CD8 T cell-mediated killing of orexinergic neurons induces a narcolepsy-like phenotype in mice

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Narcolepsy with cataplexy is a rare and severe sleep disorder caused by the destruction of orexinergic neurons in the lateral hypothalamus. The genetic and environmental factors associated with narcolepsy, together with serologic data, collectively point to an autoimmune origin. The current animal models of narcolepsy, based on either disruption of the orexinergic neurotransmission or neurons, do not allow study of the potential autoimmune etiology. Here, we sought to generate a mouse model that allows deciphering of the immune mechanisms leading to orexin$^+$ neuron loss and narcolepsy development. We generated mice expressing the hemagglutinin (HA) as a "neo-self-antigen" specifically in hypothalamic orexin$^+$ neurons (called Orex-HA), which were transferred with effector neo-self-antigen–specific T cells to assess whether an autoimmune process could be at play in narcolepsy. Given the tight association of narcolepsy with the human leukocyte antigen (HLA) *HLA-DQB1*06:02* allele, we first tested the pathogenic contribution of CD4 Th1 cells. Although these T cells readily infiltrated the hypothalamus and triggered local inflammation, they did not elicit the loss of orexin$^+$ neurons or clinical manifestations of narcolepsy. In contrast, the transfer of cytotoxic CD8 T cells (CTLs) led to both T-cell infiltration and specific destruction of orexin$^+$ neurons. This phenotype was further aggravated upon repeated injections of CTLs. In situ, CTLs interacted directly with MHC class I-expressing orexin$^+$ neurons, resulting in cytolytic granule polarization toward neurons. Finally, drastic neuronal loss caused manifestations mimicking human narcolepsy, such as cataplexy and sleep attacks. This work demonstrates the potential role of CTLs as final effectors of the immunopathological process in narcolepsy.

autoimmunity | narcolepsy | CD8 T cells | sleep disorders | orexin

Narcolepsy with cataplexy, referred to as type 1 narcolepsy (T1N), is a rare and chronic neurological disease characterized by excessive daytime sleepiness, sudden loss of muscle tone triggered by emotions (cataplexy), sleep paralysis, hypnagogic hallucinations, and fragmented nocturnal sleep (1). T1N is caused by a defective neurotransmission by the orexin/hypocretin neuropeptide and is associated with a selective and almost complete loss (85–100%) of orexinergic neurons in the hypothalamus (2, 3). The mechanisms leading to this neuronal loss are not yet elucidated, although current evidence points to an autoimmune process. Indeed, T1N is tightly associated with the human leukocyte antigen (HLA) *HLA-DQB1*06:02* allele, carried by 98.4% of patients vs. 17.7% of the general European population (4). An independent association with HLA class I alleles was recently revealed in two independent studies (5, 6). Additionally, an association with polymorphisms in the T-cell receptor (TCR) α chain locus was found and replicated (7, 8). Moreover, autoantibodies recognizing different antigenic targets expressed in the central nervous system (CNS) have been identified in the serum and cerebrospinal fluid (CSF) of narcoleptic patients (9–11). Finally, a dramatic increase in the incidence of T1N has been observed in Northern Europe during the 2009–2010 vaccination campaigns against pandemic H1N1 influenza virus using the Pandemrix vaccine (12–14). The immune mechanisms involved remain unknown, although molecular mimicry is strongly suspected (9, 15). However, recent results demonstrate that a H1N1 virus could have, by itself, a cytolytic impact on orexinergic neurons, but also on adjacent or more distant neuronal subsets (16).

To date, mouse models of T1N are based on genetic disruption of the orexinergic neurotransmission or the destruction of orexin$^+$ neurons through the expression of a deleterious gene (17–19). These models have well documented the key role of the orexinegic system for sleep/wake behavior and architecture and for muscular tonus, but they do not allow the analysis of the etiology and mechanisms of orexin$^+$ neuron destruction.

In the present work, we investigated whether an autoimmune process could lead to T1N development and deciphered the effector mechanisms responsible for the selective loss of orexin$^+$ neurons. To this end, we generated mice expressing a "neo-self-antigen" selectively in orexin$^+$ neurons and adoptively transferred neo-self-antigen–specific effector T cells in these mice. We show that both antigen-specific Th1 CD4 cells and cytotoxic CD8 T cells (CTLs) were able to cause hypothalamic inflammation. However, only CTLs were capable of triggering a selective loss of orexin$^+$ neurons mimicking human T1N. The data also support direct and antigen-dependent CTL-mediated cytotoxicity of the orexin$^+$ neurons as the mechanism of neuronal demise. Moreover, this

Significance

Narcolepsy with cataplexy is a sleep disorder characterized by excessive daytime sleepiness and sudden loss of muscle tone. These clinical manifestations are the result of selective loss of a neuronal population producing orexin. The etiology of the disease remains elusive, although converging evidence points to a key involvement of the immune system. We developed an animal model to study the autoimmune processes at play in narcolepsy. We demonstrate that cytotoxic CD8 T cells, but not Th1 CD4 cells, are able to target and destroy orexinergic neurons. This selective neuronal loss is responsible for clinical signs mimicking human narcolepsy. By identifying potential immune effectors of the immunopathological process in narcolepsy, these findings offer a rationale for the use of immunotherapies.

