Narcolepsy type 1 (NT1) is a chronic sleep disorder characterized by excessive daytime sleepiness, cataplexy, and disturbed nocturnal sleep. NT1 research has focused on loss of hypocretin-producing neurons in the hypothalamus. Notable findings supporting neural loss were decreased hypothalamic expression of neuronal activity-regulated pentraxin and dynorphin, both co-expressed by hypocretin neurons. A systematic search for involvement of hypothalamic neurons other than hypocretin neurons has never been performed in NT1.

In this study, we therefore systematically examined other hypothalamic cell groups, in a well-defined post-mortem collection of NT1 (n = 5) and controls (n = 5), including the suprachiasmatic nucleus (SCN), ventrolateral preoptic nucleus (VLPO), paraventricular nucleus (PVN), infundibular nucleus (IFN), supraoptic nucleus (SON), and nucleus basalis of Meynert (NBM).

Methods

Hypothalamic and locus coeruleus samples were obtained from the Netherlands Brain Bank (NBB). Permission for the autopsy, and use of brain material and clinical data for research purposes was obtained from the patient or next of kin. Five NT1 subjects, one of which had been treated chronically with opiates, and 5 matched controls free from neuropsychiatric disorders were included. All brains were systematically neuropathologically investigated. The patients with NT1 had been treated by experienced somnologists (authors G.J.L. and R.F.; Table 1 and Table S1). The subject with NT1 who had been treated with opiates, after which symptoms improved and cataplexy disappeared, has been reported in a prior study, where the hypocretin cell counts of NBB2008-023 and NBB2010-064 were determined. Because the median eminence of the 4 control was not available, 4 alternative controls (marked with * in the Table 1) were included. All potential confounding factors of patients and controls were matched for (see the Table 1; p ≥ 0.41).

Immunohistochemistry, Cell Counting

All formalin-fixed paraffin-embedded hypothalamsus and locus coeruleus tissue blocks were coronally serially sectioned at 6 μm. Details of antigen retrieval procedures and specificity of primary antibodies are summarized in Table S2. Immunoreactivity was visualized by the avidin-biotin complex method using DAB–nickel. Six sections were treated with silver-gold to enhance immunohistochemical staining. The coefficient of variation (SD/mean × 100%) of 20% random sampling was 7.8% (calculated by counting one control subject 3 times).

As in our group’s previous study, neuronal cell numbers were determined blind for the patient information by

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counting the nucleolus as a unique marker for each neuron. The total number of neurons on one side of the hypothalamus was determined using the Cavalieri principle. Completeness of the corticotropin-releasing hormone (CRH), SCN vasopressin (AVP), and hypocretin cell counts were confirmed by graphically presenting the number of neurons counted in every section from rostral to caudal. In contrast, due to scarcity of hypothalamic sections, AVP, oxytocin, tyrosine hydroxylase (TH), and thyrotropin releasing hormone (TRH) staining were performed only in the peak section of the PVN. The CRH peak level had an excellent correlation with total CRH cell numbers ($r = 0.926$, $p = 0.003$).
The density of neuronal populations in the VLPO, NBM, SON, and IFN was similarly determined.

Prohormone convertase 1/3 (PC1/3) and secretagogin (SCGN) have both been reported to be partially co-expressed in CRH neurons.12,13 Double labeling of CRH with PC1/3 or SCGN was confirmed by immunofluorescent confocal imaging using the Leica microscope TSA SP5 at 20 times magnification.

Integrated Optical Density Image Analysis
Two microglia markers, ionized calcium-binding adaptor molecule 1 (IBA1)14 and human leukocyte antigen (HLA), and an enzyme for cortisol synthesis (11β-hydroxysteroid-dehydrogenase [11bHSD1]) were investigated. This enzyme was tested because high local cortisol levels may inhibit CRH-expressing neurons in the PVN. Because glia cells have no nucleolus, the neuronal counting procedure could not be applied and the integrated optical density (IOD) was measured for IBA1, HLA, 11bHSD1, and CRH fibers in the median eminence. The collected images were transformed into OD images using a standard transformation curve, corrected for background. The image analysis system and procedures have been described in detail previously.15 The IOD was calculated by multiplying the percentage of the positive-stained masked area by the OD of the immunoreactivity signal in each section.

