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Epigenetic silencing of selected hypothalamic neuropeptides in narcolepsy with cataplexy

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Narcolepsy with cataplexy is a sleep disorder caused by deficiency in the hypothalamic neuropeptide hypocretin/orexin (HCRT), unanimously believed to result from autoimmune destruction of hypocretin-producing neurons. HCRT deficiency can also occur in secondary forms of narcolepsy and be only temporary, suggesting it can occur without irreversible neuronal loss. The recent discovery that narcolepsy patients also show loss of hypothalamic (corticotropin-releasing hormone) CRH-producing neurons suggests that other mechanisms than cell-specific autoimmune attack, are involved. Here, we identify the HCRT cell-colocalized neuropeptide QRFP as the best marker of HCRT neurons. We show that if HCRT neurons are ablated in mice, in addition to *Hcrt, Qrfp* transcript is also lost in the lateral hypothalamus, while in mice where only the *Hcrt* gene is inactivated *Qrfp* is unchanged. Similarly, postmortem hypothalamic tissues of narcolepsy patients show preserved *QRFP* expression, suggesting the neurons are present but fail to actively produce HCRT. We show that the promoter of the *HCRT* gene of patients exhibits hypermethylation at a methylation-sensitive and evolutionary-conserved PAX5:ETS1 transcription factor-binding site, suggesting the gene is subject to transcriptional silencing. We show also that in addition to HCRT, *CRH* and Dynorphin (*PDYN*) gene promoters, exhibit hypermethylation in the hypothalamus of patients. Altogether, we propose that *HCRT*, *PDYN*, and *CRH* are epigenetically silenced by a hypothalamic assault (inflammation) in narcolepsy patients, without concurrent cell death. Since methylation is reversible, our findings open the prospect of reversing or curing narcolepsy.

QRFP | HCRT | CRH | gene silencing | methylation

Significance

Narcolepsy with cataplexy is characterized by excessive daytime sleepiness and sudden loss of muscle tone (cataplexy) triggered by strong emotions. The best marker of the disease is low/absent hypocretin (HCRT), a hypothalamic neuropeptide. An autoimmune attack targeting these neurons is believed to cause HCRT deficiency, despite any direct evidence. We demonstrate that a substantial number of HCRT neurons are present in the brain of patients, but *HCRT* gene is silenced by methylation. The epigenetic silencing is not restricted to *HCRT* but also *CRH* and *PDYN* genes are methylated in the hypothalamus of patients. Our results strongly suggest that HCRT and CRH neurons are not destroyed but epigenetically deactivated, and possibilities exist to reactivate them to treat or even cure narcolepsy.

Narcolepsy with cataplexy (also called narcolepsy type 1 or NT1, hereafter referred to as narcolepsy) is a sleep disorder characterized by excessive daytime sleepiness and cataplexy (loss of skeletal muscle tone triggered by strong emotions) (1). Shortly after the discovery of hypocretin (HCRT) neuropeptides, the postmortem examination of hypothalamic tissue from narcolepsy patients revealed a near absence of hypocretin (*HCRT*) gene transcript, prompting the hypothesis of destruction of HCRT-producing neurons in narcolepsy (2, 3). HCRT-1 deficiency (cerebrospinal fluid [CSF] HCRT-1 levels <110 pg/mL) is presently the best biological marker of the disease (4). It is currently widely, if not unanimously, believed that HCRT neuronal loss results from an immune or autoimmune process, based on the strong association of narcolepsy with the *HLA-DQB1*06:02* allele (5), as well as the finding of a T cell receptor alpha gene polymorphism (6), and the acute increase in childhood narcolepsy that followed the 2009 to 2010 H1N1 vaccination (7). Although autoreactive T cells against various antigens expressed by HCRT neurons were reported (8, 9), direct evidence of HCRT cell death, lateral hypothalamic inflammation, or T cell infiltration, is lacking.

Based on several observations, we hypothesized that HCRT deficiency could result from the absence of *HCRT* expression, rather than from neuronal death. Evidence from patients with secondary narcolepsy (10–13) has indicated that CSF HCRT deficiency can be transient and normalize following treatment, suggesting that hypothalamic inflammatory reactions can cause reversible HCRT deficiency. Up to 48% of patients with traumatic brain injury exhibit HCRT deficiency, which recovers within 6 mo (14). Intriguingly, a 28-y-old female patient diagnosed with narcolepsy with cataplexy and undetectable CSF HCRT-1, who was placed under intravenous immunoglobulins starting as soon as 15 d after her first cataplexy attack, saw her CSF HCRT-1 level normalized, and her symptoms improved (15). Furthermore, lower CSF HCRT-1 levels, and even HCRT deficiency, were reported during hypersomnia episodes in patients with Kleine–Levin syndrome, suggesting expression levels can change without permanent cell loss (16). Aligned with these findings,

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evidence suggests that *HCRT* gene expression is finely tuned in response to both local and external environmental changes in humans and animal models.

Very recently, the mapping of hypothalamic neuropeptides in postmortem samples of narcolepsy patients led to the observation of a massive loss (88%) of corticotropin-releasing hormone (CRH) neurons in the paraventricular hypothalamic nucleus, while vasopressin, oxytocin, tyrosine hydroxylase and thyrotropin-releasing hormone neurons were unchanged (17). The cause of CRH deficiency in narcolepsy is unknown but it seems unlikely that an autoimmune attack would target both HCRT and CRH neurons, two highly specialized and unrelated cell types, while leaving other hypothalamic cells intact.

If HCRT deficiency is not caused by cellular destruction, then either loss-of-function mutations or epigenetic gene silencing are involved. Extensive genetic analyses of narcolepsy have failed to uncover causative gene mutations, with the exception of a few cases (2, 18, 19), and the disease is thought to be primarily sporadic. Epigenome-wide association studies have revealed sites of differential DNA methylation in blood and hypothalamic samples of narcolepsy patients (20, 21), but no site-specific hypermethylation associated with HCRT deficiency was found.

Results

Preserved Hypothalamic Expression of the HCRT Neuron-Coexpressed Neuropeptide QRFP in Narcolepsy. Several mouse models phenocopy narcolepsy symptoms with astonishing precision, including the *Hcrt* gene KO mouse, whose hypothalamus is known to contain cells with "HCRT-neuronal" identity (notably because they express *HCRT* promoter-driven transgenes), but they do not express *Hcrt* and several HCRT-neuron-ablated transgenic models, whose hypothalamus has lost HCRT-neurons due to expression of a neurotoxin. We previously performed RNA sequencing to identify differentially expressed genes in HCRT-neuron-ablated (Hcrt-ataxin3 transgenic) mice and in Hcrt-KO mice and their respective controls throughout several brain regions. This analysis revealed a gene, called *Qrfp* (26RFa: RFamide family 26 amino acid peptide), which displayed a striking hypothalamus-specific higher than a twofold decrease in expression in Hcrt-ataxin-3 Tg mice, whereas levels were unchanged in Hcrt-KO mice (22), and tended even to be up-regulated (Fig. 1*B*). To confirm loss of *Qrfp* expression in another HCRT-neuron-ablated model, we quantified *Qrfp* transcript in the hypothalamus of Hcrt-DTA mice, in which a bigenic system drives conditional expression of diphtheria toxin A in HCRT cells (23). The *Qrfp* transcript was found to be nearly abolished (up to 10-fold downregulation) in these mice (Fig. 1*B*), while no change was observed for *Pmch*, the MCH peptide-encoding gene, which is expressed in cells that are distinct, but intermingled with the HCRT and QRFP-producing neurons in the lateral hypothalamus (Fig. 1*A*). Similar analysis of two other neuropeptides known to colocalize with *Hcrt*, namely *Pdyn* (dynorphin) and *Nptx2* (Narp) indicated no change for *Nptx2* but around 50% decrease for *Pdyn* in neuron-ablated models (Fig. 1*B*). These findings suggested that *Qrfp* is either strongly coexpressed with *Hcrt* (and more specifically than *Pdyn* and *Nptx2*) or is dependent on HCRT cells and support the utility of *Qrfp* downregulation as a proxy for HCRT neuronal loss.

To determine whether *QRFP* expression is lost in the hypothalamus of narcolepsy patients as it is in HCRT neuron-ablated mice, RNA was extracted from the hypothalamic region of four narcolepsy and four control postmortem fresh frozen brains (24). Strikingly, the *QRFP* expression was not only preserved in the hypothalamus of narcolepsy patient (Fig. 1*C*), but also showed a very similar tendency to increase in patients (45% higher than controls), as we had found in Hcrt-KO mice (Fig. 1*B*). This may suggest competition between the two genes for the recruitment of similar transcription factors. Note that *Qrfp* and *Hcrt* are structurally and functionally related genes, their cDNAs share 50% sequence similarity and their seven-transmembrane receptors can heterodimerize (25). To next investigate QRFP peptide, we measured QRFP expression level by radioimmunoassay (RIA) in the CSF of 10 narcolepsy patients and seven control subjects. CSF-QRFP showed a significant twofold increase in patients (Fig. 1*D*), suggesting that CSF QRFP measurement might be a biomarker for narcolepsy. Altogether, our results suggest that HCRT neurons may be present in narcolepsy brains, but *HCRT* gene might be inactive.

