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Supporting Information for

Epigenetic silencing of selected hypothalamic neuropeptides in narcolepsy with cataplexy

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Supporting Information Text

Material and Methods

RT-qPCR

Mouse brains, cell culture samples and fish were collected and preserved at −80 °C until RNA extraction. Total RNA was isolated using the RNeasy Micro kit (QIAGEN 74004) or Trizol extraction method. The quantity and quality of RNA samples was checked using a Nanodrop (ND- 1000) spectrophotometer. Reverse transcription was carried out by using M-MLV reverse transcriptase (Promega) or SuperScript IV reverse transcriptase (Thermo Fisher Scientific) by random hexamer or oligo-dT primers according to the manufacturer's instructions.

RNAscope-In situ hybridization experiments

Mice were deeply anesthetized and perfused transcardially with sterile Phosphate Buffer Saline followed by ice-cold phosphate buffered 4% paraformaldehyde (PFA, pH 7.4). The brains were removed, post-fixed overnight at 4°C in sterile 4% PFA, transferred to sterile 15% sucrose (12h) and then to 30% sucrose (12 h) in 0.1 M PBS (pH 7.4) at 4°C. 12µm-thick sections,

collected every 40µm on the entire length of the hypothalamus (15 sections/hypothalamus), were cut on a cryostat and mounted on SuperFrostPlus slides and subsequently stored at -80°C. All pretreatment reagents, detection kit, and wash buffer were purchased from ACD. All incubation steps were performed at 40°C using a humidified chamber and a HybEz oven (ACD). The day of the assay, all the hybridization steps, i.e., target retrieval, dehydration, probe hybridization, amplification steps, and detection of the probe, were performed according to the online protocol for RNAscope Multifluorescent Assay. Briefly, the procedure included the following steps: slides were first submerged in Target Retrieval (ACD) heated to 98.5°C–99.5°C for 5min, followed by two brief rinses in sterile water. The slides were quickly dehydrated in 100% ethanol and allowed to air dry for 5min. The next day, the sections were incubated with Protease III for 30min (ACD). The *Pmch* probe (channel 1), the *Hcrt* probe (channel 2) and the *Qrfp* probe (channel 3) were mixed according to the manufacturer's instructions and hybridized to the sections for 2h, followed by 2min washes in wash buffer (ACD), incubation with Amp1 for 30 min, two washes, Amp2 for 30min, two washes and finally Amp3 for 15min followed by two washes. Sections were then incubated with amplification system (HRP and Opal fluorophore). The *Pmch* probe was detected with Opal 520 (Akoya Biosciences, FP1487001KT), the *Hcrt* probe with Opal 650 (Akoya Biosciences, FP1496001KT) and the *Qrfp* probe with Opal 570 (Akoya Biosciences, FP1488001KT). Sections were then immediately cover slipped with Fluoromount Aqueous Mounting (Sigma, F4680) with DAPI and stored in dark at 4°C until imaging. Images were captured using a Leica Thunder Imager 3D (DM6 B). Maximum intensity projections were made inFIJI (NIH), and images were adjusted for brightness and contrast. Quantitative analysis of neurons expressing *Qrfp* and *Hcrt* in the hypothalamic sections were performed using the Image J software (NIH, Washington, DC).

Immunofluorescence and confocal microscopy of human tissues

Hypothalamic formalin-fixed paraffin sections (5μm) were dewaxed in xylene for 15min, followed by rinsing in 99% and 96% ethanol (3 times, 5min each). Endogenous peroxidase was blocked for 20 min with methanol / 0.3% H_2O_2 followed by rinsing in 96%, 70% ethanol and 1xPBS (3 times, 5min each). Antigen retrieval was achieved by microwave heating for 3 cycles of 10sec every 5min at max 800 W up to 85-9°C in 10mM Citrate Buffer (pH 6.0), then cooling down to room temperature in the same buffer for 1 hour. Slides were washed in 1x PBS (3 times, 5min each) and incubated in bovine serum albumin (2% BSA, 0.3% Triton x-100, 5% normal donkey serum in 1xPBS) for 30min.

