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Objective: Narcolepsy type 1 (NT1) is assumed to be caused solely by a lack of hypocretin (orexin) neurotransmission. Recently, however, we found an 88% reduction in corticotropin-releasing hormone (CRH)-positive neurons in the paraventricular nucleus (PVN). We assessed the remaining CRH neurons in NT1 to determine whether they co-express vasopressin (AVP) to reflect upregulation. We also systematically assessed other wake-systems, since current NT1 treatments target histamine, dopamine, and norepinephrine pathways.

Methods: In postmortem tissue of people with NT1 and matched controls, we immunohistochemically stained and quantified neuronal populations expressing: CRH and AVP in the PVN, and CRH in the Barrington nucleus; the key neuronal histamine-synthesizing enzyme, histidine decarboxylase (HDC) in the hypothalamic tuberomammillary nucleus (TMN); the rate-limited-synthesizing enzyme, tyrosine hydroxylase (TH), for dopamine in the mid-brain and for norepinephrine in the locus coeruleus (LC).

Results: In NT1, there was: a 234% increase in the percentage of CRH cells co-expressing AVP, while there was an unchanged integrated optical density of CRH staining in the Barrington nucleus; a 36% increased number of histamine neurons expressing HDC, while the number of typical human TMN neuronal profiles was unchanged; a tendency toward an increased density of TH-positive neurons in the substantia nigra compacta; while the density of TH-positive LC neurons was unchanged.

Interpretation: Our findings suggest an upregulation of activity by histamine neurons and remaining CRH neurons in NT1. This may explain earlier reports of normal basal plasma cortisol levels but lower levels after dexamethasone suppression. Alternatively, CRH neurons co-expressing AVP neurons are less vulnerable.

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Narcolepsy type 1 (narcolepsy with cataplexy, NT1) is a rare, disabling sleep disorder characterized by excessive daytime sleepiness, cataplexy, sleep paralysis, and disturbed nocturnal sleep.[1] The disease phenotype is generally explained by a lack of hypocretin (orexin) neurotransmission.[2–4] The presumed cause is an auto-immune process leading to selective destruction of the hypothalamic hypocretin/orexin neurons.[5–7]

Unexpectedly, we recently observed in postmortem hypothalamic tissue of people with NT1 not only 97% loss of hypocretin neurons but also a 88% reduction of corticotrophin-releasing hormone (CRH) expressing neurons in the paraventricular nucleus (PVN).[8] However, *in vivo* studies have shown normal 24-h plasma cortisol levels in people with NT1.[9] In NT1, therefore, there might be an upregulation of remaining CRH neurons. The action of CRH on cortisol release is mediated by adrenocorticotropic hormone (ACTH) and strongly potentiated by vasopressin (AVP). CRH is co-expressed in

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increasing quantities when PVN neurons are chronically activated in ageing,[10] depression,[11,12] Alzheimer's disease,[13] and multiple sclerosis.[14,15] In NT1, another activated system is the histamine system, marked by the increased number of cells expressing the key synthesizing enzyme histidine decarboxylase (HDC).[16,17] The activity status of other arousal systems has not been studied, but they may also play a role in NT1 symptomatology.

In the current study, we hypothesized that there is increased activity of the remaining CRH neurons in the PVN in NT1, reflected in increased co-expression of CRH and AVP. For comparison, we also quantified CRH in the Barrington nucleus that is involved in the supraspinal regulation of micturition[18] and situated adjacent to the locus coeruleus (LC). In addition, since current NT1 treatments target the histamine,[19] dopamine and norepinephrine systems,[20] we systematically assessed whether other wake-related cell types are affected in NT1; these include the histaminergic neurons in the tuberomammillary nucleus (TMN), the dopaminergic neurons in the midbrain and the norepinephrinergic neurons in the LC.

Materials and Methods

The Netherlands Brain Bank (NBB) collected postmortem human brain tissue from clinically diagnosed and neuropathologically confirmed donors in compliance with European ethical and legal guidelines.[21,22] Informed consent for brain autopsy and using brain tissue and clinical information for scientific research was given by either the donor or the next of kin. The independent Review Board (IRB) of the Vrije University Medical Center approved the procedures of the NBB concerning "Donation of brain material for scientific research" under reference number 2009/148. The IRB of the Vrije University Medical Center in Amsterdam is registered with the US office of Human Research Protections as IRB number 00002991 under Federal wide Assurance number 00003703.

