	Automated tool [16]	Increasing, especially tuberal-inferior subunit
	Present study	**75(39:36)/87 All NT1 20-46 (31.1)**	**4-23 (11.9)**	**3 T**	**Automated tool** [16]	**Whole hypothalamus atrophy, except for tuberal-inferior segment**

Previous MRI studies on hypothalamic alterations in patients with NT1, which employed different analysis methods, MRI scanner and patients' cohort. Boldface indicates the data referring to the present study.

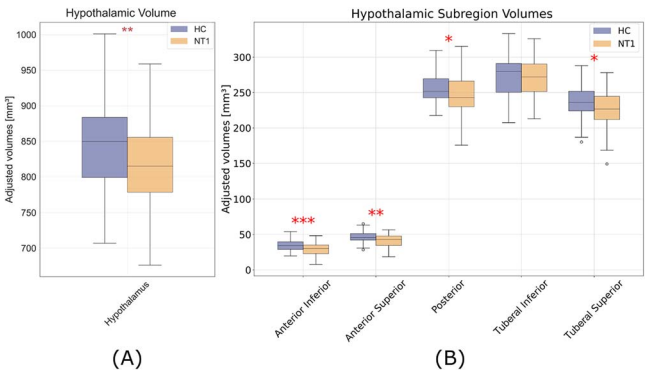


Figure 2. Group differences in hypothalamic and its subregion volumes between patients (NT1) and healthy controls (HC). These were adjusted for age, sex, and TIV. Adjusted whole hypothalamic volume (mm^3) (A). Adjusted volumes (mm^3) of specific hypothalamic subregions (B). Error bars represent the standard deviation. *p<.05, **p<.01, ***p<.001.

vaccination for H1N1 pandemic in 2009, Juvodden [15] found a larger size of the hypothalamus in patients compared to HC. The volumetric increase was more pronounced in the tuberal-inferior subunit [15] which, interestingly, in our cohort is the only region without a significant volume reduction. Nevertheless, the two samples showed relevant differences: Juvodden et al included only NT1 cases post-pandemic H1N1-vaccination, while we included consecutive patients with NT1, regardless of potential trigger (of note, *Pandemrix*® was not used in Italy); patients in the former study were younger (21.8 vs 31.1 years old), with shorter disease duration (5.8 vs 11.9 years), and some were children [15]. Thus, it is possible that the contrasting results are related to difference between triggering factor (H1N1-vaccinated narcolepsy vs sporadic NT1 cases), implying possible structural differences [28–30] as suggested by another study from Juvodden et al [31], who found different white matter abnormalities using diffusion tensor imaging in NT1 *Pandemrix*®-vaccinated compared to not vaccinated NT1.

NT1 is caused by an acquired loss of orexin-producing neurons, which are located predominantly in the perifornical area and in the posterior hypothalamus [2]. The mechanisms of this loss are still debated. Highly selective immune-mediated damage is thought to play a central role, possibly triggered by infections or vaccinations, in genetically predisposed patients [7]. Neuropathological studies have shown a disappearance of up to 95% of orexin-producing neurons in the hypothalamus of patients with NT1 [5, 6]. The absence of other markers co-expressed by orexinergic

neurons and the sparing of other subtypes of neurons such as MCH containing neurons in the lateral hypothalamus, suggest a selective neuronal death [6, 32]. Another hypothesis, based on evidence of hypermethylation of Orx gene-promoter and preserved expression of the neuropeptide co-localized in orexinergic neurons suggested an epigenetic silencing rather than killing [9].

Orexinergic neurons are congregated in the dorsal-medial nucleus and ventral-medial nucleus of the hypothalamus, in the dorsal part of parafornical nucleus, in lateral hypothalamus, and a minor component in the posterior hypothalamus [33]. The segmentation tool we employed [16] groups hypothalamic nuclei into subunits according to the maps constructed by Bocchetta [19] and Makris [20] (Table 1). With this approach, the nuclei richest in orexinergic neurons would be localized in the tuberal-superior (dorsal-medial nucleus, lateral hypothalamus) and tuberal-inferior subunits (ventral-medial nucleus, lateral tuberal nucleus) [19, 20, 33]. Therefore, the volume was mostly reduced in subunits not supposed to have significant orexinergic neuronal bodies. Instead, the tuberal-inferior subunit, which would contain a large fraction of the orexinergic cells, did not show any difference in volume with HC.