Statistical Analyses
NT1 and controls were compared using the exact Wilcoxon-Mann-Whitney U test (P) followed by Benjamini-Hochberg corrections (q). The subject with NT1 with opiates was descriptively listed. Correlations were tested by Spearman’s correlation coefficient. All p values are 2-sided with 0.05 (*) as the significance threshold using SPSS Statistics 25. Percentage changes were calculated using the median values.

Results
We confirmed the substantial loss of 97% of the total number of hypocretin-1 immunoreactive neurons in NT1 compared to the matched controls (p = 0.016*, q = 0.021*; Fig. ). In addition, most of the surviving hypocretin neurons appeared to be localized in the dorsomedial or peri-fornical area, as has been described previously.16 The subject with NT1 with chronic opiate use exhibited relatively more hypocretin neurons than the 3 typical subjects with NT1, as described before.7

The total number of CRH-positive neurons in the PVN of the patients with NT1 was significantly reduced by 88% compared to the controls (p = 0.016*, q = 0.021*; see Fig. ). In addition, the subject with NT1 with chronic opiate use clearly showed a lower number of CRH-positive neurons than the controls (see Fig. ). In agreement with the CRH-cell staining loss, CRH fibers in the median eminence were significantly reduced by 91% in patients with NT1 compared to controls (p = 0.016*, q = 0.021*; see Fig. ). In contrast, the CRH neurons in the thalamus (ie, the paraventricular and parathenial thalamic nuclei and around the medial dorsal thalamic nucleus) and in the locus coeruleus were unaffected (see Fig. ).

To test the possibility that this was a staining sensitivity issue, we performed 3 additional control experiments, none of which changed the results: (1) increasing the concentration of the same CRH antibody 10 times either with or without antigen retrieval with microwave; (2) using another CRH antibody, a rabbit polyclonal antibody Cat#5Bo; (3) using silver-gold enhancement,10 a method to enhance the visibility of immunohistochemical staining.

In contrast, densities of the other major neuronal cell types in the PVN were stable, including AVP, oxytocin, TH, and TRH. Microglia markers, IBA114 and HLA, and 11bHSD1, were similar in patients with NT1 and controls in the PVN. Additionally, all other quantified hypothalamic cell groups were stable (Table 2). Moreover, PC1/3 and SCGN staining was unaltered in the PVN of the subjects with NT1.

Discussion
We found that patients with NT1 show not only 97% loss of hypocretin neurons but also an 88% reduction of CRH-expressing neurons in the PVN compared with controls, whereas CRH-positive neurons in the thalamus and in the locus coeruleus were unaffected. It should be noted that, in the future, it is of interest to also study other CRH-containing brain regions, such as the amygdala. A 91% reduction of CRH-staining fibers in the median eminence was observed. The CRH-positive neurons in the PVN are the primary group of neurons of the HPA axis and project to the median eminence containing the portal capillaries of the pituitary.8 In contrast, the other major neuronal types intermingled with the CRH-positive neurons in the PVN were spared, including AVP-positive, oxytocin-positive, TH-positive, and TRH-positive neurons. AVP and oxytocin neurons in both the PVN and SON all were stable. No changes were observed in the neuronal densities in VLPO,17 SCN, NBM, or IFN.

Theoretically, the observed low number of CRH neurons might be due to extremely low CRH concentrations below the threshold for immunohistochemical detection. However, we tried 3 different ways to enhance the staining
(Figure legend continues on next page.)
signal, none of which changed the results. Earlier findings showed that chronic treatment with opiates can increase the number of immunohistochemically stained hypocretin cells, whereas cataplexy was ameliorated.\(^7\) Of interest, opiate treatment had no effect on the CRH-neuron number in the PVN of this subject.