Five hypothalami from people with NT1, of whom one was chronically treated with opiates, and five non-neurological controls for hypothalami matched for sex, age, postmortem delay, brain weight, and circadian clock time of death. The mesencephalon of the four NT1 cases and one NT1 with opiates, were matched for the same parameters with six mesencephalon controls. Since not all the mesencephalon controls had both the substantia nigra (SN) and LC available, we therefore had to match six mesencephalon controls containing either SN or LC to the five NT1 cases. The data of the NT1 with opiates were processed separately. Those with NT1 were diagnosed and treated by experienced neurologists/somnologists (G.J.L. and R.F.). All had a history of severe daytime sleepiness for several decades, and all four with typical NT1 had a history of cataplexy for 9–66 years. Their strongly diminished hypocretin and CRH cell counts have been reported previously.[8] The NT1 case with chronic use of opiates (NBB 2010-064) is part of another publication[23] in which we show that after chronic use of opiates cataplexy disappeared, and that in a rodent model the number of hypocretin immunoreactive neurons increases after opiate administration. This individual with chronic opiate use is thus descriptively listed and not included in the statistical comparisons.

The patient and control groups were matched for potential confounding factors, including sex, age, postmortem delay, cerebral spinal fluid (CSF)-pH, Braak stage for tangles (Alzheimer's, AD stage), amyloid stage, Braak Lewy body stage (Parkinson's, PD stage) and the clock time of death, the month of the death, as well as fixation time (see Table). A systematic and neuropathological investigation of the entire brain of both, the NT cases and controls, has been performed and is crucial.[24] Elderly "NT1" and "Controls" can also be in the preclinical stages of AD and PD diseases. Since this can affect functional activity of the systems under study, matching for AD and PD stages is crucial.

Immunohistochemistry on Human Tissue

All formalin-fixed paraffin-embedded tissue blocks were coronally serially sectioned at 6 µm thick sections. Every 100th section was used for thionin staining to determine the anatomical orientation. The TMN, ventral tegmental area (VTA), the SN pars compacta (SNC), and pars reticulata (SNR), and LC were delineated as described previously.[25–27] The CRH positive neurons forming the Barrington nucleus were intermingled on its lateral side with the LC pigmented neurons,[18] which is different from the information in the atlases showing that this nucleus is localized separately from the LC on its ventral side.[28]

In short, after deparaffinization in xylene and rehydration through a graded ethanol series, sections were rinsed in distilled water. Details regarding antigen retrieval procedures employed, as well as specific primary and secondary antibodies used in the current study, are summarized in Table S1. This table shows the specificity and characterization of antibodies with references (PMID, PubMed unique identifier).

Quantification Strategies

To minimize bias, the rater was blinded to the type of patient (control versus NT1). Following HDC staining, we systematically quantified the cell numbers of histamine-producing neurons with a visible nucleolus.[29] In the same way, the midbrain dopamine and LC norepinephrinergic neurons density were shown by immunohistochemistry for tyrosine hydroxylase (TH), the rate-limiting enzyme in synthesizing dopamine and norepinephrine. The pigmented LC-positive neurons (brown) partly intermingled with DAB-nickel-positive CRH-positive cells and fibers (black).

TABLE. Clinical-Pathological Information

NBB	Age	Sex	PMD	pH	BW	B-AD	A	B-PD	Cause of Death	Relevant Medication
Narcolepsy type 1										
2008–023	66	F	420	6.6	1,175	1	A	0	Heart failure	Amphetamine; modafinil; sodium oxybate
2018–018	82	F	330	6.5	1,165	2	O	0	Pneumonia	Modafinil
2018–091	71	F	395	6.3	1,215	4	A	0	Heart failure, renal insufficiency	Sodium oxybate; modafinil
2021–046	83	M	442	6.9	1,355	NA	NA	NA	Severe heart failure, died within 24 h after fall with hip fracture	Sodium oxybate, paroxetine
Narcolepsy type 1 with chronic opiates										
2010–064	85	F	220	6.8	1,206	1	O	0	Chronic pain syndrome with palliative sedation	Morphine (9 yr); modafinil; Midazolam (last 24 h)
Control										
2012–052 Hyp	64	F	340	6.4	1,221	0	O	0	Legal euthanasia	Oxycodone, morphine, fentanyl, prednisolone, promethazine, codeine, oxazepam
2000–022 Hyp LC	83	F	470	6.5	1,072	2	O	0	Heart failure, Cachexia	-
2012–005 Hyp	84	F	336	6.7	1,027	2	A	0	Heart failure	Gabapentin, oxycodone, haloperidol
2009–001 Hyp	88	M	283	6.2	1,418	2	A	0	Gastro-intestinal bleeding	-
1998–104 Hyp	74	F	445	7.0	1,167	2	O	0	Cachexia in endstage pancreas carcinoma	Promethazine
1998–036 SN	69	F	375	6.6	1,229	1	NA	0	Cardiogenic shock	Levothyroxine, lorazepam
2017–131 SN	71	F	375	NA	1,175	2	NA	NA	Heart failure	Duloxetine, fentanyl, codeine, pregabaline, quetiapine, temazepam, zopiclone
2012–094 SN	82	F	315	6.3	1,221	2	B	NA	Thoracic aortic dissection	Levothyroxine, temazepam
2018–105 SN	86	M	405	6.39	1,340	3	O	0	Intra-abdominal leakage of fluid causing abdominal infection	-
2011–021 SN LC	85	F	425	NA	1,007	1	B	0	Terminal renal insufficiency	Clonazepam, diazepam, amitriptyline, morphine, zopiclone, tramadol, pramipexol, fentanyl, midazolam
2016–080 SN	83	M	305	7.12	1,108	2	C	2	Legal euthanasia	-
2001–094 LC	82	F	315	6.3	1,221	2	B	NA	Thoracic aortic dissection	Finasteride
2001–139 LC	73	F	815	6.30	1,231	2	C	0	Respiratory insufficiency	Oxazepam, paroxetine, prednisolone, haloperidol, ketanserine
2011–072 LC	76	F	435	6.87	1,072	2	O	0	Hepatic failure and colon carcinoma	Prednisolone, codeine, fentanyl, temazepam
2017–093 LC	82	M	345	7.28	1,195	2	A	0	Legal Euthanasia	Codeine, prednisolone