DNA methylation analysis

Two methods were used to quantify CpG methylation. In the first method PCR amplicons were gel purified followed by Sanger sequencing. Sequence trace files were analyzed by ESME software (1) resulting in average methylation at each CpG. In the second method purified PCR amplicons (10ng) were used to prepare sequencing libraries by TruSeq Nano DNA LT Library Preparation Kit (Illumina) with the following modifications. Purified bisulfite PCR products were not sheared, but directly subjected to end repair and then cleaned up with a single round of purification beads at a 1.8X ratio. The standard protocol was strictly followed for the subsequent steps. Libraries were quantified with a fluorimetric method (QubIT, Thermo Fisher) and their size pattern evaluated with the Fragment Analyzer (Agilent). An equimolar pool of each library was prepared and loaded at 10pM (with 25% PhiX spike in for generating sequencing diversity) for a 150 cycle paired-end run on a MiSeq instrument (Illumina) using a v2 reagent kit. Sequencing data were demultiplexed using the bcl2fastq conversion software (version 2.20, Illumina). Quality control was performed using the NGmerge tool (2) to keep only read-pairs that could be reconstructed (between 59 and 67% of the reads). On average,

160194 reads (range 10824-349712 reads) were obtained par fragment and per subject. A weight matrix profile was prepared from the reference sequence to take into account the C to T alterations caused by bisulfite treatment and the reads were aligned to the profile using pfsearch from pftools (3). A summary table counting the number of occurrences of each nucleotide along the fragment of interest was produced and the ratio of methylated C nucleotides was computed as a percentage of methylation for each position.

Electrophoretic mobility shift assay (EMSA)

All oligos were resuspend in Tris-EDTA pH 8.0 at 100µM. For annealing of the double stranded probes, 5µL of forward oligonucleotides and 5µL of reverse were pooled and placed on a heat block at 100°C for 5min and then cooled down to room temperature (RT). Subsequent dilution was performed using Ambion Nuclease-Free Water (Invitrogen, AM9938) to prepare working solutions at 100nM. EMSA was performed following Odyssey® EMSA Kit protocol (Li-Cor, 829-07910). Briefly, a binding reaction was prepared with final concentration of 1x Binding buffer, 2.5mM DTT/0.25% Tween 20, 5% glycerol, 25ng/µl Salmon Sperm DNA and 0.25% Ficoll. ETS1 (LSbio, G26390) and/or PAX5 (Cloud-Clone Corp, RPH445Hu01) full-length recombinant proteins were added to this mix at various concentrations as indicated in Fig. legends. For super-shift assays, either anti-PAX5 (LSbio, LS-C352634) or anti-ETS1 (Cell Signaling Technology, 14069) antibody was diluted 1:20 directly in the reaction mix and incubated for 15min at RT. Finally, 5fM of probes were added and the reactions incubated for 30min in the dark at RT. 2µl of 10X Orange Loading Dye (provided in Li-Cor kit) were added to the samples before gel loading. Separation of protein-DNA complexes was performed by loading the samples on 7.5% Mini-PROTEAN® TGX™ Precast Protein native polyacrylamide gels (Biorad, 4561024). Electrophoresis was performed for 1h15min at 100V at 4°C in the dark in the absence of antibodies and first 5min at 100V followed by 1h30min running at 80V at

4°C in the presence of antibodies. Resulting gels were imaged with the Odyssey Imaging System (Li-Cor).

Chromatin immunoprecipitation-qPCR

Briefly, hypothalami were dissected from 17 Hcrt-Cre transgenic mice (4) and half of the samples were pooled together and disrupted using a Dispomix Drive Homogenizer (Medic Tools) directly in fixation buffer containing 1% formaldehyde (Sigma, 252549) for 45 sec. The suspension was incubated for 5min at RT with gentle shaking. Chromatin was sheared by sonication using an E220 (Covaris) with following parameters: peak incidence power: 140W; cycles per burst: 200; duty factor: 4%; Time 20min.

Morpholino study in zebrafish

Locomotion tracking were performed on wild-type AB line. Embryos and larvae were maintained in an incubator at 28.5°C on 14/10h light/dark cycle and staged as described by Kimmel *et al*. (5) Rest-activity quantification was performed according to Seifinejad *et al*. (6) Briefly, larvae were recorded from 5 to 7dpf in 96-well plates, one fish per well, in 300µl of egg water. Individual activity was measured with the quantification mode of Zebrabox (viewpoint) set with the following parameters: Freeze: 3, Burst: 29, Threshold: 15, Bin: 1min, Light intensity: 7%. Any 1min with less than 0.1s of movement was counted as 1min of sleep.