Various explanations for these results may be hypothesized. Reduction in orexin production, either due to the death of producing neurons or epigenetic silencing, leads to a lack of its binding with orexin receptors OX1R/OX2R, located in the hypothalamus and widespread in the brain. Previous works have shown that one third of all hypothalamic neurons express orexin receptors that mediate an excitatory function [34, 35]. The failure of orexin-receptor binding, resulting in reduced stimulation to multiple hypothalamic nuclei, could progressively lead to a "functional" atrophy. Alternatively, the death of orexinergic neurons could lead to degeneration of axons and terminals, known to loaded with orexin-containing vesicle. These projections are widespread within hypothalamus [34], and neuronal loss could result in the atrophy of the other hypothalamic subunits in concordance with previous histopathological work [8]. In agreement with this hypothesis, studies on diffusion-tensor imaging have found lower fractional anisotropy and higher radial diffusivity in the whole brain of 12 patients with NT1 compared to HC. This could be explained as a lower axonal density or myelination secondary to the loss of orexinergic cells [36]. In this direction, it is notable that all regions of the hypothalamus and the pons have a very high content of orexin peptide by gram of tissue [5]. Therefore, one must consider orexin cell bodies as representing only a very small surface areas of the total volume of these neurons that have many projections loaded with peptides.

The normal volume of the lower tuberal area could be explained by its relative sparing, or by increased hypothalamic histaminergic neurons. Indeed, Thannickal et al. [8] showed an increase in GFAP-labelled astrocytes at the tuberomammillary nuclei (+184% compared to healthy controls), arcuate nucleus (+150%), ventromedial nucleus (+127.3%) and supraoptic nucleus (+57.8%). Such glial cell infiltrate could result in a slight volumetric increase in the region masking the atrophy due to the loss of orexinergic neurons. However, some postmortem autopsy studies [5, 37] have not shown a gliotic response with cellular infiltrate.

Alternatively, the lack of change could be due to the concomitant increase of histaminergic hypothalamic neurons in NT1. These neurons are also involved in the regulation of the sleep–wake cycle and tend to localize adjacent to orexin-secreting neurons especially in the posterior hypothalamic portions [38]. Two studies [39, 40] have highlighted a strong increase in the number of histamine neurons in the tuberomammillary nucleus (TMN) of the hypothalamus, from 64 to 94%. The TMN seems to be localized in the tuberal-inferior and posterior segments. So, in our study, a possible increase of histamine neurons in NT1 patients could determine the non-significant volumetric alteration of tuberal-inferior part, compensating the loss of orexin-secreting neurons.

We observed a lack of correlation between hypothalamic volumes (either whole organ or subunits) and disease duration. This negative finding, consistent with previous evidence [15], supports the hypothesis that hypothalamic atrophy in NT1 represents an early and largely irreversible structural consequence of disease onset rather than a progressive neurodegenerative process, as mirrored by the stable disease course over time. In NT1, converging immunological and clinical evidences suggest that the autoimmune-mediated loss of orexin neurons occurs rapidly around disease onset. Within this framework, the reduced hypothalamic volumes observed in our study are more consistent with a residual structural "scar" established early in the disease course, followed by a long-term plateau phase in which further volume loss is minimal or absent.

Taken together, these findings provide evidence of persistent hypothalamic tissue alteration in long-standing NT1.

The absence of an association between hypothalamic volumes and CSF orexin levels suggests that volumetric MRI measures and neurochemical alterations may reflect partially distinct aspects of hypothalamic involvement in NT1, without supporting a progressive structure–function relationship over time. It is also reasonable that both MRI volumetric analysis and CSF orexin measurement by RIA are not sensitive enough to quantify internal differences and correlation within a cohort of patients with orexin deficiency, due to a floor effect. Moreover, it is known that the CSF orexin level, despite being the diagnostic gold standard, is not an accurate measure of orexinergic neuronal loss when analyzed below the threshold of orexin deficiency, and that studies employing high-performance liquid chromatography have shown that only <10% of the values provided by RIA are the intact form of orexin, suggesting that RIA also measures immunoreactive orexin-related metabolites not binding the orexin-receptors, without a clear pathogenic meaning [41].