Abbreviations: ∼ = around that time; A = amyloid; BW = brain weight gram; B-AD = Braak Alzheimer's disease tangle stages (PMID, 8307060); B-PD = Braak Parkinson's disease Lewy body's stages (PMID, 12498954); F = female; Hyp = control for hypothalamus; LC = control for locus coeruleus; M = male; NA = not available; NBB = Netherlands Brain Bank identification number; PMD = postmortem delay in minutes; SN = control for substantia nigra.

There was an exceptional high amount of CRH fibers present in the LC area. Counting cells with a nucleolus does not give information on CRH fibers. The integrated optical density (IOD) of CRH-positive cells in the Barrington nucleus and the CRH fibers in the LC were determined.

Stereological Analysis

Light microscopy neuronal counting was performed using a Zeiss Axioskop Plan-NEOFLUAR Zeiss objectives (Carl Zeiss GmbH, Jena, Germany) with a motorized scanning stage (Märzhäuser, Wetzlar, Germany), that was connected to a color camera (Micropublisher RTV, QImaging, Surrey, British Columbia, Canada). The software used for quantification was Image Pro Plus 6.3 (Media Cybernetics, Bethesda, USA). Sections were sampled at 600 µm intervals for immunohistochemistry to determine the borders precisely. A low-magnification image (2.5 x objective) covering the areas of interest was obtained from each section. The areas of interest were outlined manually using the overview images. The Image Pro software subsequently generated a grid of rectangular fields covering the outlines, which were subsequently analyzed at a higher magnification (40 x objective) by random systematic sampling of 20% of the fields. The coefficient of variation (SD/mean x 100%) of this method was 7.8% (calculated by counting three counts per control, for two controls).

Neuronal cell numbers were determined by counting only neurons with a visible nucleolus, serving as a unique marker for each neuron and preventing double counting.[30] To prevent bias, the rater was blinded to the type of sample (control versus narcolepsy). The total number of monoaminergic neurons on one side of the hypothalamus or midbrain was determined using the Cavalieri principle as previously described.[29] Human histamine neurons are characterized by their typical lipofuscin load, with intense thionin staining of the endoplasmic reticulum localized in the periphery of the cytoplasm interspersed with typical irregularities in the cell membrane.[31] Ericson et al.'s morphological analysis of the magnocellular neurons in TMN in the rat brain showed that they were HDC immunoreactive positive.[32] In line with this observation, Panula et al. confirmed that the histamine-immunoreactivity cell bodies corresponded to "typical" TMN neuronal profiles.[33] We counted the total number of HDC staining and thionin TMN neuronal profiles with a nucleolus in both control and NT1 samples. Completeness of the cell counting was confirmed by graphically presenting the actual number of neurons counted in every section from rostral to caudal in order to review the typical rostro-caudal distribution pattern (Fig. S1). A part of the midbrain and LC was used for neuropathological staging of Parkinson's disease. Therefore, it was not complete and the density of the TH positive neurons with a nucleolus in the central part of the VTA, SNC, SNR, and LC densities (cells/mm^3) were determined in 6–10 sections.