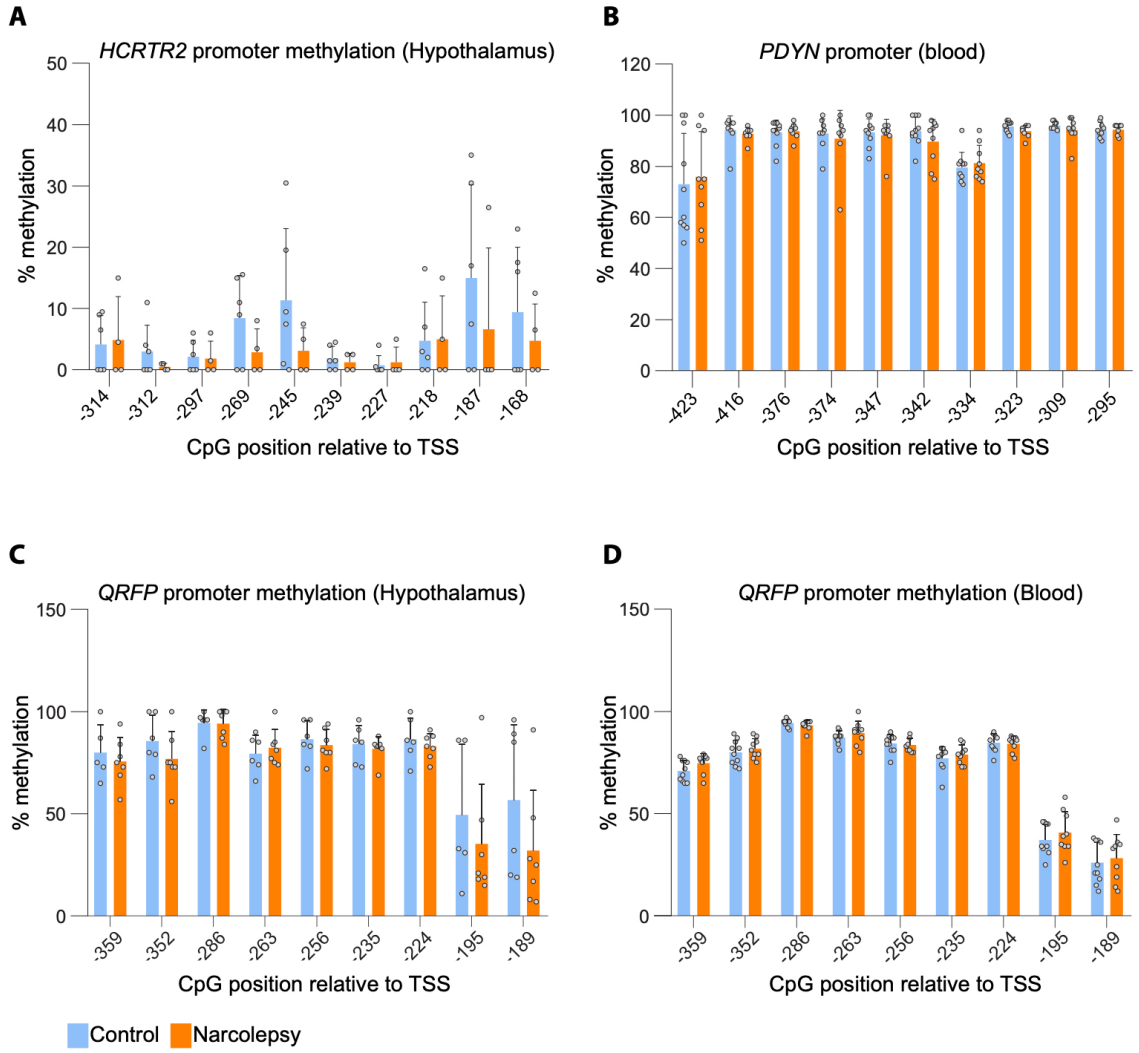

Fig. S1: Methylation status of candidate genes in hypothalamus and blood of narcolepsy and control subjects. (A) *HCRTR2* promoter is hypomethylated in the hypothalamus of both patients and control subject, suggesting normal expression. **(B)** *PDYN* promoter is hypermethylated in blood cells of both patients and controls, consistent with the lack of expression. **(C** and **D)** *QRFP* promoter methylation shows no difference between patients and controls and is hypomethylated at the last 2 CpGs (PAX5:ETS1 binding site), suggesting expression both in the hypothalamus and peripheral tissues.

Table S1: Nested primers used for bisulfite sequencing.