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neuronal loss leads to a narcoleptic-like phenotype. Our results thus emphasize that CTLs could play a central role in the final steps of narcolepsy immunopathogenesis.

Results

Expression of HA as a Neo-Self-Antigen Selectively in Orexin$^+$ Neurons. To test a potential autoimmune basis of T1N, we generated a mouse line, named Orex-HA, expressing the H1N1 influenza virus HA as a neo-self-antigen specifically in orexinergic neurons. To this end, the Rosa26$^{tm(HA)1Lib}$ mice (20) were crossed with the Orex-Cre animals, expressing Cre under the control of the human orexin promoter (21).

To evaluate the expression pattern of HA in the Orex-HA mice, we monitored the transcription of HA in different parts of the brain by quantitative RT-PCR (Fig. S1*A*). HA mRNA was highly enriched in the hypothalamus and the basal forebrain, a site of intense projection from orexinergic neurons (22, 23), compared with the rest of the brain (Fig. S1*A*). HA expression was not detected in any other tissue tested by RT-PCR, except for the heart. As expected, HA was not expressed in wild-type (WT) littermate control mice (Fig. S1*A*). HA protein expression was detected by immunohistofluorescence in ∼75% of orexin$^+$ neurons from Orex-HA mice, but not in WT littermates (Fig. S1 *B* and *C*). Only few hypothalamic cells expressing HA (≤7%) were not immunostained for orexin (Fig. S1*D*). Collectively, these results show that HA is expressed as a neo-self-antigen selectively in the orexin$^+$ neurons of Orex-HA mice.

The Orex-HA mice presented a normal phenotype and exhibited a sleep architecture and numbers of orexin$^+$ neurons similar to those of WT littermates.

Neo-Self-Antigen–Specific Th1 CD4 T Cells Infiltrate the Hypothalamus of Orex-HA Mice, but Fail to Elicit Neuronal Demise in the Absence of Autoantibodies. A remarkable association has been found worldwide between T1N and the *HLA-DQB1*06:02* allele, reaching an odds ratio of >250 (4), suggesting the contribution of CD4 T cells to the disease process. Furthermore, recent studies reported an increase in IFN-γ or IFN-γ–induced chemokines in the serum or CSF of T1N patients (24, 25). We therefore evaluated the ability of in vitro differentiated neo-self-antigen–specific Th1 cells to trigger the destruction of orexin$^+$ neurons in Orex-HA mice by adoptive transfer experiments (Fig. S2 *A* and *B*).

To determine whether the antigen-specific Th1 cells could reach the antigen-expressing region of the brain, we analyzed CD3 and Orexin-A staining on the hypothalamus of Orex-HA and control mice 8 d after transfer. We detected CD3$^+$ T cells in the hypothalamus of Orex-HA (Fig. 1 *A* and *C*), but not control (Fig. 1 *B* and *D*), mice, indicating that the presence of the neo-self-antigen was required for the infiltration and/or retention of T cells in the parenchyma. This T-cell infiltration spared the rest of the brain (Fig. S3 *A*–*D*) and was accompanied by marked microglial activation (Fig. 1 *E* and *F*), with up-regulation of major histocompatibility complex (MHC) class II molecules (Fig. 1*J*). No significant recruitment of dendritic cells or macrophages was noted (Fig. 1*I* and Fig. S4). To evaluate the kinetics of T-cell infiltration, we quantified the number of CD3$^+$ T cells in the hypothalamus of Orex-HA mice at different time points after Th1 injection and noted that T-cell infiltration peaked at day 8 after transfer and declined progressively thereafter (Fig. 1*G*). Based on the CD45.1 congenic marker exclusively expressed by transferred T cells, we demonstrated that antigen-specific Th1 cells were among the CNS-infiltrating T cells in Orex-HA mice (Fig. 1*H*). Interestingly, localized inflammation due to both T cells (Fig. S5*A*) and activated microglia (Fig. S5*C*) persisted for >60 d in close vicinity to the hypothalamic orexin$^+$ neurons. Despite this chronic inflammation, there was surprisingly no reduction in the number of orexin$^+$ neurons in Orex-HA (Fig. 1 *K* and *M*) compared with WT mice (Fig. 1 *L* and *M*). The number of hypothalamic melanin-concentrating hormone$^+$ (MCH) neurons, which are intermingled with but different from orexin$^+$ neurons, also remained unchanged (Fig. 1 *K*, *L*, and *N*).

Collectively, these data indicate that autoreactive Th1 cells can infiltrate the hypothalamus, but are unable to cause the destruction of orexin$^+$ neurons.