There are conflicting reports on whether *Qrfp* and *Hcrt* are coexpressed in the same neurons in mice. One study reported two distinct hypothalamic neuronal populations in adult mice (26), while another study using single-cell RNA-sequencing of hypothalamic neurons of juvenile mice indicated colocalization (27). Hierarchical clustering defined by molecular fingerprints suggested that *Hcrt*- and *Qrfp*-expressing neurons share a similar neuronal lineage (27). Since no commercially available QRFP antibody shows specificity in mice, we tested if *Hcrt* and *Qrfp* transcripts are colocalized in the mouse hypothalamus by triple fluorescent in situ hybridization using RNAscope® (28) with probe sets covering the full-length *Hcrt*, *Qrfp,* and *Pmch* transcripts (Fig. 1 *E* and *F*). Quantification of RNAscope data indicated that 94.3 ± 10% of *Hcrt*-expressing neurons coexpressed *Qrfp*, while 82.5 ± 20.4% of *Qrfp*-expressing neurons coexpressed *Hcrt* (N = 5), with no colocalization with *Pmch* (Fig. 1*G*). Also, recently, Takahashi et al. reported the generation of a knockin mouse line expressing iCre from the endogenous *Qrfp* gene and observed low levels of iCre expression in HCRT immunoreactive neurons, supporting *Hcrt*/*Qrfp* coexpression (29).

To test whether HCRT and QRFP peptides colocalize in humans, we performed double immunostaining of postmortem hypothalamic sections (Fig. 1*H*). Immunostaining of four lateral hypothalamic sections from four control subjects and four sections from 2 narcolepsy patients revealed, as expected, over 90% reduction in the number of HCRT-immunoreactive neurons (n = 553 in controls versus n = 50 in patients). The same analysis for QRFP-immunoreactive neurons indicated no difference between patients and controls (n = 328 in controls and n = 278 in patients). In controls, we found that among QRFP-positive neurons, 62.0% were also HCRT-positive, and 38.7% of HCRT-positive cells appeared QRFP-positive, indicating significant colocalization. Next, among the very few and low-intensity HCRT-positive neurons found in patients (N = 50), 82% (n = 41) were QRFP-positive. Affecting these neuronal counts, we made the additional observation that the intensity of HCRT and QRFP immunostaining appeared inversely related, i.e., neurons with low HCRT staining displayed high QRFP signal, and vice versa (Fig. 1*H*, arrowheads). This finding aligns with our observations above at the mRNA level, showing that *QRFP* transcript levels tend to increase in narcolepsy patients, and suggests the existence of an *HCRT*–*QRFP* gene interaction. Altogether these data confirm that QRFP mRNA and protein are preserved in narcolepsy and largely colocalize with HCRT, supporting survival of a substantial number of these cells.

***HCRT* Gene Promoter Shows Site-Specific Epigenetic Alteration in Narcolepsy.** If indeed human narcolepsy brains contain HCRT neurons but similarly to Hcrt-KO mice do not express *HCRT*, and because *HCRT* gene mutations are not found in human narcolepsy, lack of expression may be the result of epigenetic dysregulation. Epigenetic control of gene expression can operate