A stack of multiple images on the fluorescence channels of Alexa 488 and Cy5 were collected using a 20x objective by a tissue scanner Axio Scan 2.0 (Carl Zeiss GmbH, Jena, Germany). The profile of CRH-expressing neurons in the PVN expressing either green fluorescence CRH (Alexa 488) or red fluorescence AVP (Cy5) were counted under Qupath version 0.3.2. software assistance.[34]

IOD Measurements

The images were obtained by scanning the sections in the tissue scanner Axio Scan 2.0 (Zeiss) with the same light condition. A stack of multiple images on 20x objective on the brightfield camera setting were collected. The image analysis was performed with Qupath version 0.3.2. On each image an outline was placed manually, surrounding separately the pigmented cells of the LC and CRH cells of the Barrington nucleus from rostral to caudal. A script was programmed in Qupath version 0.3.2. that can separate the pixel classifiers made for the pigmented cells, CRH cell bodies, and the CRH fibers. The immunoreactivity area and the optical density (OD) of these signals have been described into detail in our previous work.[35–37] In short, the background was defined in the adjacent area with absence of positive staining on the same section. OD values two times above background were considered as positive signals. The integrate optical density (IOD) was calculated by multiplying the percentage of the positive stained area by the OD of immunoreactivity signal in each section, where this IOD measurement is positively correlated with protein levels.[35–37]

Statistical Analyses

Data were not normally distributed as tested by the Komogorow–Smirnov and Shapiro–Wilk tests. NT1 and controls were, therefore, compared using the exact Wilcoxon–Mann–Whitney U test (P). Spearman's correlation coefficient tested correlations. The false discovery rate was corrected by Benjamini-Hochberg corrections (q). Intergroup differences in clock time and month of death were evaluated using the Mardia–Watson–Wheeler test. All P values are two-sided. All q values have <0.05 (*), < 0.01 (**) as the significance thresholds. Percentage changes were calculated using the median values. Data of the groups are expressed as mean ± standard error of the mean (SEM). Statistical analyses were carried out using SPSS Statistics version 25.0 (SPSS Inc, Chicago, IL). The figures were made by GraphPad Prism version 8.2 (GraphPad Software, San Diego, CA, USA).

Results

Increased co-Expression of CRH and AVP in the PVN

The number of CRH neurons co-expressing AVP in the PVN was not different between NT1 and control samples ($P = 0.806$, $q = 1.000$, Fig. 1H). In NT1, however, the percentage of CRH neurons co-expressing AVP in the PVN was significantly (234%) higher than in matched controls ($P = 0.014$*, $q = 0.024$*, Fig. 1D). The individual with NT1 and chronic use of opiates (NBB2010-064) also exhibited a higher percentage of CRH neurons co-expressing AVP in the PVN than controls.

No Difference of CRH Neurons and Fibers in Barrington Nucleus

The IOD of CRH-positive neurons and fibers in that part of Barrington's nucleus that overlapped with the pigmented neurons of the LC was not different from controls (neurons [$P = 0.476$, $q = 0.476$]; fibers [$P = 0.257$, $q = 0.476$]).

Increased Number of Histaminergic Neurons in the TMN

In NT1, there were significantly (36%) more HDC-positive neurons found in the TMN than in the matched controls ($P = 0.016$*, $q = 0.024$*, Fig. 2C). In contrast, the number of typical TMN neuronal profiles with thionin staining was similar to that of controls ($P = 1.000$, $q = 1.000$, Fig. 2F). The individual with NT1 and chronic use of opiates (NBB2010-064) had a lower number of HDC positive neurons and a lower number of typical histamine neuronal profiles than the other NT1 cases and the controls.

The Dopaminergic Neurons in the Mesencephalon

The density of TH-positive neurons tended to be higher in the SNC ($P = 0.019$*, $q = 0.076$, Fig. 3B) in NT1 than in controls, while it was not different from controls in both the VTA and SNR ($P = 0.114$, $q = 0.228$, Fig. 3A and $P = 0.257$, $q = 0.257$, Fig 3C). In the individual with NT1 and chronic use of opiates (NBB2010-064), there was no different density of TH-positive neurons compared to the controls or NT1 in the three assessed areas.

The Norepinephrinergic Neurons in the LC

The density of TH positive neurons was the same ($P = 0.257$, $q = 0.257$, Fig. 4) in the LC of NT1 as in controls. The individual with NT1 and chronic use of opiates showed a similar TH positive neuron density as controls and NT1 in this area.

Discussion

We found an increased percentage of paraventricular CRH neurons co-expressing AVP in NT1. This supports the hypothesis that there is increased activity of the remaining CRH neurons in this disorder. In addition, we confirmed that the number of histamine neurons expressing HDC in the posterior hypothalamus is significantly increased in NT1 (36% here, compared to 64%–94% in other studies[16,17]). In contrast, the total number of typical Nissl stained TMN neurons was not changed in NT1. This implies an upregulation of HDC by existing, but not active, histamine neurons in NT1. We also demonstrated that the density of dopamine neurons, marked by TH, in the midbrain tended to be increased in the SNC but was stable in both the VTA and SNR. Also, the density of TH-positive LC neurons and CRH immunoreactivity in the Barrington nucleus was stable in NT1.