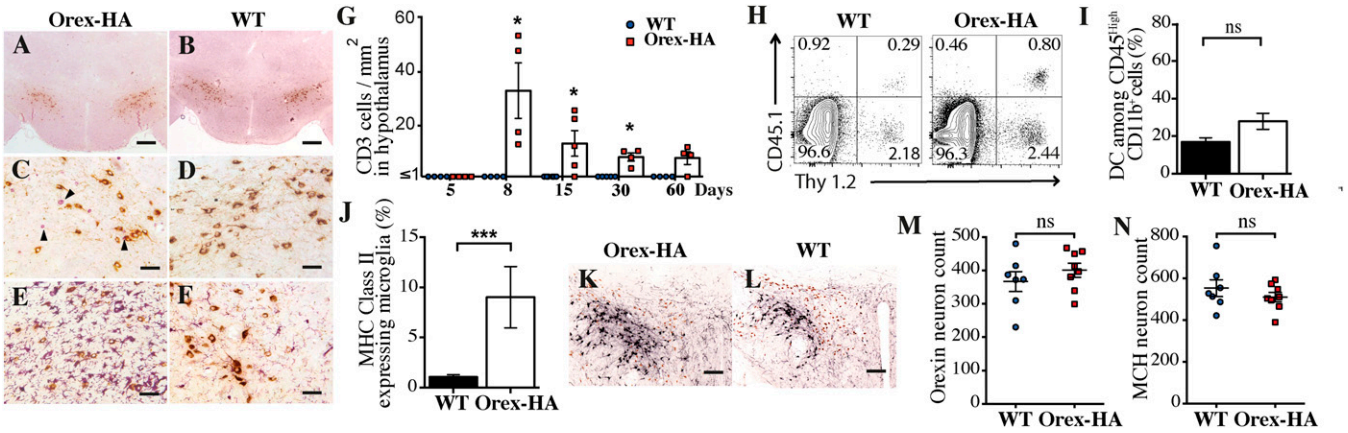

Fig. 1. Neo-self-antigen–specific Th1 cells trigger focal hypothalamic inflammation but no loss of orexinergic neurons. (*A*–*D*) Immunohistochemistry staining for CD3 (violet) and orexin-A (brown) in Orex-HA mice (*A* and *C*) and WT littermate control mice (*B* and *D*) 8 d after transfer of 3 × 10^7 neo-self-antigen–specific Th1 cells. (*E* and *F*) Immunohistochemistry staining for Iba-1 (violet) and orexin$^+$ neurons (brown) in Orex-HA (*E*) and WT (*F*) mice. Representative results from four or five mice per group are shown. Arrowheads in *C* point to infiltrating CD3$^+$ cells. [Scale bars: 200 μm (*A* and *B*) or 50 μm (*C*–*F*).] (*G*) Quantification of CD3$^+$ T cells in the hypothalamus of WT or Orex-HA mice at different time points after Th1 injection. Each symbol represents an individual mouse. Results are expressed as mean ± SEM of four or five mice per group for each time point. (*H*) Representative FACS plots of brain-infiltrating cells from WT or Orex-HA animals at day 8 after Th1 transfer. Representative results from three independent experiments are shown. (*I* and *J*) Frequency of CD11c$^+$ among CD45^{high} CD11b$^+$ cells was assessed by flow cytometry (*I*) and MHC class II expression on CD45^{dim} CD11b$^+$ Thy1.2$^-$ microglia (*J*). Results are expressed as mean ± SEM of 12 mice per group from three independent experiments. (*K* and *L*) At 60 d after Th1 cell transfer, double immunohistochemistry, staining for orexin$^+$ neurons (black) and MCH$^+$ neurons (brown) in Orex-HA mice (*K*) and WT controls (*L*). Representative results from seven or eight mice per group are shown. [Scale bars: 125 μm (*K* and *L*).] (*M* and *N*) The density of orexin$^+$ neurons (*M*) and MCH$^+$ neurons (*N*) in Orex-HA and WT mice is shown at day 60 after Th1 injection. Results are expressed as mean ± SEM of seven or eight mice per group from two independent experiments. Statistical analysis was performed by using the Mann–Whitney *u* test. ns, not significant. **P* < 0.05; ****P* < 0.001.

Several studies (9–11) described the presence of circulating autoantibodies directed against hypothalamic neurons in narcolepsy. We thus investigated if antibodies directed against HA could trigger neuronal death in the context hypothalamic inflammation induced by Th1 cells. Orex-HA mice received either anti-HA antibodies or control IgG between day 5 and 15 after the transfer of neo-self-antigen–specific Th1 cells. A mild loss of orexin$^+$ neurons (mean = 22.6%, range 8.3–49.2%) was observed 60 d after Th1 cell transfer in mice receiving anti-HA antibodies, in contrast to mice injected with control IgG (Fig. S6 *A*, *C*, and *D*). MCH$^+$ neurons were unaffected (Fig. S6*B*). This result suggests that synergy between cellular and humoral immunity could contribute to the pathogenesis of narcolepsy.

Cytotoxic CTLs Induce Selective Loss of Orexin$^+$ Neurons. Because CTLs have been implicated in neuronal destruction in other neurological disorders (26), we investigated whether autoreactive CTLs could be the immune effectors of orexin$^+$ neuron destruction in T1N. To this aim, in vitro-differentiated neo-self-antigen–specific CTLs were transferred into Orex-HA and WT littermates (Fig. S2 *A* and *B*). Histological analyses revealed a dense T-cell infiltration in the hypothalamus of Orex-HA mice (Fig. 2 *A* and *C*), but not in controls (Fig. 2 *B* and *D*). This robust T-cell infiltration peaked early, at day 5–8 after transfer (Fig. 2*G*), and scarcely involved CNS structures other than the hypothalamus (Fig. S3 *E–H*). The vast majority of brain-infiltrating T cells were the transferred antigen-specific CTLs marked by the CD45.1 congenic surface molecule (Fig. 2*H*). Marked activation of microglia (Fig. 2 *E*, *F*, and *J*) and recruitment of dendritic cells (Fig. 2*I*) were also salient features of this model. However, no significant increase in the number of CD45^{high} CD11b$^+$ macrophages was noted (Fig. S4).