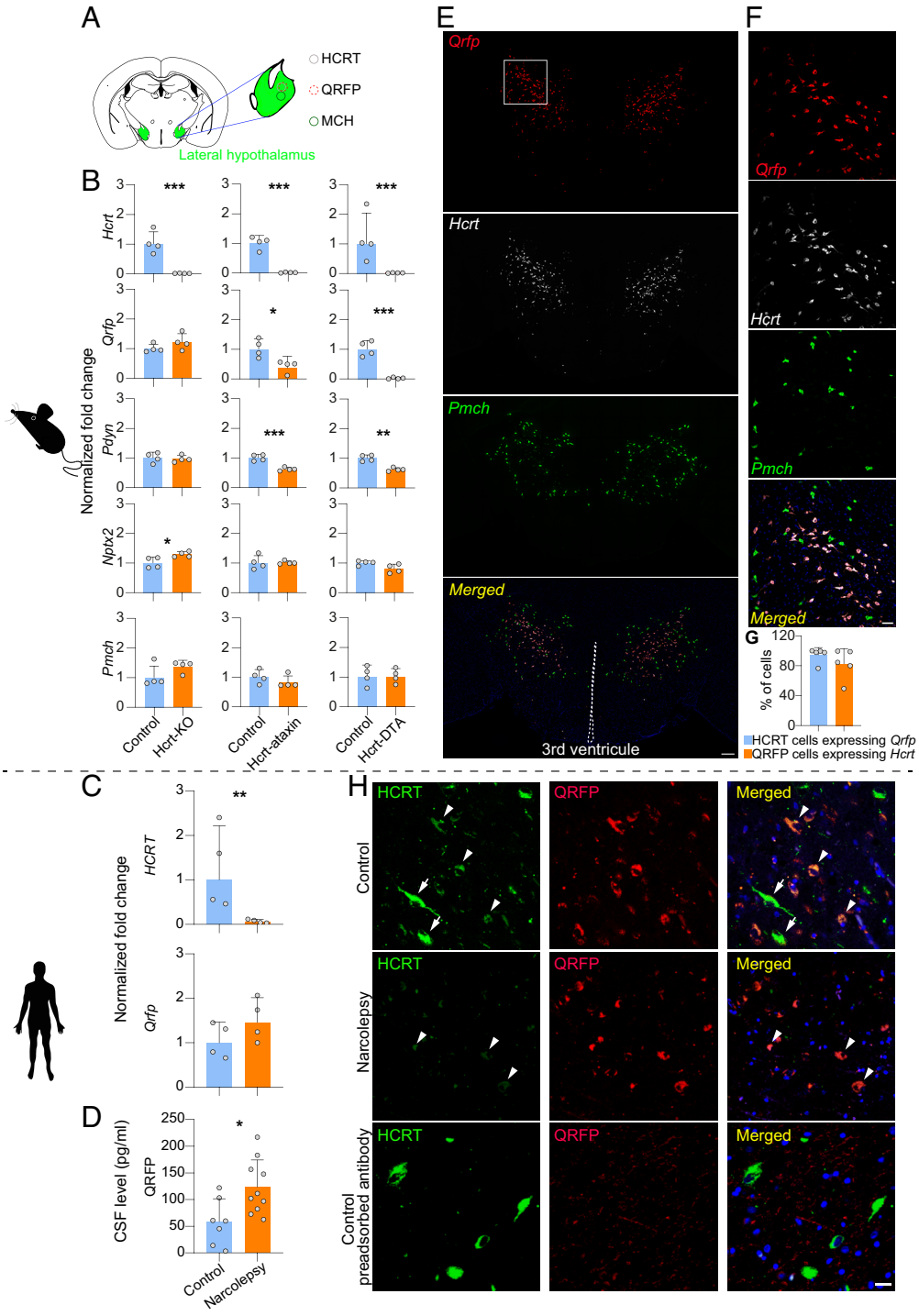

Fig. 1. Expression of the HCRT-neuron coexpressed QRFP neuropeptide is intact in the hypothalamus of narcolepsy patients. (*A*) Schematic presentation of the lateral hypothalamus with major neuropeptidergic cell groups. (*B*) Expression levels of *Hcrt*, *Qrfp*, *Pdyn*, *Nptx2*, and *Pmch* transcripts in the hypothalamus of Hcrt-KO mice and two HCRT neuron-ablated transgenic lines, Hcrt-ataxin and Hcrt-DTA mice ($n = 4$ in each group). As expected, *Hcrt* expression is dramatically reduced in all three narcolepsy mouse models as compared to their respective controls. *Qrfp* expression is unchanged (or tends to increase) in Hcrt-KO mice, but is profoundly decreased in both neuron-ablated models. *Pdyn* expression is also substantially decreased in cell-ablated mice, although less than *Qrfp*, while *Nptx2* does not seem to be affected. Also, *Pmch* is unchanged in all three mouse models. (*C*) The *HCRT* transcript is also dramatically decreased in the hypothalamus of narcolepsy patients, as expected, while *QRFP* expression tends to be increased, similar to Hcrt-KO mice. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, *t* tests on log transformed normalized fold change of mutated mice compared to their respective controls, $n = 4$. Bars represent geometric mean + geometric SD. (*D*) Quantification by RIA of the QRFP peptide in the CSF of narcolepsy ($n = 10$) and control subjects ($n = 7$) indicates increased QRFP levels in narcolepsy patients. $^*P < 0.05$, *t* test. Bars represent mean + SD. (*E*) RNAscope in situ hybridization analysis of coronal sections through the mouse hypothalamus depicts a dense population of *Qrfp*, *Hcrt*, and *Pmch* expressing neurons in the lateral hypothalamic area. The merge photomicrograph shows extensive coexpression of *Qrfp* and *Hcrt* mRNA, while *Pmch*-expressing neurons surround but do not overlap with *Qrfp*+*Hcrt* neurons. (*F*) Higher magnification images (corresponding to the region highlighted with the white rectangle on top left) show coexpression of *Qrfp* and *Hcrt* mRNAs (pink), and absence of coexpression with the *Pmch* transcript, which is expressed in independent neurons (green). (Scale bars: A = 200 µm; B = 50 µm.) (*G*) Cell number quantification of *Hcrt* neurons expressing *Qrfp* and *Qrfp* neurons expressing *Hcrt* throughout the entire lateral hypothalamus ($n = 5$). (*H*) Postmortem human hypothalamic sections subjected to immunofluorescence staining for HCRT, QRFP, and merged immunofluorescence images are shown for a control subject (*Upper*), a narcolepsy patient (*Middle*), or a control subject after preadsorption of the antiserum with QRFP peptide (*Lower*). Note that neurons with low HCRT immunoreactivity (arrowheads) appear QRFP-positive, while neurons with high HCRT immunoreactivity (arrows) do not. In the patient, again neurons with residual, low-intensity staining for HCRT show strong QRFP staining. The number of QRFP-positive neurons in the patient and control subject appears similar. Lower panels of a control subject with QRFP peptide-preadsorbed QRFP antibody confirm QRFP antibody specificity (scale bars: 20 µm).

via different mechanisms, including DNA methylation, histone modification, chromatin structural change, and microRNAs. DNA methylation and histone modification are major mechanisms for gene silencing, which mainly occur in regulatory regions of genes such as promoters and enhancers.

Transgenic analysis of the human *HCRT* promoter using *lacZ* reporter mice identified a 3.2-kb fragment upstream of the transcription start site that fully recapitulates expression in mice (30). Extensive deletion analysis of this fragment revealed that a 57-bp core region in the proximal promoter is critical for *HCRT* expression and represents the main regulatory region for the binding of transcription factors (31). To determine the methylation sensitivity of the human *HCRT* gene, we cloned a 1,800-bp proximal promoter in a CpG-free luciferase reporter vector (pCpGL) and performed luciferase assays. Two different cell lines (HEK 293FT and PC12) were transfected with intact unmethylated human *HCRT* promoter-luciferase pCpGL construct, or the same construct that had been preexposed to M.SssI methyltransferase in vitro. Methylation of the *HCRT* promoter decreased the expression of luciferase by 60 to 90% ($P < 0.05$) (Fig. 2*A*), confirming that methylation of the *HCRT* promoter inhibits its expression.

To determine whether promoter methylation is involved in HCRT deficiency of narcolepsy patients, we next evaluated *HCRT* gene promoter methylation in DNA samples extracted from postmortem lateral hypothalami of seven patients and seven control subjects. The proximal human *HCRT* promoter contains 13 CpGs (Fig. 2*B*). We applied bisulfite treatment, followed by either of two sequencing methods to quantify the CpG methylation status (*SI Appendix*, Table S1). The first method relies on direct Sanger sequencing of PCR amplicons and measures the average methylation of DNA molecules in a tissue. When sufficient high-quality DNA was available, we also performed targeted next-generation bisulfite sequencing. Twelve of the 13 CpG sites within the proximal *HCRT* promoter (all except the most 5′ CpG in Fig. 2*B*) were tested. A higher methylation level was observed in narcolepsy patients as compared to controls at eight of these twelve CpGs, and one of them was significantly hypermethylated both by Sanger and targeted sequencing (CpG −239, methylation 93.9% in patients vs. 69.93% in controls, $P < 0.01$, Fig. 2 *C*, *Left*). Interestingly, the 57-bp core regulatory region of the proximal human *HCRT* promoter contains only 2 CpGs: CpG −291 and the patient-hypermethylated CpG-239. This core region was shown to be one of the 2 subregions essential for correct *HCRT* expression and transcription factor binding in vivo (31). Therefore, the patient differentially methylated residue is positioned at a functionally critical region.

Previous postmortem analysis of narcolepsy patients' brains indicated that immunostaining for two other neuropeptides, PDYN (Dynorphin) and NPTX2 (Narp), which partially colocalize with HCRT, are also lost in the lateral hypothalamus (32, 33). In silico analysis of the promoter regions of these two genes revealed that both harbor a CpG island, suggesting that DNA methylation may affect their expression. The *NPTX2* promoter contains a very high-density CpG island hindering bisulfite sequencing. We therefore focused on the methylation status of the *PDYN* promoter by applying the same methods as for the *HCRT* promoter (*SI Appendix*, Table S1). All 11 CpGs tested showed higher methylation in the hypothalamus of patients than in controls, and 7 from these 11 were significantly hypermethylated (N = 5 in each group, $P < 0.05$; Fig. 2*D*). Therefore, loss of PDYN signal in narcolepsy might also be due to promoter methylation rather than cell loss.

To determine whether differential methylation in narcolepsy patients compared to controls is specific to HCRT neurons, we tested the methylation status of the *HCRT* receptor 2 gene (*HCRTR2*). *HCRTR2* is widely expressed in the hypothalamus including the lateral hypothalamus but we have shown that HCRT neurons do not express *HCRTR2* (34). The *HCRTR2* promoter contains a CpG island, in which we evaluated 10 CpGs for methylation level (*SI Appendix*, Table S1). Surprisingly, we found very low methylation (all CpGs had a methylation level below 35%), and no difference between narcolepsy patients and controls was observed ($n = 4$ narcolepsy patients and 6 controls, *SI Appendix*, Fig. S1*A*). This suggests that promoter hypermethylation within the lateral hypothalamus of narcolepsy patients has cell- and gene-specificity.

As DNA methylation patterns are tissue-specific, we tested methylation of the *HCRT* and *PDYN* promoters in DNA samples extracted from the peripheral blood cells of narcolepsy patients and controls. Eleven out of the same 12 CpGs of *HCRT* as tested in the brain samples were also tested by Sanger sequencing. Similar to the hypothalamus DNA, methylation at these 11 CpGs varied between 68 and 100% in the blood DNA (Fig. 2 *C*, *Right*). We however found that the CpG -239 that was hypermethylated in the hypothalamus of narcolepsy patients compared to controls was methylated at 100% in peripheral blood of all subjects, in patients ($n = 9$) or controls ($n = 10$) (Fig. 2 *C*, *Right*), consistent with the brain-specific expression of these genes. The fact that *HCRT* methylation level is not dramatically different in the brain of patients (although the pattern is), likely reflects that our hypothalamic samples contain heterogeneous populations of cells and the fraction of the relevant HCRT-producing neurons is small, leading to dilution of the signal. The same analysis was also performed for *PDYN* DNA from blood samples, which likewise indicated high methylation and no difference between narcolepsy patients and controls ($n = 9$ narcolepsy patients and 10 controls, *SI Appendix*, Fig. S1*B*). We next examined the CpG methylation levels of the *QRFP* gene promoter, which features 9 CpGs (*SI Appendix*, Table S1). We found no changes either in the hypothalamus or blood of narcolepsy patients as compared to controls ($n = 7$ narcolepsy patients and 6 controls for the hypothalamus, $n = 9$ narcolepsy and 10 controls for the blood, *SI Appendix*, Fig. S1 *C* and *D*), consistently with our observation above that QRFP transcript and protein are intact or increased in narcolepsy. Altogether these results suggest that the DNA-methylation alterations we find in narcolepsy are both cell type- and gene specific.

During the preparation of this manuscript, we made an unexpected discovery in an independent study aimed at mapping expression of hypothalamic neuropeptides in human narcolepsy. Analysis of CRH-expressing neurons revealed a severe loss of CRH signal in the paraventricular hypothalamus area of narcolepsy patients (17). Since *CRH* and *HCRT* are not coexpressed, we hypothesized that similar to *HCRT*, the *CRH* gene expression might be inhibited by promoter methylation, rather than CRH neuron-specific autoimmune attack. The *CRH* promoter is known to contain a CpG island whose expression is sensitive to methylation, specifically at a cAMP response element (CRE) bound by the CREB protein (35, 36). We thus performed bisulfite DNA sequencing of the proximal *CRH* promoter (*SI Appendix*, Table S1) using postmortem hypothalamic samples from 6 narcolepsy patients and 6 controls and found hypermethylation in narcolepsy patients at several CpGs including CpG −224, which is located in the CREB-binding site (Fig. 2*E*). Our finding of *CRH* promoter hypermethylation in narcolepsy is consistent with our hypothesis that expression loss is due to *CRH* gene silencing. The alternate hypothesis that an autoimmune attack electively targets HCRT and CRH neurons, two very different cell types, seems less likely.