Remaining CRH Neurons in NT1

The percentage of CRH neurons co-expressing AVP was shown to be a reliable measure of activation of these neurons in both humans[38] and rodents.[39,40] Paraventricular CRH neurons co-expressing AVP were found to be increased in animals with a hyperactive hypothalamic–pituitary–adrenal (HPA) axis.[39–42] In humans, the number of CRH neurons co-expressing AVP increases with ageing,[10] in Alzheimer's disease,[10] in depression[12] and in multiple sclerosis,[14,43] accompanied by higher serum or CSF cortisol levels.[15,38] In NT1 a marked 88% reduction of CRH-expressing neurons in the PVN was observed.[8] Paraventricular CRH-positive neurons were found to be the major population directly innervating hypocretin (orexin) neurons with monosynaptic contacts.[44] The questions as to whether the hypocretin or CRH neurons were first to disappear in the NT1 process and whether one of the cell types influenced the other in the process of degeneration cannot be answered at present. Our findings of many more paraventricular CRH neurons co-expressing AVP in NT1, support the hypothesis that the remaining CRH neurons are hyperactive in NT1; this may at least partly explain the normal *in vivo* basal plasma cortisol level described in these individuals.[9] These activated CRH neurons may also affect central processes, such as the sleep–wake cycle.[44,45] It should be noted that the fact that, in NT1 a strongly decreased number of CRH immunoreactive cells is present, does not prove that the cells have disappeared. The may contain CRH under the detection level, either due to decreased production or enhanced transport and release. The absolute number of CRH neurons co-expressing AVP in NT1 was similar to controls. An alternative explanation may be that these neurons selectively persevered in NT1 because of their hyperactivity, as was also proposed for other active neurons in a neurodegenerative disorder such as Alzheimer's disease.[46] The alternative possible explanations warrant future studies.

Other Wake-Systems in NT1

In line with earlier reports,[16,17] we found that the number of TMN neurons expressing HDC in the posterior hypothalamus was significantly increased in NT1. Interestingly, the total number of typical Nissl stained TMN neurons was not different in NT1 and controls. Almost all NT1 cases showed a significantly higher proportion of HDC cell counts than the number of typical Nissl stained TMN