T-cell infiltration recessed from day 8 onward (Fig. 2*G*), although local inflammation lasted for at least 60 d in the vicinity of orexin$^+$ neuronal cell bodies (Fig. S5 *E–H*). At this late time point, a profound loss in orexin$^+$ neurons (mean = 68.8%; range 54.5–86.3%) was revealed in Orex-HA animals (Fig. 2 *K* and *M*), compared with WT mice (Fig. 2 *L* and *M*). A concomitant decrease of orexin and HA transcripts was found in the hypothalamus of these Orex-HA mice, arguing further in favor of the orexin$^+$ neuron death, rather than a defect in orexin expression (Fig. 2*O*). Importantly, MCH$^+$ neurons were not destroyed, underlining the antigen-specific and selective nature of the neuronal destruction (Fig. 2 *K*, *L*, and *N*).

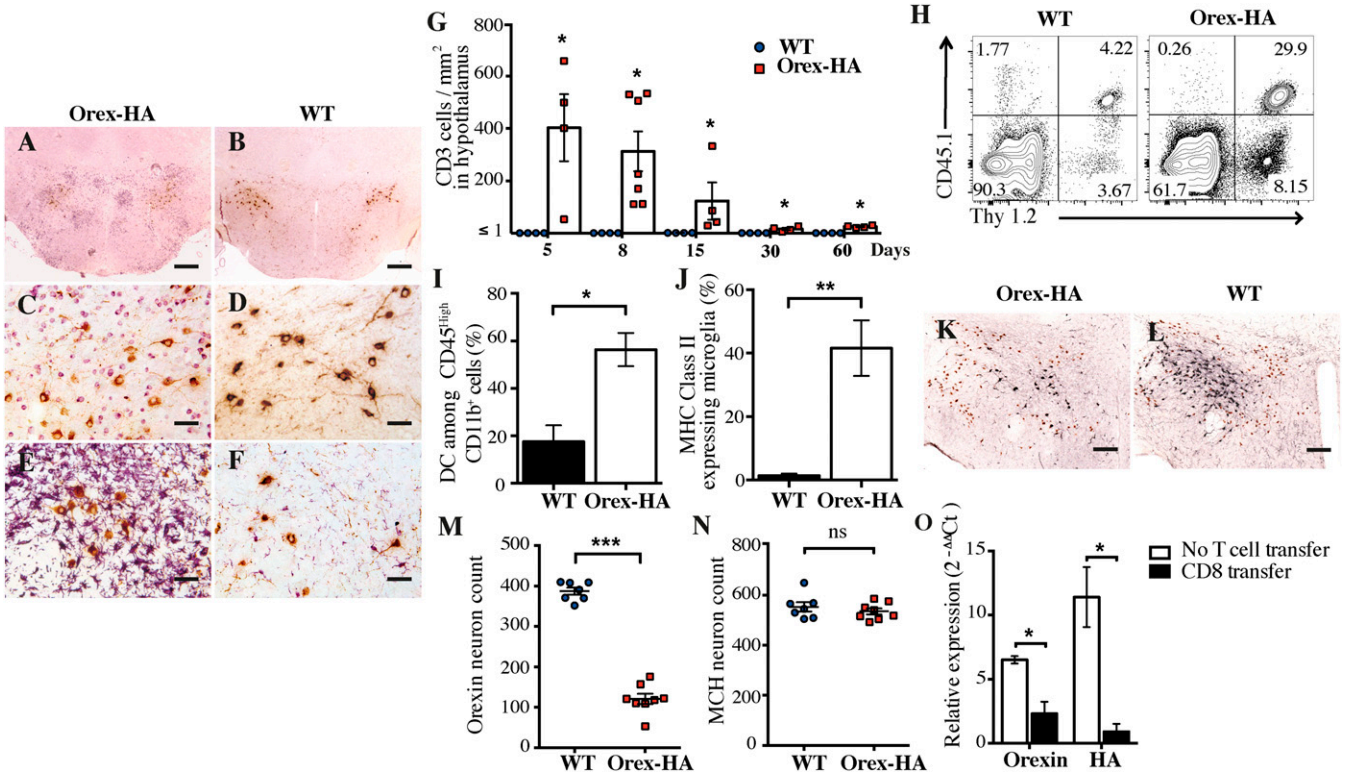

Fig. 2. Orex-HA animals develop massive hypothalamic inflammation and marked orexin neuron loss after transfer of neo-self-antigen–specific CTLs. (*A–D*) Immunohistochemistry staining for CD3 (violet) and orexin-A (brown) in Orex-HA mice (*A* and *C*) and WT mice (*B* and *D*) 8 d after adoptive transfer of 3×10^7 CTLs. (*E* and *F*) Immunohistochemistry staining of microglial (Iba1; violet) and orexin$^+$ neurons (brown) in Orex-HA (*E*) and WT (*F*) mice. Representative results from four to seven mice per group are shown. [Scale bars: 200 μm (*A* and *B*) or 50 μm (*C–F*).] (*G*) Quantification of T cells in the hypothalamus of Orex-HA and WT mice at different time points after CTL injection. Results are expressed as mean ± SEM of four to seven mice per group for each time point. (*H*) Representative FACS plots of brain-infiltrating cells from Orex-HA and WT littermates on day 8 after CTL transfer. (*I* and *J*) The proportion of CD11c$^+$ among CD45^{high} CD11b$^+$ cells (*I*) and the expression of MHC class II molecules on CD45^{dim} CD11b$^+$ Thy1.2$^-$ cells microglia (*J*) were assessed by flow cytometry. Results are expressed as mean ± SEM of six to eight mice per group from three independent experiments. Statistical analyses were performed by using the Mann–Whitney *u* test. $^*P < 0.05$; $^{**}P < 0.01$, comparing the Orex-HA group with the respective WT controls. (*K* and *L*) At 60 d after CTL transfer, immunohistochemistry staining for orexin$^+$ neurons (black) and MCH$^+$ neurons (brown) in Orex-HA animals (*K*) and WT mice (*L*). [Scale bars: 125 μm (*K* and *L*).] (*M* and *N*) Quantification of orexin$^+$ (*M*) and MCH$^+$ (*N*) neuronal cell bodies in the hypothalamus of Orex-HA mice compared with WT animals 60 d after CTL transfer. Results are expressed as mean ± SEM of seven or eight mice per group from two independent experiments. (*O*) Expression of orexin and HA mRNA in the hypothalamus of Orex-HA mice that received neo-self-antigen–specific CTLs or have been left untreated. Results are expressed as mean ± SEM of four or five mice per group from two independent experiments. Statistical analyses were performed by using the Mann–Whitney *u* test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ns, not significant.