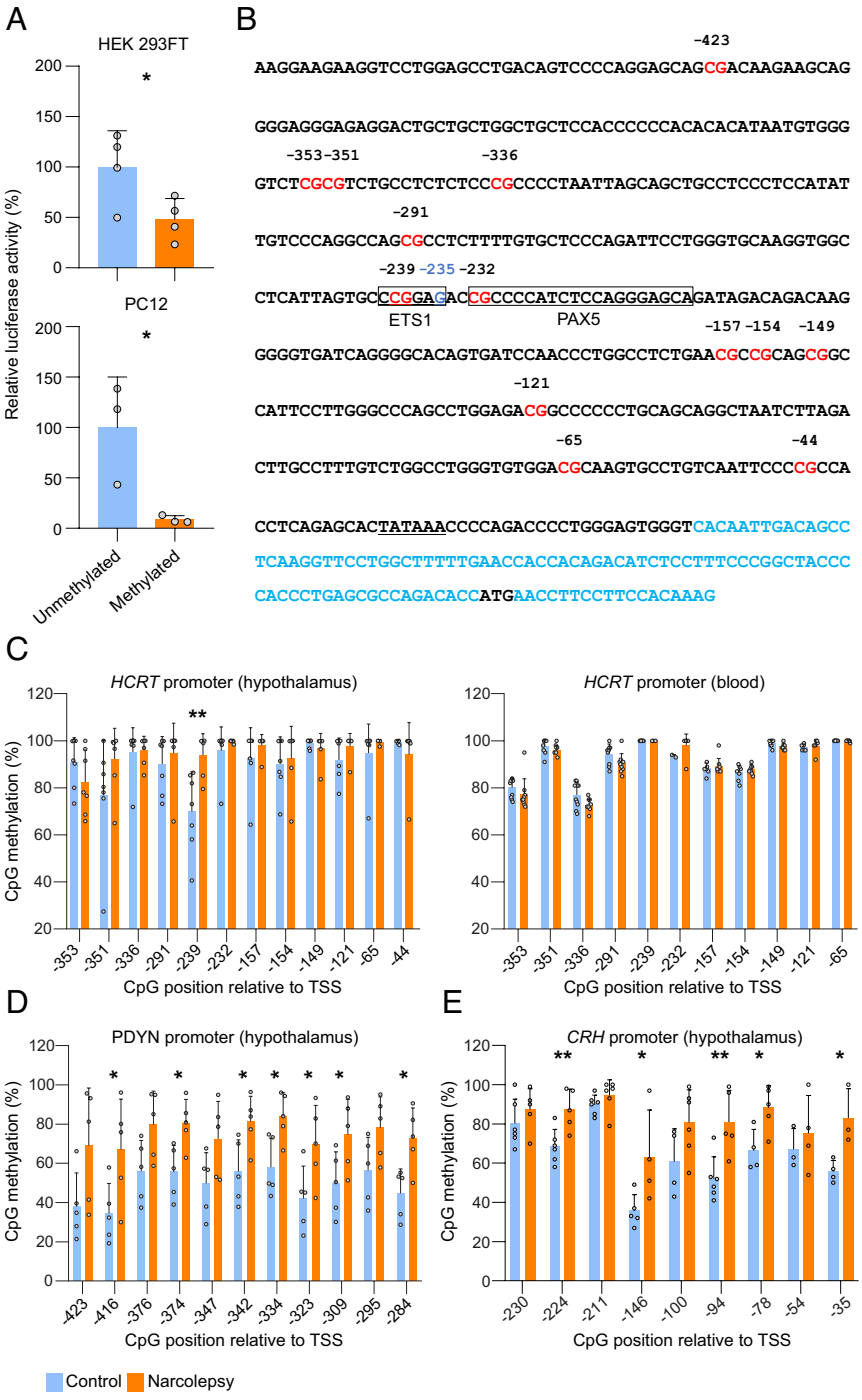

Fig. 2. Selective hypermethylation of the *HCRT* gene promoter at a PAX5:ETS1-binding site in the hypothalamus of narcolepsy patients. (*A*) Expression of a human *HCRT* gene promoter-driven luciferase reporter in two different cell lines is suppressed by methylation. Both cultures were transfected with a *HCRT*-promoter-driven luciferase construct either methylated or unmethylated. Values are averages of three wells measured in duplicate over three different transfections, and reported as a percentage of the reference unmethylated vector. $*P < 0.05$, *t* tests of methylated compared to unmethylated, $n = 4$ (HEK 293FT) and $n = 3$ (PC12). Bars represent mean + SD. (*B*) Sequence of the human *HCRT* promoter. Shown are 500 nucleotides upstream of the transcription start site, together with the first exon (in blue), with 5′ untranslated region and the translation start site (ATG, black). CpGs are highlighted in red. The CCCGAG sequence in the PAX5:ETS1 binding site is boxed with a G (instead of *A* in the consensus ETS1 sequence) in blue (−235). The TATA box is underlined. (*C*, *Left*) Methylation status at the 12 most proximal CpGs of the *HCRT* promoter (see *B*) in hypothalamic sections in seven narcolepsy patients and seven controls. One of the 12 CpGs is significantly hypermethylated in narcolepsy patients relative to controls (−239 CpG). (*C*, *Right*) Methylation status of 11 out of the 12 most proximal CpGs of the *HCRT* promoter from peripheral blood samples of nine narcolepsy and 10 control subjects. (*D*) Methylation status of the *PDYN* promoter from hypothalamic sections of five narcolepsy and five control subjects shows significant hypermethylation at 7 out of the 11 CpGs relative to controls. (*E*) Methylation status of the *CRH* promoter from brain hypothalamic sections in five narcolepsy and five control subjects shows significant hypermethylation at 5 out of the 9 CpGs relative to controls. Bisulfite sequencing of hypothalamic *HCRT* and *PDYN* promoters was performed by targeted next-generation sequencing, while data for blood and *CRH* derive from Sanger sequencing (percentage + SD). $*P < 0.05$ and $**P < 0.005$, *t* tests between narcolepsy patients and controls. CpG positions are indicated relative to the transcriptional start site (TSS).

A Methylation-Sensitive PAX5:ETS1 Transcription Factor Complex Binds and Regulates the *HCRT* Gene Promoter. The hypermethylated CpG −239 residue of the *HCRT* promoter is located within a suboptimal ETS1-binding site (5′-CCGGAG-3′), a sequence originally identified in the promoter of the early B cell-specific *MB-1* (*CD79A*) gene, encoding the Ig-alpha protein (37). ETS1 binding at this site was reported to be repressed by methylation (38, 39). Monomeric ETS1 is expressed in an autoinhibited form with very low affinity for its binding site, but it can bind either as a dimer at two head-to-head ETS1-binding sites, or cooperatively with another transcription factor, such as PAX5 or RUNX1. To determine whether this site in the human *HCRT* promoter recruits ETS1, we mutated the binding sequence to create an optimal ETS1-binding site (5′-CCGGAA-3′) in our *HCRT* promoter-driven luciferase reporter plasmid. This mutation caused a significantly increase in luciferase activity (Fig. 3*A*), indicating that the identified binding site is important for *HCRT* promoter-driven expression.

To assess the significance of the ETS1-binding site for endogenous *Hcrt* gene-expression in vivo, we next quantified *Hcrt* transcripts in the hypothalamus of Ets1-KO mice. A significant decrease in *Hcrt* gene expression was observed as compared to their wild-type littermates (Fig. 3*B*), suggesting that ETS1 controls the expression of the mouse *Hcrt* gene in vivo, consistently with our in vitro data above using the human *HCRT* promoter sequence. The 57-bp core regulatory region of the human HCRT promoter is well conserved in the mouse (82.5% base identity), although the mouse promoter shows an optimal ETS1-binding site (5′-CGGAA-3′) instead of the suboptimal human sequence (5′-CCGGAG-3′). *Qrfp* and *Pmch* expressions were also significantly decreased in the hypothalamus of

Ets1-KO mice (Fig. 3*B*), suggesting that the ETS1 transcription factor controls the expression of multiple hypothalamic neuropeptides in mice. Consistently, both *Qrfp* and *Pmch* promoters were found to contain an optimal ETS1-binding site, similar to the mouse *Hcrt* promoter, at a similar position relative to the transcription start site.

Interestingly, we found that methylation levels of the last 2 CpG residues of the *QRFP* promoter were substantially lower than the one of all other CpGs of the *QRFP* promoter, in hypothalamus and blood of narcolepsy patients, or control subjects (*SI Appendix*, Fig. S1 *C* and *D*). These 2 CpGs overlap a 5′-CGGAAGCCG-3′ sequence, which contains an ETS1-binding site (underlined). This is consistent with regulation of the *QRFP* promoter by ETS1, and the relative hypomethylation of its ETS1-binding site may indicate ETS1 occupancy, reflecting active *QRFP* expression in the hypothalamus of narcolepsy patients.