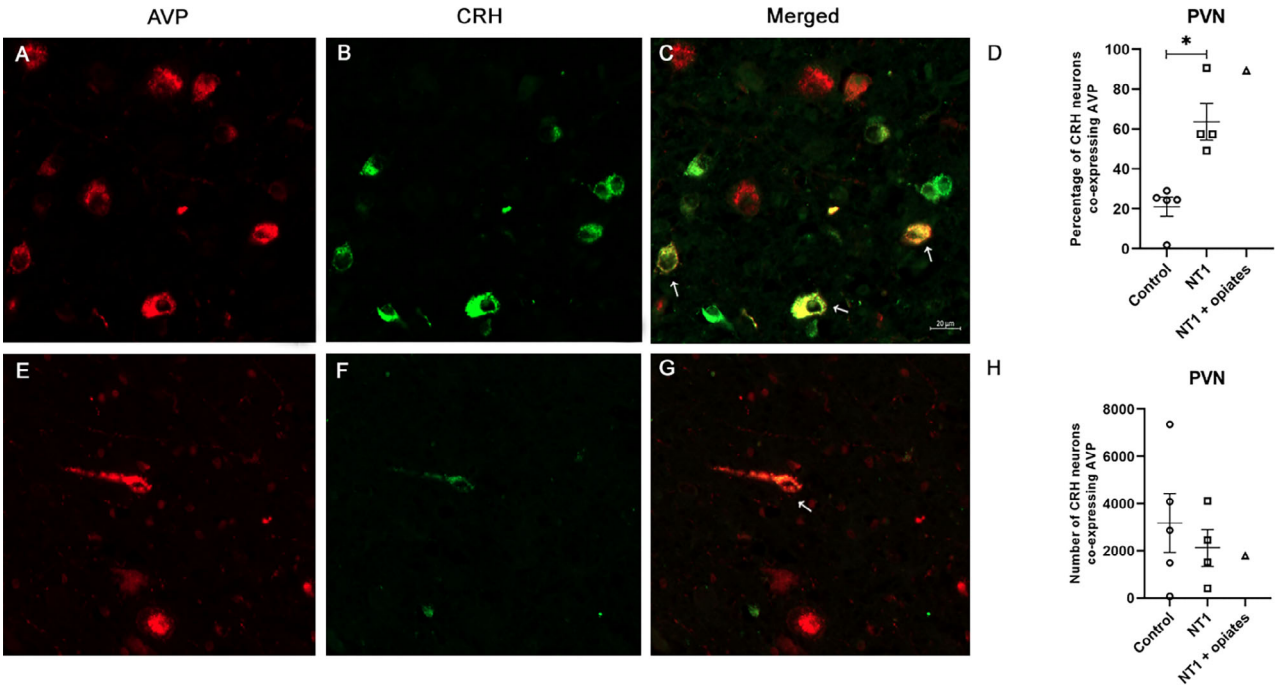

FIGURE 1: Increase in proportion of corticotropin-releasing hormone (CRH) positive neurons co-express vasopressin (AVP) in the paraventricular nucleus (PVN). In controls, (A–C) few (24.5%) CRH positive neurons (green) co-localized (indicated by white arrows) with AVP (red) and stain yellow. In the PVN of people with NT1 (E–G), the few remaining CRH positive neurons (green) largely (57.5%) co-localize (indicated by white arrows) with AVP (red). The percentage of CRH neurons co-expressing AVP (D) was significantly increased in NT1 compared to controls. The individual with NT1 and chronic opiate use (NT1 + opiates) showed a similar percentage of CRH neurons that co-expressed AVP as the others with NT1. Scale bar in C represents 20 μm for A–C and E–G. In the (D) (H), the error bars show the mean ± SEM, Mann–Whitney *U* test followed by Benjamini-Hochberg correction *q*. * 0.01 < *q* < 0.05. [Color figure can be viewed at www.annalsofneurology.org]

neurons, whereas this was the case in only two out of five controls (Fig. S1). The distribution of HDC-positive neurons overlapped with that of the Nissl stained TMN neuronal profiles in all NT1 and control cases (Fig. S1). This means that in NT1 HDC is also stained in TMN neurons that do not have the typical microscopic

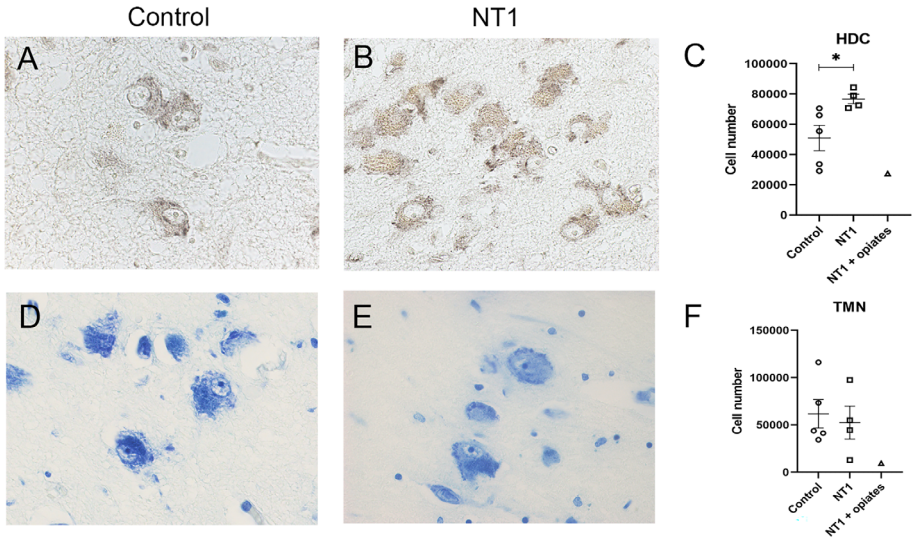

FIGURE 2: Increase in the number of histidine decarboxylase (HDC) positive neurons but not in the number of tuberomammillary nucleus (TMN) neurons in Narcolepsy type 1 (NT1). (A–C) The total number of HDC positive neurons is 36% higher in NT1 than in controls. (D–F) The total number of typical TMN neuronal profiles in Nissl staining is similar in NT1 and controls. Representative photomicrographs are from controls (A, D) and NT1 (B, E). The quantitative data are presented in C and F. The scale bar represents 10 μm. The error bars show the mean ± SEM. The *P* value was tested by the Mann–Whitney *U* test between NT1 and controls, which was corrected by the Benjamini-Hochberg test *q*. * 0.01 < *q* < 0.05. The individual with NT1 and chronic opiate use (NT1 + opiates) is shown separately. [Color figure can be viewed at www.annalsofneurology.org]