Mechanism of CTL-Mediated Loss of Orexin$^+$ Neurons. In favor of a direct killing of neurons by the neo-self-antigen–specific CTLs, CTLs were localized at early time points after transfer in close contact to orexin$^+$ neurons (Fig. 3*A*), which expressed MHC class I molecules (Fig. 3*B*). We also revealed that this interaction led to the polarization of the CTL cytolytic granules toward orexin$^+$ neurons (Fig. 3*C*). To further investigate the T-cell/neuron interactions in situ, we used two-photon laser-scanning microscopy on explanted brain tissue of Orex-HA mice, also expressing a green fluorescent protein in orexin$^+$ neurons, after transfer of fluorescently labeled antigen-specific CTLs. Dynamic as well as static physical interactions between neo-self-antigen–specific CTLs and orexin$^+$ neurons were revealed (Movie S1). When CTL motility was assessed in the presence of a monoclonal antibody blocking the MHC class I-presenting molecule K^d, we noted enhanced mean track velocities (Fig. 3*D*) and decreased arrest coefficient (Fig. 3*E*), revealing a clear inhibition of CTL/orexin$^+$ neuron interactions. These data indicate that the direct interaction between CTLs and orexin$^+$ neurons are antigen-specific and MHC class I-dependent.

We next tested whether Th1 cells could potentiate the neuronal destruction mediated by CTLs. We thus transferred neo-self-antigen–specific Th1 cells in addition to neo-self-antigen–specific CTLs in Orex-HA mice, either concomitantly or 15 d apart. In both conditions, however, the level of orexin$^+$ neuron loss was of similar magnitude (mean = 63.6%; range 41.4–85.9%) than when CTLs were transferred alone (Fig. S7 *A* and *B*), suggesting that effector Th1 cells do not play a significant role in the demise of orexin$^+$ neurons. To investigate whether repeated waves of effector immune cells could potentiate destruction of orexin$^+$ neurons, Orex-HA mice received CTLs twice, 15 d apart. This experimental approach led to a profound loss of orexin$^+$ neurons (mean = 73.8%, range 23.7–92.5%) in Orex-HA animals (Fig. 4*A*). Here again, no significant loss of MCH$^+$ neurons was observed (Fig. 4*B*). These data strongly argue for the key contribution of effector CTLs, but not of Th1 cells, in the final pathogenic steps of this immune-mediated narcolepsy model.