The present study has some limitations. Firstly, this is a cross-sectional study, so with a limited capability of assessing temporal dynamics or causality in hypothalamic volume changes, compared to a longitudinal study. However, the employed tool for hypothalamic segmentation and volumetric analysis has been made available only in recent years and, nevertheless, this consecutive recruitment allowed for the recruitment of a large cohort of people with a rare and under-recognized disease, the largest as far as we know. Moreover, this design allowed for studying hypothalamic volumetry in patients at different stages of the disease course, which is, in our opinion, a strength of the project. Secondly, the cohort of patient is heterogeneous in term of disease duration and ongoing treatment, with some patients studied at the diagnosis and some during follow up, but we did not find any difference in treated vs untreated patients and further excluded a role of age, disease duration and treatment on our findings. No correlations between ongoing treatments and MRI findings appeared. Therefore, we do not believe that the findings, including the lack of correlations with clinical variables, are influenced by treatment status. While the lack of correlation between volumetric alterations and disease duration could suggest that the timing of the MRI evaluation did not influence our findings, we did not evaluate patients very close to disease onset possibly investigating morphological changes in the period when orexinergic system damage could be still ongoing. No children were included in this

study, and this population deserves further attention. Studying children's cohort would be of great interest and would provide additional insights on the neuroradiological features of the disease, possibly closer to the typical disease onset.

This study focused on hypothalamic subunits, but the analysis of other cerebral areas involved in the orexin pathway, and the use of other advanced neuroimaging techniques would help to better understand in-vivo brain changes of patients with NT1. Finally, it is to acknowledge that hypothalamic segmentation was based on gross anatomy meant for MRI images, that's not comparable to the resolution of histological methods, in the assessment of possible pathological alterations in hypothalamic neurons. Nevertheless, MRI segmentation enjoys the advantage of permitting a quantitative and non-invasive approach for studying the hypothalamus, in a large cohort of patients in vivo.

In conclusion, in adult patients with NT1 we found a volume reduction of the whole hypothalamus but the tuberal-inferior, compared to healthy controls. These findings may relate to a pathological process that extends throughout the hypothalamus, beyond the orexin neurons. The sparing of tuberal-inferior region might be linked to neuroinflammatory gliosis or a compensatory increase in histaminergic neurons, a hypothesis calling for future anatomical studies.

Other imaging studies are needed to contribute to understanding the underlying pathological process in NT1 and to clarify if quantitative imaging studies could become a potential biomarker of NT1 disease for the future.

Supplementary material

Supplementary material is available at *SLEEP* online.

Acknowledgments

We are indebted to all the participants of the study, most notably the Italian association of patients with narcolepsy (Associazione Italiana Narcolettici ed Ipersonni, ONLUS). Without their contribution, this study would not have been possible. This work was supported by #NEXTGENERATIONEU (NGEU) - Ministry of University and Research (MUR), National Recovery and Resilience Plan (NRRP), project MNESYS (PE0000006). A multiscale integrated approach to the study of the nervous system in health and disease (DN. 1553 11.10.2022).

Funding

The publication of this article was supported by the "Ricerca Corrente" funding from the Italian Ministry of Health.

Disclosure statement

Financial disclosure: F. Biscarini received support for attending congresses from Bioprojet. F. Pizza received support for attending congresses from Bioprojet and honoraria for lectures from Jazz and Bioprojet. E. Mignot received research and clinical trial support from Jazz Pharmaceuticals, Takeda, and Alkermes; consulting fees and travel reimbursements from Takeda and Paladin; and owns stocks in Centessa. The other authors report no financial conflicts of interest.

Non-financial disclosure: F. Pizza participated on boards for Takeda. G. Plazzi is a member of the Advisory Board for Jazz, Bioprojet, Centessa, Alkermes, Takeda, and Idorsia. The other authors report no non-financial conflicts of interest.

Data availability

The per-subject data underlying this article will be shared at reasonable request to the corresponding author.

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https://doi.org/10.1093/sleep/zsag087
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