To test if, similarly to what was reported in the *MB*-1 promoter, PAX5 recruits ETS1 at the CpG −239 of the human *HCRT* promoter, we performed in vitro electrophoretic mobility shift assays (EMSA) with full-length human recombinant PAX5 protein and a probe including 43 bp (−255 to −213) of the human *HCRT* promoter, or a 35-bp *MB-1* probe (37) (*SI Appendix*, Table S2). PAX5 protein titration with both probes confirmed binding, although with lower affinity for the *HCRT* than for the MB-1 probe. Additionally, at concentrations higher than 100 nM, PAX5 was found to bind both probes as a dimer. As expected, the full-length human recombinant ETS1 showed very limited binding (trace binding at over 1-μg protein concentration). However, when PAX5 was added in the binding reaction, a ternary complex was detected (Fig. 3*C*, lane 4). Interestingly, although PAX5 alone binds *HCRT* probe as a dimer, in the presence of ETS1, the ternary complex seems to contain monomeric PAX5, leading to a similarly sized complex. The addition of ETS1 or PAX5 antibody to the binding reaction confirmed that the complex contains both proteins (Fig. 3*C*, lanes 5 and 6). The ternary complex formation could be completely removed with 200-fold excess unlabeled *HCRT* probe as a specific competitor (Fig. 3*C*, lane 7). Next, we tested the effect on binding of site-specific methylation by using the same *HCRT* probe but with the CpG −239 residue being methylated on the sense strand (*SI Appendix*, Table S2). Methylation dramatically reduced the ternary complex formation (Fig. 3*C*, lane 8), confirming the methylation-sensitive binding of ETS1. These results confirm that, similar to the *MB-1* promoter (Fig. 3*C*, lanes 9 and 10), the *HCRT* promoter can cooperatively bind PAX5 and ETS1 in a site-specific and methylation-dependent manner. Our data further evidence structural differences in the PAX5:ETS1 complex at the *HCRT* and *MB-1* promoters, with PAX5 binding the *HCRT* sequence as a dimer and at higher concentrations. PAX5 was first identified as a B cell-specific transcription factor, but it also plays important functions in the brain. Full-body *Pax5* KO in mice leads to severe structural defects in the embryonic brain (40). The function of *Pax5* in the postnatal brain is less well understood, but the gene was recently reported to be up-regulated in specific hypothalamic neuronal subtypes shortly after birth (41), and a role in the adult brain was suggested by the observation that its expression is down-regulated in the hippocampus of patients with bipolar disorder (42).

To determine whether PAX5:ETS1 cooperative binding is conserved across species, we performed EMSA with probes targeting the promoter of the mouse *Hcrt*, and the zebrafish *hcrt* gene (*SI Appendix*, Table S2), both containing putative PAX5 and ETS1-binding sites, with zebrafish *hcrt* gene containing several ETS1-binding sites. Both the mouse and the zebrafish probes showed PAX5 and PAX5:ETS1 cooperative binding using the human recombinant proteins (Fig. 3*D*), and the ternary complex could be supershifted with ETS1 and PAX5 antibodies, confirming that the cooperative PAX5:ETS1 binding to the *Hcrt* gene is evolutionary conserved.

To confirm recruitment of ETS1 at both the human *HCRT* and mouse *Hcrt* promoters in vivo, we performed chromatin immunoprecipitation (ChIP) with hypothalamic extracts of Hcrt-Cre transgenic mice (43), which contain both the endogenous *Hcrt* gene and the human *HCRT*-driven Cre-expressing transgene, and an anti-ETS1 antibody. ChIP-qPCR demonstrated efficient immunoprecipitation of both human and mouse promoters by the ETS1 antibody, with fold enrichments of 5.4 ± 0.6 for the human and 3.6 ± 0.2 for the mouse promoter (Fig. 3 *E* and *F*), indicating ETS1 protein is bound at both promoters in vivo.

We next knocked down both *pax5* and *ets1* genes by injecting morpholino oligonucleotides in zebrafish embryos at the one-cell stage and assessed fish endogenous *hcrt* transcript levels. Fish with normal morphogenetic development (*SI Appendix*, Table S3) were selected for gene expression and activity/sleep analysis. Use of a transgenic line expressing a zebrafish *hcrt* promoter-driven eGFP reporter (44) allowed us to show that while *hcrt*-reporter expression was diminished by *pax5*-*ets*1 knockdown, the number of hcrt-positive cells was unchanged (Fig. 4*A*). Fish with *pax5* and *ets1* knockdown showed decrease *hcrt* transcript levels, relative to fish injected with a control morpholino oligonucleotide, while the expression of *qrfp* was unchanged (Fig. 4*B*). Furthermore, rest-activity analysis revealed that *pax5* and *ets1* knockdown fish exhibited a significant decrease in spontaneous locomotor activity during the light period (Fig. 4*C*), while sleep was decreased (Fig. 4*D*) and largely fragmented during the major sleep period (dark) (Fig. 4*E*), a trait commonly seen in narcolepsy (45). Prober et al., (46) showed that overexpression of *hcrt* in zebrafish leads to increased locomotor activity and reduced sleep, while Elbaz et al., (44) produced a zebrafish model where hcrt neurons are ablated, and found increased sleep and sleep fragmentation similar to our observations, although they did not find decreased locomotor activity. Altogether, our results suggest that PAX5:ETS1 may have a conserved function in controlling *HCRT* gene expression in man, mouse, and fish.

Discussion

In this study, we demonstrate the use of the coexpressed QRFP neuropeptide as a marker of a subpopulation of HCRT neurons, that is independent of concomitant *HCRT* expression, and has better specificity than previously identified coexpressed genes. Several studies sought to discover gene products enriched in HCRT neurons, with the aim of identifying potential self-antigens acting as targets of a hypothetical autoimmune process. Different approaches were used and yielded a number of candidate HCRT cell coexpressed genes (24, 47, 48). However, none of these genes are specific to HCRT neurons and are expressed in other forebrain cell types. We have used RNA-sequencing as a sensitive gene expression analytical tool to differentiate the hypothalamus of HCRT cell-ablated with the one of control mice, and did not find the previously reported genes but discovered *Qrfp* as the most significant differentially expressed gene, lost specifically within the hypothalamus of Hcrt-ataxin mice but not in other brain areas (22). We here confirm and extend this observation by showing that over 95% of *Qrfp* transcript is lost in HCRT neuron-ablated Hcrt-DTA mice. Loss of *Qrfp* expression when HCRT neurons are destroyed may be due to *Qrfp* transcript colocalization, or alternatively indicate that HCRT neurons are necessary for *Qrfp* gene expression. Because there is no mechanistic evidence that HCRT neurons would control *Qrfp* expression in other cells, colocalization of the two transcripts is the most parsimonious