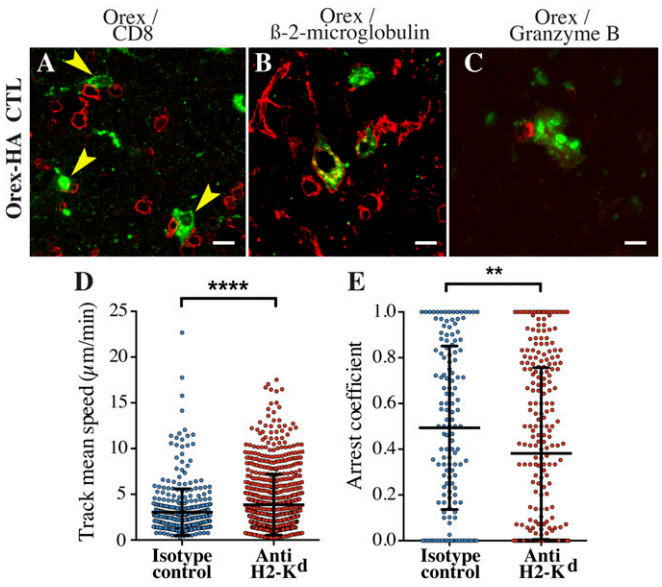

Fig. 3. CTL-mediated cytotoxicity contributes to orexin$^+$ neuron loss in Orex-HA mice. (*A* and *B*) Representative confocal micrographs of CTLs (anti-CD8; red) and orexin$^+$ neurons (anti–orexin-A; green) (*A*) or MHC class I expression (anti–β2-microglobulin; red) and orexin$^+$ neuron (anti-orexin A; green) in Orex-HA mice (*B*) 8 days after CTL transfer. (*C*) Immunofluorescence analysis of granzyme B-containing granules (red) and orexin$^+$ neurons (green) in Orex-HA mice. [Scale bars: 10 μm (*A*–*C*).] Analysis of the behavior of CellTrace violet-labeled neo-self-antigen–specific CTLs interacting with orexinergic neurons from ZsGreen Orex-HA mice by two-photon laser scanning microscopy in ex vivo slices of hypothalamus. (*D*) The mean speed of labeled T cells was analyzed in the presence of anti-H2-K^d mAb (red circles; 776 cells) or isotype control IgG (blue circles; 403 cells). (*E*) The proportion of time spent arrested (<2 μm/min) in contact with ZsGreen$^+$ orexinergic neurons was determined for the CTLs that have once been in contact with ZsGreen$^+$ orexinergic neuron cell bodies. This subgroup analysis included 263 cells in the presence of anti–H2-K^d mAb and 139 cells in the presence of isotype control IgG. Circles represent individual CTLs in five mice per group from two independent experiments. Results are expressed as mean ± SD, and statistical analyses were performed by using the unpaired Student *t* test. $^{**}P < 0.01$; $^{****}P < 0.0001$.

Orex-HA Mice Exhibit a Narcoleptic-Like Phenotype After Cytotoxic CTL Transfer. Given the similarity between the histopathological features observed in our mouse model and those of patients with T1N (2, 3), we next determined whether Orex-HA mice that had received neo-self-antigen–specific T cells develop a narcolepsy–cataplexy phenotype. At 60 d after T-cell transfer, we monitored electroencephalography (EEG)/electromyography (EMG) activities concomitantly with video recording over a 24-h period in Orex-HA and control mice. Orex-HA mice that received CTLs alone or in combination with Th1 cells exhibited pathologic behavioral arrests. These behavioral arrests were composed of cataplexy (Fig. 4 *C* and *E*) and sleep attack (Fig. 4*D*), as defined (27). These narcoleptic-like events were present in 86% of animals (three of four mice injected with CTLs once; five of six mice injected with CTL+Th1; and all four mice injected twice with CTLs) (Fig. 4 *C* and *D* and Fig. S7*C*). Interestingly, the severe loss of orexin$^+$ neurons, achieved with two injections of CTLs, was accompanied by a marked increase in the number of cataplectic events (Fig. 4*C*).

Discussion

We have established a mouse model to investigate the immunopathogenesis of T1N. In this model, antigen-specific CTLs triggered the specific destruction of orexin$^+$ neurons, whereas antigen-specific Th1 cells did not. In addition, the loss of orexin$^+$ neurons after CTL transfer in Orex-HA mice was associated with both sleep attacks and cataplectic episodes.

Surprisingly, although effector Th1 cells migrated to the hypothalamus in Orex-HA mice, this did not promote any loss of orexin$^+$ neurons. This result may be due to the lack of MHC class II expression on neurons, leading to the absence of cognate interactions between specific CD4 T cells and the neurons expressing HA as a neo-self-antigen. However, myelin-specific Th1 cells induce experimental autoimmune encephalomyelitis (28). Thus, the pathogenic contribution of Th1 in CNS autoimmune diseases may depend on the considered model. Indeed, the pathogenic impact of myelin-specific Th1 was shown to depend strongly on the recruitment of macrophages (29), a feature absent in our model. Interestingly, a tendency for higher levels of Th1-related cytokines/chemokines was detected in the CSF or serum of narcoleptic patients, in particular soon after disease onset (24, 25). The data have been interpreted as pointing to the importance of Th1 cells in narcolepsy, although other immune cell types could secrete these mediators. It may also be important to assess the involvement of other Th subtypes, such as Th17 cells that play important roles in other neuroinflammatory diseases, such as multiple sclerosis and neuromyelitis optica (30, 31), although no evidence currently points to their implication in narcolepsy.