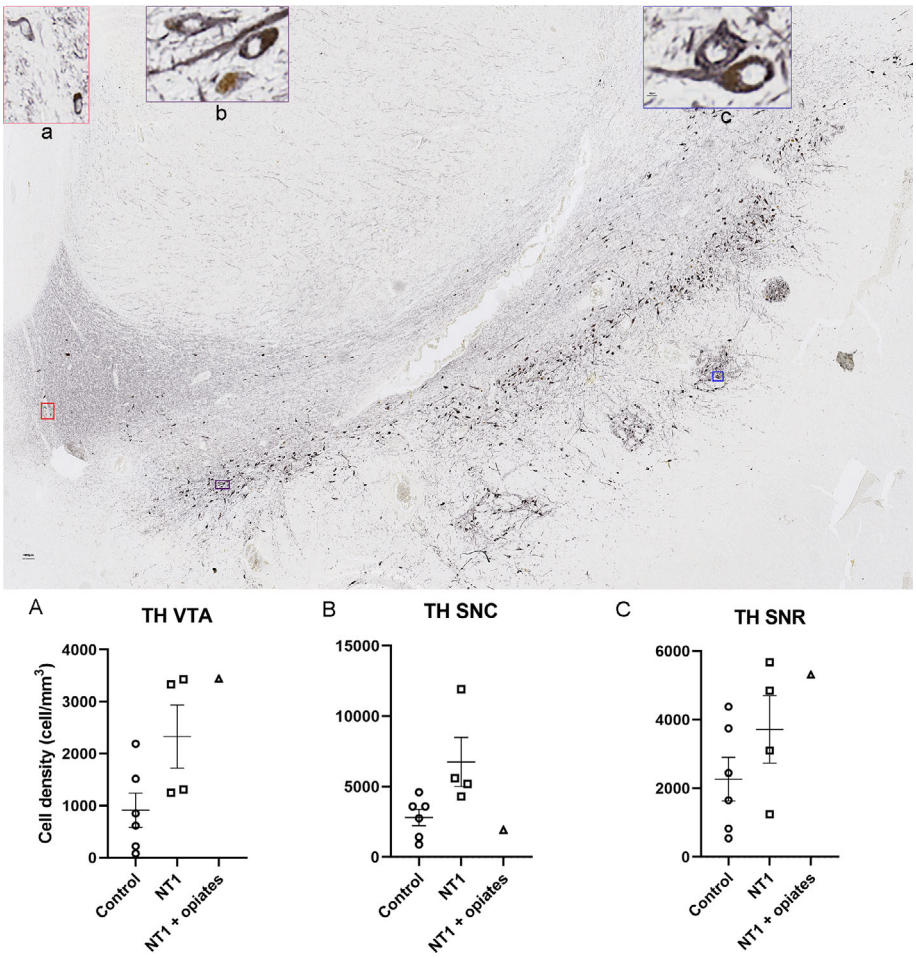

FIGURE 3: Unaltered density of tyrosine hydroxylase (TH) positive neurons in the mesencephalon. Representative photomicrographs from TH positive neurons in a control and higher magnifications of (A) the ventral tegmental area (VTA), (B) the substantia nigra pars compacta (SNC), or (C) the substantia nigra pars reticulata (SNR). The quantitative data (A–C). Scale bar represents 1,000 μm for B and 10 μm for inserts in C. The error bars show the mean ± SEM. The Mann–Whitney *U* test was corrected by the Benjamini-Hochberg test *q*. $q \geq 0.076$. The individual with Narcolepsy type 1 (NT1) and chronic opiate use (NT1 + opiates) is shown separately. [Color figure can be viewed at www.annalsofneurology.org]

appearance of TMN neurons and have in controls a lower than the immunohistochemical detection level of HDC. Diurnal fluctuations of HDC-mRNA and protein have been reported in humans and rodents.[47,48] In controls, HDC-mRNA expression was significantly (37%) higher in those with a clock time of death during daytime than in those who died during the night.[47] However, it should be noted that the patients and controls in our study were matched for the time of death. In nocturnal mice, the number of neurons containing HDC was 34% greater in the active phase than in the rest phase.[49] These changes are all in the same order of magnitude as the 36% increase we found in the number of HDC neurons in people with NT1. The observations above support the hypothesis that there is an upregulation of HDC by existing histamine cells in NT1, rather than generation of new histaminergic neurons. We observed the density of TH positive neurons in the SNC tends to be increased, but not in the VTA and SNR. Together with the significant higher number of HDC positive neurons this may perhaps be seen as a compensatory mechanism after loss of hypocretin signaling, which may—at least partly—contribute to the frequent arousals from sleep seen in NT1.[50]

Limitations

One limitation of the present study is the low number of cases. As NT1 is a rare disorder, there are few postmortem brains from people with narcolepsy in brain banks worldwide. Yet, even with five NT1 and five control cases, we confirm the greatly reduced number of hypocretin cells and increased histaminergic neurons as reported in other studies.[2,3,8,16,17] An alternative explanation for the percentage of CRH neurons co-expressing AVP in NT1 could be narcolepsy medication, such as modafinil or sodium oxybate. We are unaware of any evidence that anti-narcoleptic drugs could stimulate CRH neurons.