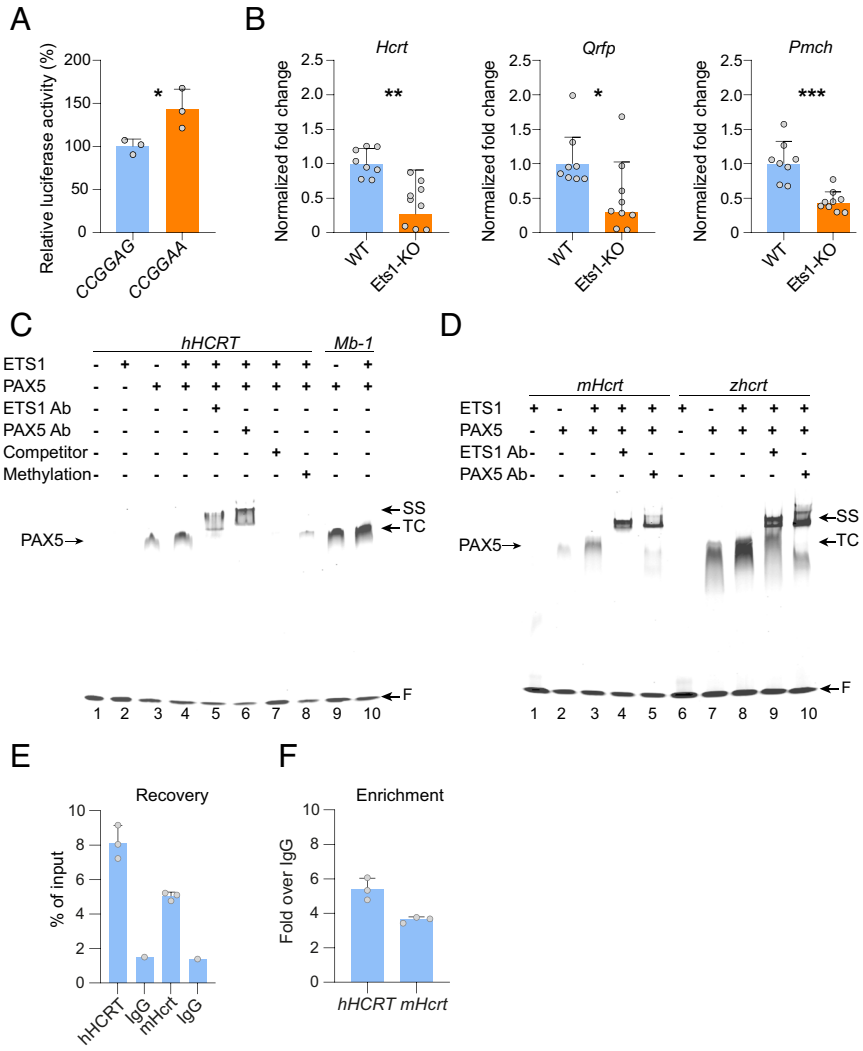

Fig. 3. A PAX5:ETS1 complex regulates *HCRT* expression. (*A*) Mutagenesis of a suboptimal 5′-CCGGAG-3′ site of the human *HCRT* promoter into the optimized ETS1 binding site (5′-CCGGAA-3′) leads to a significant increase in expression of a luciferase reporter. $*P < 0.05$, *t* tests on CCGGAA compared to CCGGAG, $n = 3$. (*B*) The expression of the *Hcrt* transcript is significantly decreased in the hypothalamus of Ets1-KO mice. Levels of the *Pmch* and *Qrfp* transcripts are also significantly decreased, suggesting ETS1 may regulate expression of several lateral hypothalamic neuropeptide genes. $*P < 0.05$, and $***P < 0.001$, *t* tests on log transformed normalized fold change of Ets1-KO mice compared to wild-type controls, $n = 8$ wild-type and $n = 9$ Ets1-KO. Bars represent geometric mean + geometric SD. (*C*) Inverted grayscale fluorescence micrograph of an EMSA gel showing the cooperative binding of PAX5 and ETS1 to the human *HCRT* (lanes 1 to 8) and *Mb-1* (lane 9 to 10) promoters. In lane 8, the *HCRT* probe was synthesized to contain a methylated Cytosine at the −239 CpG residue of the sense strand, resulting in severely reduced binding. For *HCRT*: recombinant human ETS1 at 200 nM, PAX5 at 100 nM, for *Mb-1*: ETS1 and PAX5 at 100 nM, ETS1 and PAX5 antibodies (Ab) at 1:20, unlabeled specific competitor at 200X. (*D*) EMSA evidence of conserved cooperative binding of PAX5 and ETS1 to the mouse (lanes 1 to 5) and zebrafish proximal *Hcrt/hcrt* promoters (lanes 6 to 10). ETS1 at 200 nM, PAX5 at 100 nM, ETS1 and PAX5 antibodies at 1:20. TC: ternary complex, SS: super-shift, F: free probe. (*E*) ChIP-qPCR performed on hypothalamic tissue of 17 Hcrt-Cre transgenic mice and an anti-ETS1 antibody yielded substantial recovery (as % of input) of both the human and mouse hypocretin promoters, with fold enrichments (*F*) over the control IgG IP.

explanation. Additionally, we found that loss of *Hcrt* expression in Hcrt-KO mice leads to upregulation of *Qrfp* expression, possibly reflecting an interaction between the two genes within the same cells, for instance by competition for recruitment of common transcription factors. Most directly, we used RNAscope in mice to demonstrate that over 94% of *Hcrt*-expressing neurons in the lateral hypothalamus coexpress *Qrfp*. Reciprocally 82% of *Qrfp*-expressing neurons express *Hcrt*. The 18% of *Qrfp*-expressing neurons that do not express *Hcrt* are mainly located outside the lateral hypothalamus. Although we cannot directly transpose mouse findings to humans, or two different detection techniques (RNAscope versus immunohistochemistry), we show that 38.7% of HCRT-immunoreactive cells in the brain of human control subjects are immunoreactive for QRFP, and 62% of QRFP-positive neurons are HCRT-positive. The lower figures in humans as compared to mice may stem from several factors, most notably the lower sensitivity of immunohistochemistry compared to in-situ RNA hybridization. Additionally, these data are confounded by our observation that when HCRT-immunoreactivity is low, QRFP-immunoreactivity is high. Consistently we observe that among the few remaining and low-expressing HCRT neurons in the brain of narcolepsy patients, 82% are QRFP-positive.

Even if only 38.7% of HCRT neurons appear to coexpress QRFP in controls, the loss of HCRT neurons by a hypothetical immune attack would be expected to lead to a significant decrease in QRFP. In contrast, we show that not only it is not the case but QRFP expression is increased in the brain of patients. Altogether, these results support that QRFP is a marker of HCRT neurons and because its expression is not decreased but increased, a substantial number of HCRT neurons must be preserved in narcolepsy patients but do not productively express HCRT.

The second important finding of this study is the mechanism by which HCRT neurons are silenced in narcolepsy. If these neurons are not destroyed but do not express HCRT, then either there is a physiological downregulation or epigenetic mechanisms are involved. In contrast to Hcrt-KO mice, human narcolepsy patients carry an intact *HCRT* gene. In addition, none of the narcolepsy transcriptome studies found loss of any critical gene that could control *HCRT* expression, such as a signaling molecule or a transcription factor. We do not exclude that at disease onset, a local immune reaction (inflammation) caused a certain level of cell loss. Accordingly, both epigenetic gene silencing and cell death may occur in narcolepsy. We show here that not only the human *HCRT* (as well as *Pdyn* and *CRH*) promoter is hypermethylated in the hypothalamus of narcolepsy patients but that the methylation occurs at a highly critical and phylogenetically conserved CpG within the PAX5:ETS1 binding site. One may argue that if HCRT neurons are destroyed then our methylation analysis is based on non-HCRT-expressing neurons, that could be hypermethylated. First, we clearly show in the first part that HCRT-neurons could not be absent in the brain of narcolepsy patients. Second, we show that the pattern of methylation in the hypothalamus is different