Our hypothesis that CTLs play a key role in the destruction of orexin$^+$ neurons (32) is supported by data showing that infiltration of the hypothalamus by CTLs is prominent in rare cases of "symptomatic" T1N, such as anti-Ma2 antibody-associated encephalitis (33). In addition, HLA class I alleles have recently been associated with narcolepsy (5, 6). Consistent with this hypothesis,

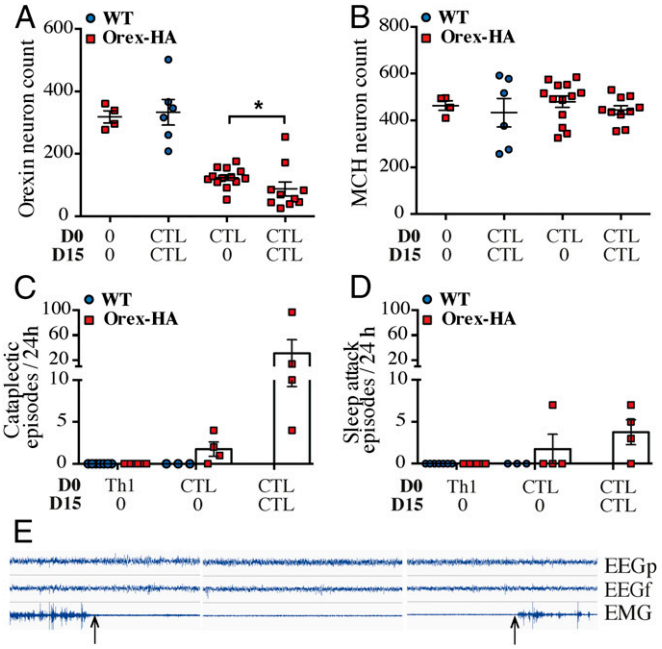

Fig. 4. Repeated injections of CTLs aggravate the loss of orexin$^+$ neurons in Orex-HA animals and lead to a narcolepsy-like phenotype. (*A* and *B*) Quantification of orexin$^+$ neurons (*A*) and MCH$^+$ neurons (*B*) in Orex-HA mice (red squares) that received no injection, CTLs once, or CTLs twice (15 d apart) 60 days after adoptive transfer. The results of WT mice (blue circles) that received CTLs twice (15 d apart) are also shown. Results for Orex-HA mice receiving CTLs once are already partially depicted in Fig. 2. Results are expressed as mean ± SEM of 4–13 mice per group. Statistical analyses were performed by using the Mann–Whitney *u* test. *$P < 0.05$. (*C* and *D*) Enumeration of cataplexy episodes (*C*) and sleep attacks (*D*) during 24 h in Orex-HA animals (red squares) and WT mice (blue circles) that were injected with Th1 cells or with CTLs once or twice (15 d apart). The behavioral arrests were subdivided into sleep attacks or cataplexy according to their EEG/EMG features. Results are expressed as mean ± SEM of four mice per group. (*E*) Example of cataplexy with typical EEG/EMG characteristics. Arrows demarcate the onset and termination of the cataplexy episode.

we showed that neo-self-antigen–specific CTLs could readily infiltrate into the hypothalamus and induce a specific loss of orexin$^+$ neurons. This neuronal loss likely involves a cognate interaction between granzyme B$^+$ CTLs and orexin$^+$ neurons (34), although we cannot rule out a possible contribution of additional cytotoxic pathways such as Fas/FasL or TNF/TNFR1. In any event, the neuronal destruction is clearly antigen-dependent, because the intermingled MCH$^+$ neurons were spared. Our demonstration of the expression of MHC class I molecules by neurons, likely driven by IFN-γ and TNF-α released from the CTLs (and possibly activated microglia), as well as the inhibitory effect of K^d-blocking antibodies, strongly argues for a direct presentation of neo-self-antigen peptide by the orexin$^+$ neurons to the antigen-specific CTLs.

CD4 T cells contribute at several steps to the development of an optimal antigen-specific CTL response. First, naive CTLs are attracted to sites of productive interaction between CD4$^+$ T cells and antigen-presenting dendritic cells through the local release of CCL3 and CCL4 chemokines (35). In addition, after interaction with activated CD4 T cells, antigen-presenting dendritic cells upregulate CD80/CD86 costimulatory molecules and produce cytokines, thereby enhancing CTL activation and differentiation (36–38). Finally, antigen-specific CD4 T cells are at the center of an elaborate immune cell interaction, resulting in optimal development of effector CD8$^+$ T-cell responses and the acquisition of robust immune memory (39). As such, CD4 T cells could make a key contribution in T1N pathogenesis, by initiating and maintaining pathogenic effector CTL (and possibly also B-cell) responses, without behaving as final effectors.

The drastic loss of orexin$^+$ neurons led to clear narcoleptic episodes (i.e., cataplexy and sleep attack) in most Orex-HA mice that received neo-self-antigen–specific CTLs. The phenotype was particularly striking in mice transferred twice with CTLs, in which ~90% of orexin$^+$ neurons were lost. This finding is reminiscent of the clinical observations made in T1N patients, which may result from a chronic multistep process. Indeed, most patients first develop excessive daytime sleepiness, whereas cataplectic episodes appear months or years later (40).