**TABLE 2. Immunocytochemistry and Quantification Procedures**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Primary antibody</th>
<th>Q</th>
<th>NT1 (opiates)</th>
<th>Control</th>
<th>NT1 (opiates)</th>
<th>R (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN</td>
<td>AVP TC</td>
<td>11,749 ± 7,679</td>
<td>11,786 ± 3,926</td>
<td>18,700</td>
<td>1.00 (0.08 1.93)</td>
<td></td>
</tr>
<tr>
<td>VLPO</td>
<td>Galanin CD</td>
<td>44 ± 17</td>
<td>35 ± 16</td>
<td>15</td>
<td>0.80 (0.20 1.81)</td>
<td></td>
</tr>
<tr>
<td>MLH</td>
<td>Hcrt TC</td>
<td>4,175 ± 2,639</td>
<td>41,493 ± 5,931</td>
<td>16,384</td>
<td>13.10 (7.90 18.29)</td>
<td></td>
</tr>
<tr>
<td>NBM</td>
<td>ChAT CD</td>
<td>5,155 ± 189</td>
<td>4,954 ± 797</td>
<td>3,713</td>
<td>0.96 (0.47 1.45)</td>
<td></td>
</tr>
<tr>
<td>PVN</td>
<td>CRH TC</td>
<td>2,862 ± 1,704</td>
<td>21,197 ± 5,517</td>
<td>0</td>
<td>9.02 (2.50 15.54)</td>
<td></td>
</tr>
<tr>
<td>PVN/SON</td>
<td>AVP CD</td>
<td>PVN (70 ± 30)</td>
<td>PVN (49 ± 11)</td>
<td>PVN (86)</td>
<td>1.34 (0.30 2.37)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxy CD</td>
<td>PVN (34 ± 4)</td>
<td>PVN (48 ± 10)</td>
<td>PVN (16)</td>
<td>2.75 (0.51 5.00)</td>
<td></td>
</tr>
<tr>
<td>PVN</td>
<td>Th CD</td>
<td>14 ± 6</td>
<td>27 ± 9</td>
<td>4</td>
<td>1.89 (0.22 3.56)</td>
<td></td>
</tr>
<tr>
<td>PVN</td>
<td>SCG CD</td>
<td>2 ± 2</td>
<td>15 ± 12</td>
<td>0.010</td>
<td>6.12 (8.11 20.35)</td>
<td></td>
</tr>
<tr>
<td>PVN</td>
<td>PC1/3 CD</td>
<td>7 ± 3</td>
<td>14 ± 9</td>
<td>0.002</td>
<td>2.10 (1.53 5.73)</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>NPY CD</td>
<td>10 ± 3</td>
<td>16 ± 6</td>
<td>35</td>
<td>1.59 (0.03 3.15)</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>POMC CD</td>
<td>16 ± 6</td>
<td>28 ± 11</td>
<td>40</td>
<td>2.01 (0.25 4.27)</td>
<td></td>
</tr>
<tr>
<td>PVN</td>
<td>11βHSD1 IOD</td>
<td>0.03 ± 0.001</td>
<td>0.003 ± 0.001</td>
<td>0.003</td>
<td>1.11 (0.28 2.50)</td>
<td></td>
</tr>
<tr>
<td>PVN</td>
<td>HLA IOD</td>
<td>0.026 ± 0.006</td>
<td>0.030 ± 0.020</td>
<td>0.015</td>
<td>1.01 (0.86 2.88)</td>
<td></td>
</tr>
<tr>
<td>PVN</td>
<td>IBA1 IOD</td>
<td>0.010 ± 0.003</td>
<td>0.010 ± 0.004</td>
<td>0.018</td>
<td>1.00 (0.11 2.11)</td>
<td></td>
</tr>
</tbody>
</table>

Data of the groups are expressed as mean ± standard error of the mean (SEM); 11βHSD1 = 11β-hydroxysteroid-dehydrogenase; AVP = arginine vasopressin; ChAT = choline acetyltransferase; CD = cell density (cell/mm\(^2\)); CRH = corticotropin-releasing hormone; Hcrt = hypocretin-1 /orexin A; HLA = human leukocyte antigen-DP, DQ, DR; IBA1 = Ionized calcium binding adaptor molecule 1; IFN = infundibular nucleus; IOD = integrated optical density; NBM = nucleus basalis of Meynert; NPY = neuropeptide Y; NT1 = narcolepsy type 1 (narcolepsy with cataplexy); NT1 with opiates = NBB2010-064; Oxy = oxytocin; PC1/3 = prohormone convertase 1/3; SCN = suprachiasmatic nucleus; SCG = secretagogin double stain with CRH; SON = supraoptic nucleus; TC = total cell number; Th = tyrosine hydroxylase; TRH = thyrotropin releasing hormone; MLH = medial lateral hypothalamus; VLPO = ventrolateral preoptic nucleus; R = mean of ratio (controls/NT1 mean); 95% CI = 95% confidence interval (lower bound upper bound).