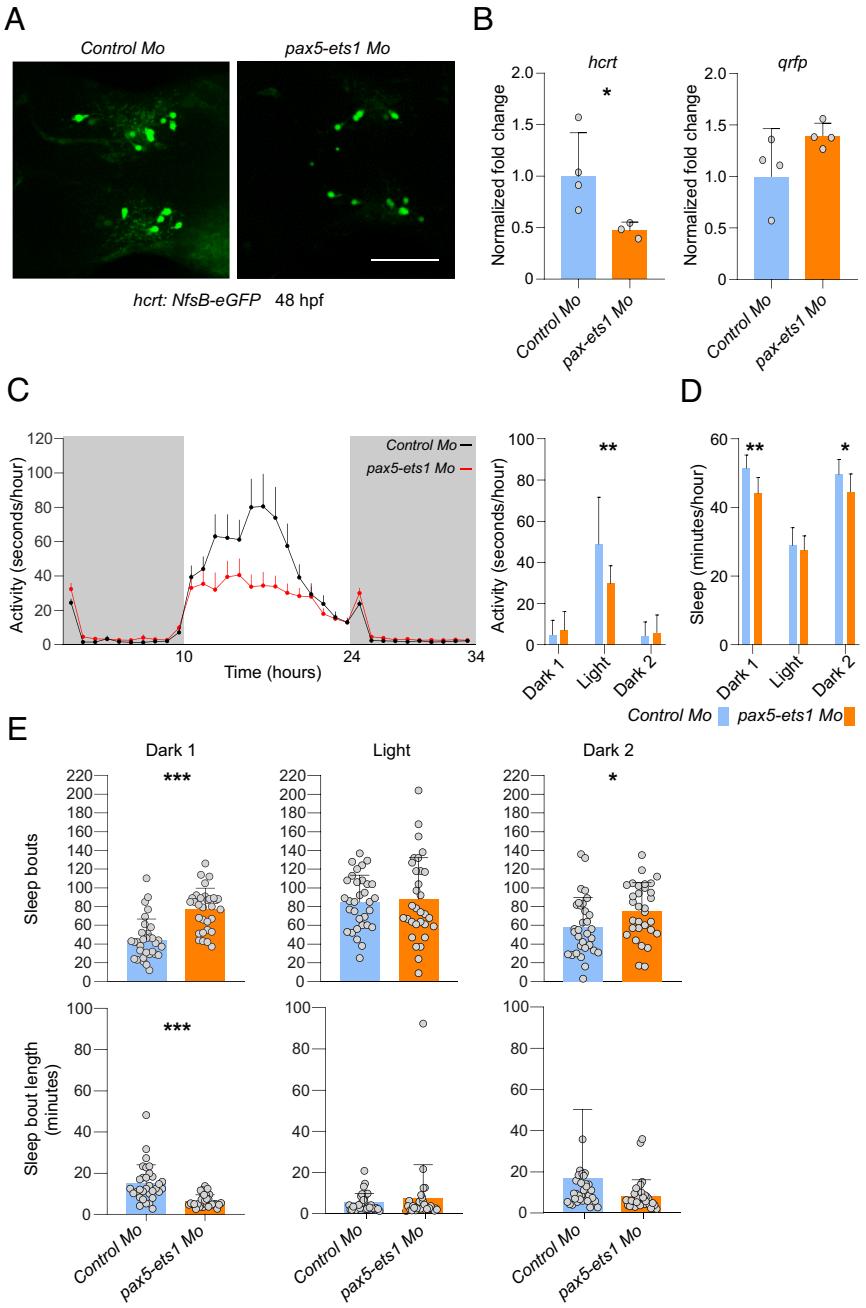

Fig. 4. PAX5:ETS1 regulates the zebrafish *hcrt* expression and sleep. (*A*) Zebrafish larvae injected with morpholinos (Mo) against both *ets1* and *pax5* exhibit a substantial decrease in *hcrt:nfs-eGFP* expression (GFP immunoreactivity 48 h postfertilization (hpf), scale bar: 50 μm), and *hcrt* mRNA level (*B*), relative to fish injected with a control morpholinowhile the level of expression of *qrfp* is not changed or tends to increase. **P* < 0.05, *t* test on log transformed normalized fold change of *pax5* and *ets1* morpholinos-injected fish compared to control morpholino-injected fish (*n* = 4 RNA samples extracted from pooled 60 fish at 5dpf each). (*C*) Knockdown of *ets1* and *pax5* also leads to decreased spontaneous locomotor activity during the light period (dark periods are indicated in gray), (*D*) decreased sleep during the dark period, and (*E*) increased sleep fragmentation in the dark period (i.e., a higher number of sleep bouts, of shorter average duration). Recording corresponds to fish at 5 to 6 dpf. N = 32, mean + SD; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *t* tests.

from that of blood cells, which do not express HCRT. For instance, CpG-336 is nearly 100% methylated in the lateral hypothalamus while its methylation is below 80% in blood cells (Fig. 2*C*) and the differences are even larger for *Pdyn* promoter methylation between the hypothalamus and blood cells (Fig. 2*D* and *SI Appendix*, Fig. S1*B*). Finally, non-HCRT hypothalamic cells may not be fully methylated but only partially leading to undetectable HCRT peptide (for instance by immunohistochemistry), and these neurons can express detectable HCRT under certain circumstances, as previously reported (see below) (49). The % DNA methylation values we obtain from our hypothalamic samples need here to be put in context. Clearly our samples contain heterogeneous populations of cells, and HCRT neurons (estimated to number about 70,000 in humans) represent only a small fraction of them (evaluated to grossly represent about 2%). Yet the difference in methylation level between patients and controls that we observe (with ~95% methylation in patients vs. ~70% in controls at CpG −239, i.e., approximatively 25%) appears about an order of magnitude greater than the number of DNA molecules plausibly contributed by HCRT neurons in the tissue sample. We find that the CpG −239 of the *HCRT* promoter is overall about 70% methylated in the hypothalamus of control subjects, indicating that this site is not always methylated in cells that do not express *HCRT*, and mechanisms other than DNA methylation are also implicated in the regulation of *HCRT* expression in different cell types. In the hypothalamus of patients, methylation at this site approaches 95%. A 25% *HCRT* DNA methylation increase in narcolepsy patients strongly suggests that methylation has occurred within HCRT neurons, as well as a fraction of non-HCRT cells. Thus, the *HCRT* gene methylation changes that our values reflect must be accounted by a sizable population of non-HCRT cells in addition to HCRT neurons, and the same applies to *CRH* gene DNA methylation.

Other epigenetic mechanisms (histone modifications and chromatin remodeling enzymes, microRNAs and long noncoding RNAs), were not analyzed in our limited postmortem material in this study, but we recently reported that the evolutionary-conserved

miRNA-137 is also involved in *Hcrt* expression (50). Whether in addition to methylation, histone modifications (acetylation, methylation, or phosphorylation) are involved cannot be excluded, especially because of a crosstalk exists between the two mechanisms. For instance, DNA methylation can guide histone modifications and DNA methylation can also take its cues from histone modifications (51, 52).

Epigenetic silencing of HCRT neurons might be facilitated by genetic predispositions (the most significant being the HLA-*DQB1*06:02* allele) that make individuals particularly susceptible to immune-related processes triggered by an inflammatory insult within the lateral hypothalamus, for instance a viral infection that causes local release of proinflammatory cytokines within the lateral hypothalamus. Systemic inflammation has been linked to reduced HCRT CSF levels in patients and rats (53), through non-identified mechanisms. Further supporting a link between inflammation and reduced HCRT expression, a polymorphism in the proinflammatory TNF-alpha gene was reported to be significantly associated with narcolepsy (54, 55), and narcolepsy patients are reported to have increased serum TNF-alpha levels (56, 57). Moreover, TNF-alpha was found to dramatically decrease *HCRT* expression in vitro (58). Importantly, prolonged exposure to TNF-alpha was reported to induce promoter methylation in cell lines (59). The unexplained recent discovery of a marked increase in the number of HCRT-immunoreactive neurons in the hypothalamus of heroin addicts, later reproduced in mice and shown to be independent of neurogenesis (49), is another example of changes in *HCRT* gene expression that could be explained by epigenetic mechanisms operating at the *HCRT* gene promoter. *HCRT* gene expression malleability in response to various external cues would be consistent with HCRT prime role as a global environmental integrator, and therefore susceptible to epigenetic modifications as in narcolepsy. Altogether we propose that human narcolepsy is not a neurodegenerative disease, but rather results from an epigenetic remodeling of a number of hypothalamic tissue- and cell-specific genes. The disease is therefore neither appropriately modeled by the Hcrt-KO mice, since in addition to loss of *HCRT* expression it also features loss of hypothalamic *NPTX2* and *PDYN* transcripts, nor by HCRT-neuron ablation, since other hypothalamic cell types (e.g., CRH-producing cells) are also affected.

Intriguingly, a rare condition called autosomal dominant cerebellar ataxia, deafness and narcolepsy, is caused by dominant mutations in the gene encoding the maintenance DNA methyltransferase 1 (*DNMT1*) (60, 61). Dominant mutations in DNMT1 were associated with increased methylation at normally unmethylated regions of promoters and CpG islands, leading to gene silencing (62), strengthening the notion of a causal link between DNA hypermethylation and narcolepsy. Partial or transient epigenetic modulation of HCRT expression might also be involved in narcolepsy without cataplexy, idiopathic hypersomnia or Kleine–Levin syndrome. Furthermore, the gradual decrease in the number of HCRT-immunoreactive neurons with aging (63, 64) might also be due to epigenetic downregulation of *HCRT* gene expression, leading to profound changes in sleep and sleepiness in aged subjects.

Narcolepsy is increasingly recognized as a hypothalamic disorder, rather than exclusively a HCRT disease, involving not only sleep-wake, but also motor, psychiatric, emotional, cognitive, metabolic, and autonomic abnormalities (1). Together with other studies, our results strongly suggest that narcolepsy results from dysregulations, including epigenetic alterations, in several other neuropeptidergic systems (PDYN, NPTX2, CRH, and QRFP) with diverse functions.

Methylation being reversible, our findings open the avenue for therapeutic interventions based on DNA demethylation, leading to treatment or even cure of narcolepsy. Although DNA methylation/demethylation events were initially thought to be restricted to dividing cells, recent evidence indicates that they also occur in postmitotic cells, including in neurons (65). Epigenetic editing tools, including pharmacological compounds, or site-specific demethylating genetic reagents, are becoming available (66). Beyond narcolepsy, epigenetic silencing may represent a key causative factor in several other immune, autoimmune, neurodegenerative, or neuropsychiatric diseases.

Materials and Methods

Animals. Hcrt-KO and Hcrt-ataxin mice were bred locally on C57BL/6J background. Brains of Hcrt-DTA (23), Ets1-KO (67) and their controls were provided by international collaborators. Brains of Hcrt-DTA mice were sampled 4 wk after doxycycline removal from the diet, while their controls were maintained under doxycycline. All animal procedures followed Swiss federal laws and were approved by the State of Vaud Veterinary Office. At all times, care was taken to minimize animal discomfort and avoid pain.

Human Tissue Samples. Hypothalamic RNA samples from four narcolepsy patients and four controls were available in the laboratory of M. Honda (24) and were tested locally by qPCR with TaqMan assays Hs01891339_s1 (*HCRT*), Hs01650960_s1 (*QRFP*), and reference genes Hs00744842_sH (*TUBA1B*), Hs01375212_g1 (*RPS18*), and Hs04185005_g1 (*RPS27*). Formalin fixed sections (40 μm, 2 to 4 sections/subject) through the lateral hypothalamus from narcolepsy patients and controls were obtained through international collaborators. All tissues samples were from postmortem brains previously published (17, 49, 68–71). The research protocol and the use of human biological material was approved by the local ethics committee (SwissEthics). Formalin fixed sections were washed with PBS, and genomic DNA was extracted with the AllPrep DNA FFPE Tissue kit (Qiagen). CSF samples from narcolepsy and control subjects were provided by the laboratory of GJ. Lammers.