Our data indicate that immune effector mechanisms other than CTLs, in particular autoantibodies, could also contribute to T1N pathogenesis, especially in the context of T-cell–triggered hypothalamic inflammation. Antibodies directed against several intracellular or membrane-bound neuronal autoantigens have been detected in the serum of narcolepsy patients (9–11). In particular, a recent study reported that 85% of Finnish T1N patients carrying the *HLA-DQB1*06:02* allele and vaccinated with Pandemrix presented antibodies against the orexin receptor type 2. Importantly, these autoantibodies also cross-reacted with the H1N1 influenza nucleoprotein (9). However, these autoantibodies were also highly prevalent (55%) in a cohort of Finnish children with neither T1N nor pandemic H1N1 vaccination (8). Another recent study has shown the presence of autoantibodies in the sera of some narcoleptic patients reacting to hypothalamic antigens on rat brain slices (10). Although these autoantibodies did not target orexin$^+$ neurons, intracerebral injection of autoantibody-containing IgG induced alterations in the sleep architecture, but not a narcolepsy-like phenotype. Antibodies directed against Trib2, found in autoimmune uveitis, have also been described in narcolepsy. Our results underline the potential pathogenic relevance of circulating autoantibodies in an inflammatory context (41). Further studies are needed, however, to narrow down the contribution of B cells and autoantibodies in the pathogenesis of T1N, especially in the post-H1N1 vaccine context. In addition, it is currently unknown whether autoantibodies precede or are the consequence of brain tissue destruction in narcolepsy (42).

To conclude, our study highlights the central role of CTLs as final effectors in immune-mediated narcolepsy. This observation does not negate the contribution of other immune cell types in the disease process. Indeed, activated CD4 T cells could have a major impact on the initiation and development of the pathogenic immune response, involving CTLs and/or autoantibodies. In that respect, the development of hypothalamic autoimmunity in response to influenza virus vaccination remains to be investigated. By providing direct evidence that immune targeting of orexin$^+$ neurons can model clinical and neuropathological aspects of T1N, our study uncovers important clues. Further studies should address whether T1N patients, related or not with Pandemrix vaccine, also exhibit altered cellular immune responses.

Materials and Methods

Mouse Strains. The (BALB/cJ × C57BL/6J) F1-Orex-HA mice were generated by crossing the BALB/cJ-Rosa26$^{tm(HA)1Lib}$ mice (20) with the C57BL/6J Orex-Cre mice, a gift from T. Sakurai, Kanazawa University, Kanazawa, Japan (21). In the resulting double-transgenic Orex-HA mice, the expression of HA of an H1N1 influenza virus, which is otherwise prevented by an upstream floxed Stop cassette, was induced because of the expression of the Cre recombinase specifically in orexin$^+$ neurons. The (BALB/cJ × C57BL/6J) F1-CL4-TCR and F1-6.5-TCR mice have been described (43, 44). To perform two-photon laser microscopy experiments, we generated Orex-HA-ZsGreen triple-transgenic mice by the cross of C57BL/6J Orex-Cre mice, C57BL/6J.Cg-Gt(ROSA)26$^{Sortm6(CAG-ZsGreen1)Hze}$ (The Jackson Laboratory) (45), and BALB/cJ-Rosa26$^{tm(HA)1Lib}$. These mice were kept in pathogen-free conditions within the UMS006 animal facility, Toulouse, France. All animal experiments were performed in accordance with the European Union guidelines following approval of the local ethics committee (13-U1043 RL/RV-16).

T-Cell Differentiation and T-Cell Transfer. HA-specific $CD8^+$ CTL and HA-specific CD4+ Th1 were generated from F1 CL4-TCR mice and F1 6.5-TCR mice, respectively, as described (46, 47). After culture, living cells were collected by Ficoll density separation. CTL and Th1 preparations routinely contained >95% of $CD8^+V\beta8^+$ or $CD4^+V\beta8^+$, respectively, and >70% were IFN-γ/TNF-α producers when assessed by flow cytometry. Then, 3×10^7 CTLs and/or Th1 were adoptively transferred i.v. to Orex-HA mice or WT littermate controls.

Histopathology. Tissues were fixed in 4% (wt/vol) paraformaldehyde, conserved in 70% (vol/vol) ethanol, and embedded in paraffin. Immunohistochemical or immunofluorescence staining of 5-μm-thick coronal sections was performed by using the following antibodies: rabbit anti-orexin A (Phoenix Pharmaceuticals), rabbit anti-MCH (Phoenix Pharmaceuticals), rabbit anti-CD3 (SP7; Zytomed), rat anti-CD8a (4SM15; eBioscience), rabbit anti–Iba-1 (Wako), anti-granzyme B (ab4059, AbCam), anti–β2-microglobulin, and anti-HA hybridoma (37, 38) supernatant. Image acquisition was performed with confocal microscope Leica SP8 (Leica Microsystems) or 3DHistec Panoramic 250 slide scanner (3DHistec Ltd). For neuronal enumeration, brains were fixed in 4% paraformaldehyde and 30-μm-thick coronal free-floating sections performed at cryostat. Immunohistochemical detection of $orexin^+$ and MCH^+ neurons was performed by using the antibodies described above. Neuron enumeration was performed on 1/12th of the entire hypothalamus (seven sections per brain) by using Mercator software (Explora Nova).

Statistical Analyses. All statistical analyses were made by using GraphPad Prism software (Version 6.0). Results were compared by using one-way ANOVA, Mann–Whitney *u* test, or unpaired Student *t* test. Statistical significance was expressed as $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and $****P < 0.0001$.

SI Material and Methods. Details regarding RT-PCR, purification of CNS-infiltrating mononuclear cells, FACS analyses, injection of anti-HA antibodies, two-photon laser scanning microscopy, and polysomnographic analyses are displayed in *Supporting Information*.

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