**FIGURE:** Patients with narcolepsy type 1 (NT1) have not only a loss of 97% of hypocretin/orexin (Hcrt) neurons but also an 88% reduction of corticotropin-releasing hormone (CRH) expressing neurons in the paraventricular nucleus (pvn) and a 91% reduction of CRH-positive fiber staining in the median eminence (me). (A) Control and (B) NT1 Hcrt immunoreactive cells. (C) The total number of Hcrt neurons is more than 97% reduced in patients with NT1 than in controls. (D) Control and (E) NT1 CRH immunoreactive cells in the pvn. (F) The total number of CRH neurons in the PVN is 88% lower in patients with NT1 than in controls. The subject with NT1 with chronic opiates (NT1 + opiates) also showed few CRH neurons. (G) Control and (H) NT1 fiber immunoreactivity in the median eminence. (I) The total optical density of CRH in the peak level of median eminence is 91% lower in NT1 than in controls. (J, K) Photomicrographs showing pigmented locus coeruleus neurons (indicated with an arrow head) and (J) an example of the positive CRH staining (indicated with an arrow) in the locus coeruleus of control, (K) a positive CRH staining (indicated with an arrow) in the locus coeruleus of the subject with NT1. The 6 μm sections of the locus coeruleus area contained up to 20 CRH neurons, in both, the controls and the patients with NT1. Scale bar represents 10 μm for A; 100 μm for D and 10 μm for insert; 200 μm for G and H, and 20 μm for K. Bar plots show the mean and the lower Bound-Upper Bound of the 95% confidence intervals in C, F, and I. [Color figure can be viewed at www.annalsofneurology.org]
A limitation of the present study is the low number of subjects. Because NT1 is a rare disorder,1 there are very few postmortem brains available for research. However, it is unlikely that this has influenced our conclusions, because the reduction in CRH neurons was so striking. In addition, even with this small size, we confirmed the greatly reduced number of hypocretin cells.2,3 In addition, it is unclear whether current narcolepsy animal models have diminished CRH neuron numbers in the PVN. Another question is whether the loss of hypocretin-expressing and CRH-expressing neurons are functionally related.

In conclusion, 2 decades ago, the discovery that hypocretin production is almost completely lost in human NT12,3 led to the successful establishment of the diagnostic standard (i.e. low/undetectable hypocretin-1 levels in CSP).18 Our study indicates that besides hypocretin deficiency, there is also a marked loss of CRH-positive neurons selectively in the PVN. This may explain earlier findings, such as a 60% reduction in the basal secretion of ACTH in patients with NT1,19 and lower plasma levels of cortisol after dexamethasone suppression.20 The unaffected number of AVP neurons in the biological clock may explain the normal circadian rhythm of adrenocorticotropic hormone.19 The marked loss of CRH-positive neurons in the PVN may provide a novel target for diagnostics and therapeutic interventions, and for identifying the target of the presumed auto-immune cause of narcolepsy.

Acknowledgments
L.S. has received funding from the European Union’s Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant (Agreement No. 707404) and has been awarded the Young Scientist Award 2020 by the European Narcolepsy Network (supported by the Klaus-Grawe Foundation). D.F.S. and L.S. were supported by Friends of the Netherlands Institute for Neuroscience Foundation. This work was partially supported by research grants from the Bioprojet-Biotech, Saint Gregoire, France. The funding agencies had no role in the design of the study, nor in the collection, analysis, or interpretation of data.

The authors are greatly indebted to the Netherlands Brain Bank and the following persons for their invaluable support: R.W.H. Verwer, J. Coppens, J. van Heerikhuize, M. Kooreman, M. Groot, J.R. Zhou, J. Shen, S. Linssen, Z. Harteman, and P. Reinbold. The authors are grateful to Professor I. Huitinga from the Netherlands Institute for Neuroscience for providing the brain samples and material from multiple sclerosis brain tissue that served as positive controls. Dr. D. Roelen from the LUMC did the HLA genotyping. We thank Professors G. Martens and J. Homberg from the Radboud University for the suggestions and valuable discussion.

Author Contributions
L.S., R.F., G.J.L., and D.F.S contributed to the conception and design of the study. L.S., D.F.S., R.B., R.F., and G.J.L. contributed to the acquisition and 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 in the treatment of excessive sleepiness) and Bioprojet (Pitolisant, manufactured by Bioprojet, is used in the treatment of narcolepsy). R.F. and G.J.L. have received consultancy fees from Bioprojet.

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