RT-qPCR. RNA extraction, quantification, and processing are detailed in *SI Appendix*. Amplification was carried out with TaqMan assay kits Mm01964030_s1 (*Hcrt*), Mm01701538_m1 (*Qrfp*), Mm0124886_g1 (*Pmch*), Mm00457573_m1 (*Pdyn*), Mm00479438_m1 (*Nptx2*), and reference genes Mm01973893_g1 (*Eef1a1*), Mm00850060_s1 (*Rps9*). qPCR for fish samples were performed by primers in combination with Power SYBR Green (Thermo Fisher Scientific) using a ViiA7 real-time PCR system (Thermo Fisher Scientific). Primers were: *hcrt*-F 5′-CTCCTGCAAACTCTACGAGATG-3′, *hcrt*-R 5′-GTCGTTGTTGAGATGCACTAAA-3′, *qrfp*-F 5′-AATGCTGCCACACCAACCAA-3′, *qrfp*-R 5′-TCCCAGGTCCTGAAACAAAGC-3′, and the reference gene *18s*-F 5′- AGCGTGCGGGAAACCACGAG-3′ and *18s*-R 5′-AAGCCGCAGGCTCCACTCCT-3′. Normalized fold expression was calculated according to Taylor et al. (72).

QRFP RIA. Quantification of QRFP in human CSF samples was carried out using a specific RIA, previously described in detail (73). CSF samples were pumped at a flow rate of 1.5 mL/min through one Sep-Pak C_{18} cartridge. Bound material was eluted with acetonitrile/water/TFA (50:49.9:0.1; v/v/v), and acetonitrile was evaporated under reduced pressure. Finally, the dried extracts were resuspended in PBS 0.1M and assayed for QRFP. Data represent the mean of duplicates for each sample.

RNAscope-In Situ Hybridization Experiments. Tissue processing is detailed in *SI Appendix*. Fluorescent in situ hybridization for the simultaneous detection of the *Qrfp*, *Hcrt*, and *Pmch* transcripts was performed using RNAscope (ACD; Advanced Cell Diagnostics). The *Hcrt* probe targeted the region 2 to 577 (accession number NM_010410.2; ACD, Cat. No. 490461-C2). The *Qrfp* probe targeted the region 112 to 1,081 (accession number NM_183424.4; ACD, Cat No. 464341-C3). The *Pmch* probe targeted the region 4 to 652 (accession number NM_029971.2; ACD, Cat. No. 478721-C1). Negative and positive control probes recognizing dihydrodipicolinate reductase, DapB (a bacterial transcript) and PolR2A (C1 channel), PPIB (C2 channel) and UBC (C3 channel), respectively, were processed in parallel with the target probes to ensure tissue RNA integrity and optimal assay performance.

Immunofluorescence and Confocal Microscopy of Human Tissues. Tissue processing is detailed in *SI Appendix*. QRFP antibody (73) at 1:400 was applied overnight at room temperature, followed by a second incubation with QRFP antibody 1:400 and HCRT antibody 1:500 (goat HCRT anti HCRT, Santa Cruz Biotechnology, SC-8070) at 4 °C for 24 h. After rinsing with 1xPBS for 3 times (10 min each), secondary antibodies were applied (donkey IgGs coupled to Alexa-594, or -488 fluorophores) in 1:500 dilutions for 2 h at room temperature followed by rinsing with 1xPBS for 3 times (10 min each). To test the specificity of QRFP antibody, QRFP antibody was blocked with QRFP (NM_198180) Human Over-expression Lysate (2 μg/mL, 1%BSA) for 2 h at room temperature before use in the above-described immunofluorescence assay. Images were acquired on an inverted Zeiss LSM780 confocal laser-scanning microscope (405-, 488-, and 561-nm lasers) using a 40× oil objective (EC plan-Neofluar 40×/1.30 Oil DIC M 27). For each human section used for cell quantification, confocal images covering the HCRT positive cell field were acquired at 8-bit image depth and a frame of 1,024 × 1,024 pixels and tiled together at size of 2,125.48 × 2,125.48 μm using ZEN software. Immunoreactive cell counts were evaluated within that frame using ImageJ software.

***HCRT* Promotor Cloning.** 1,800 base pairs of the human *HCRT* promoter upstream of the transcription start site were cloned in multiple cloning sites of the pCpG-basic vector (74) upstream of the firefly luciferase sequence. The cloned plasmids were transformed in a Pir1 bacterial strain and sequenced for verification. The bacterial colonies were cultured with Zeocin selection at large scale and maxiplasmid preparations were carried out using the HiSpeed Plasmid Maxi Kit (Qiagen, 12662). CpG Methyltransferase, M.SssI (New England Biolabs, M0226s) was used to methylate the CpG sites on the *HCRT* promoter.

Luciferase Assay. HEK 293FT and PC12 cell lines were cultured at 1.5-2*10^5 density in 24-well plates in DMEM medium containing 10% FBS. Cultures were transfected with methylated and unmethylated plasmids. The transfection was performed using X-tremeGENE™ HP DNA Transfection Reagent (Sigma, 6366244001) with the DNA to reagent ration of 1:3. The transfected plasmids were mixed with Firefly and Renilla luciferases with ratio of 19:1. Then, 48 h after transfection the cells were lysed and prepared for the luciferase activity measurement according to Promega kit (E1910). Luciferase activity was measured using the GloMax 96 microplate luminometer from Promega. The firefly luciferase activity was normalized to the Renilla activity.

DNA Methylation Analysis. Using the EpiTect Fast DNA Bisulfite kit (Qiagen), 500 ng DNA was treated with sodium bisulfite. Primers for nested PCR (2 fragments for *HCRT*, 1 for *PDYN and HCRTR2*) were designed with MethPrimer (75) (*SI Appendix*, Table S1). Methods for methylation quantification are detailed in *SI Appendix*.

Electrophoretic Mobility Shift Assay (EMSA). Oligonucleotides probes (*SI Appendix*, Table S2) were purchased from Microsynth AG (Balgach, Switzerland). To test the effect on binding of selective methylation at the CpG-239 residue, the *HCRT* probe was synthesized with a methyl-Cytosine at this position on the sense strand (Fig. 3*C*, lane 8). Probes were 3′ labeled with Dyomics 781. Unlabeled oligos were used for competition assays. Assay procedure is detailed in *SI Appendix*.

ChIP-qPCR. ChIP was performed using iDeal ChIP-seq kit for Transcription Factors (Diagenode, C01010054) following the provided protocol. Tissue processing and chromatin preparation is detailed in *SI Appendix*. Sheared chromatin was subjected to magnetic immunoprecipitation using 1 μg anti-ETS1 (Cell Signaling Technology, D8O8A) and anti-IgG in ChIP reaction provided by the kit. Quantification of ChIP was performed by qPCR using Power SYBR™ Green PCR Master Mix (Fisher Scientific, 4367659) using primers as listed in *SI Appendix*, Table S2. The % recovery was determined as 2^((Ct(input)-6.64)-Ct(IP))*100, where 6.64 is the adjusting factor to correct for the input dilution (1:100), and the fold enrichment of target over the control sequences were calculated as 2^(Ct(target)-Ct(IgG)).

Morpholino Study in Zebrafish. Morpholino antisense oligos targeting *ets1* and *pax5* transcripts were designed according to Pham et al. (76) and Kwak et al. (77), respectively. Subsequently, 0.25 pmol *ets1* (5′-GTCATGGTCACGCATTCAAACGTAC-3′) or a combination of 0.375 pmol *pax5* TB1 (5′-CAGTGGATTTCCATCTGTTTTAAA-3′) and 0.375 pmol *pax5* TB2 (5′- CTCGGATCTCCCAGGCAACATGGT-3′) was injected in 1 to 2 cell zygotes in a total volume of 1 nL. The control morpholino corresponds to the standard sequence proposed by the manufacturer (5′-CCTCTTACCTCAGTTACAATTTATA-3′). Morpholino synthesis was performed by Gene-tools (http://www.gene-tools.com). Visualization of Hcrt neurons was performed using the *Tg(hcrt:nfsB-EGFP)* transgenic line, in which a 2-kb fragment of the zebrafish hcrt promoter drives expression of a nfsB-EGFP fusion protein (44). Imaging was performed on homozygous embryos, using a LSM710 confocal microscope (Zeiss). Locomotion tracking is detailed in *SI Appendix*. Larvae exhibiting morphological defects (*SI Appendix*, Table S3) were excluded from analysis to control against motor impairments.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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