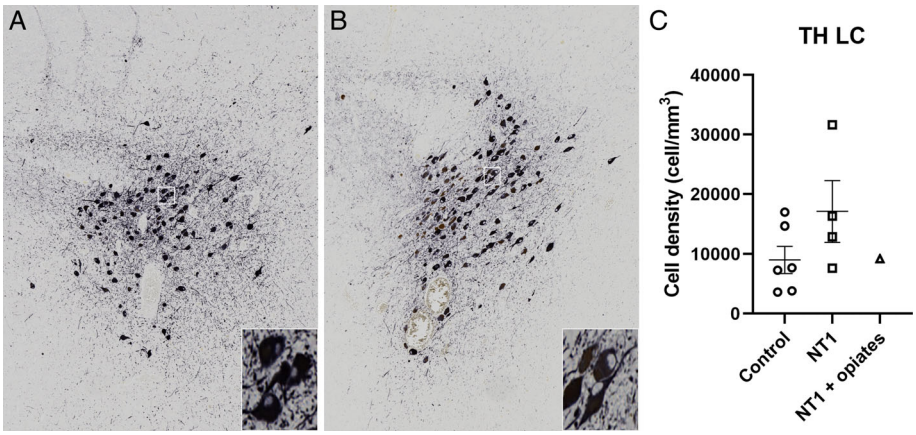

FIGURE 4: Unaltered tyrosine hydroxylase (TH) neuronal density in the locus coeruleus (LC). Representative photomicrographs from (A) a control and (B) an individual with Narcolepsy type 1 (NT1), including a higher magnification of TH positive neurons. Scale bar represents 100 µm for A and 10 µm for insert. (C) The TH positive neurons in quantitative data are presented. The error bars show the mean ± SEM. The difference was tested with Mann–Whitney U ($P \geq 0.257$). The individual with NT1 and chronic opiate use (NT1 + opiates) is shown separately. [Color figure can be viewed at www.annalsofneurology.org]

The individual with NT1 and chronic use of opiates (NBB2010-064), who had stopped modafinil 2 years before death and never used sodium oxybate, still showed a significantly higher percentage of CRH neurons co-expressing AVP, falling well within the range of those with NT1. This suggests that it is not likely that anti-narcoleptic treatment has influenced the percentage of CRH-positive neurons co-expressing AVP. One of the assessed controls had used corticosteroids months before death (N2012-052). This did not significantly impact the percentage of CRH neurons co-expressing AVP. Last, in the individual with NT1 who had chronically used opiates, it appears to have led to an increase in the number of hypocretin immunoreactive neurons.[23] The use of opiates may also have had unknown effects on other neuronal populations, such as a lower number of HDC-positive neurons and typical histamine neuronal profiles than the other NT1 cases and controls.

In conclusion, our evidence points toward the increased activity of remaining CRH neurons in NT1. This may explain—at least partly—earlier clinical findings in NT1, such as normal 24-h secretion levels of plasma cortisol,[9] and lower levels after dexamethasone suppression,[51] suggesting a partial compensation of HPA-axis activity. An alternative or additional, explanation for our findings is that CRH neurons co-expressing AVP are less vulnerable and selectively remain in NT1. Furthermore, there is an upregulation of HDC immunoreactivity by existing histamine cells. The number of dopamine neurons in the SNC tends to be increased. Alterations in not only the hypocretin system, but also in dopamine, histamine, and CRH systems may all contribute to the symptomatology of NT1. Currently, NT1 treatments target either the hypocretin,[52] the histamine,[19] or the dopamine and norepinephrine systems.[20] Our findings support the notion that additionally targeting the CRH and HPA-axis may also help to improve the symptoms of NT1.

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Author Contributions

L.S., R.F., G.J.L., and D.F.S. conceptualized and designed the study. L.S., D.F.S., S.L., Z.H., Fd.D., N.B., and J.A., acquisition the analysis of data. L.S., R.F., G.J.L., and D.F.S. contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

L.S., R.F., and G.J.L. have received research support from Jazz Pharma (Solriamfetol is a Jazz Pharma medication used to treat excessive sleepiness) and Bioprojet (Pitolisant, manufactured by Bioprojet, is used in the treatment of narcolepsy). R.F., L.S., and G.J.L. have received consultancy fees from Takeda. R.F. and G.J.L. have received consultancy fees from Bioprojet and Jazz Pharmaceuticals. G.J.L. have received consultancy fees from UCB. R.F. have received consultancy fees from Lundbeck, Lilly, Novartis and TEVA. The other authors have no conflict to disclose.

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