HCRT	
First fragment: F1	5'-GAAGAAGGTTTTGGAGTTTGATAGTT-3'
First fragment: R1	5'-CTATACCCCTAATCACCCCCTTAT-3'
First fragment: F2	5'-TTGGGTGTAAGGTGGTTTTATTAGT-3'
First fragment: R2	5'-TTATAACCCACTCCCAAAAATCTAA-3'
Second fragment: F1	5'-GGATTGTTGTTGGTTGTTTTATTTT-3'
Second fragment: R1	5'-CTATACCCCTAATCACCCCCTTAT-3'
Second fragment: F2	5'-ATAAGGGGGTGATTAGGGGTATAG-3'
Second fragment: R2	5'-TTATAACCCACTCCCAAAAATCTAA-3'
PDYN	
F1	5'-GTTGTGTTTTTGTTAGGGTTAGTGT-3'
R1	5'-CAAAAACTAAAACAATCTTCTAATCAC-3'
F2	5'-GTTTGGTATTAGTTTAGGTATGTATTAGAG-3'
R2	5'-CAAAAACTAAAACAATCTTCTAATCAC-3'
HCRTR2	
F1	5'-GGTGTTATTGTTGTAGTTTTTAGTGT-3'
R1	5'-AACTCTTAAATTTCATTCAACTCC-3'
F2	5'-GAGGTATTGGTTTAGTAATTTTTTA-3'
R2	5'-AACTCTTAAATTTCATTCAACTCC-3'
QRFP	
F1	5'-TTGTAATGATATGGTTATTTTATAATAAGA-3'
R1	5'-AAAAAACAAAATAACAAACTACACAC-3'
F2	5'-TTGTAATGATATGGTTATTTTATAATAAGA-3'
R2	5'-AAAAAACAAAATAACAAACTACACAC-3'
CRH	
F1	5'-TTTGGGAAATTTTATTTAAGAATTTTT-3'
R1	5'-CTAAATTTCTCCACTCCAAAACCTA-3'
F2	5'-GTTAATGGATAAGTTATAAGAAGTTTTT-3'
R2	5'-TCCACTCCAAAACCTAAAATAAAAT-3'

Table S2: Sequences of the fluorescent probes used in EMSA and qPCR primers used in ChIP.

EMSA	
hHCRT-F	5'-TGGCCTCATTAGTGCCCGGAGACCGCCCCATCTCCAGGGAGCA-3'
hHCRT-R	5'-TGCTCCCTGGAGATGGGGCGGTCTCCGGGCACTAATGAGGCCA-3'
Methylated h*HCRT*-F	5'-TGGCCTCATTAGTGCCC(CH3)GGAGACCGCCCCATCTCCAGGGAGCA-3'
Mb-1-F	5'-AGTAAGGGCCACTGGAGCCCATCTCCGGCACGGCT-3'
Mb-1-R	5'-AGCCGTGCCGGAGATGGGCTCCAGTGGCCCTTACT-3'
mHcrt-F	5'-TAGTACTCGGAAACTGCCCTATCTCCAAGGAACAGGCAGA-3'
mHcrt-R	5' TCTGCCTGTTCCTTGGAGATAGGGCAGTTTCCGAGTACTA-3'
zhcrt-F	5'-CCGGGATACAGGTAATCCTGGTTACACGCTAATGACAAAG-3'
zhcrt-R	5'-CTTTGTCATTAGCGTGTAACCAGGATTACCTGTATCCCGG-3'
ChIP	
mHcrt-F	5'-TCCTTCCAGTGTCCCAGCCA-3'
mHcrt-R	5'-GGTCCCCAGCTTGTCTGTCT-3'
hHCRT-F	5'-ATTAGCAGCTGCCTCCCTCC-3'
hHCRT-R	5'-AGGGTTGGATCACTGTGCCC-3'

Table S3: Normality and developmental assessment of the morphant fish.

	Injection 1		Injection 2		Injection 3		Injection 4	
	Ctrl Mo	Mo Pax5-Ets1	Ctrl Mo	Mo Pax5- Ets1	Ctrl Mo	Mo Pax5- Ets1	Ctrl Mo	Mo Pax5-Ets1
Normal Morphology	70	36	86	82	37	42	56	78
Deformities	0	7	1	10	1	5	0	12
% Deformities	0.00%	16.28%	1.15%	10.87%	2.63%	10.64%	0.00%	13.33%

SI references

1. J. Lewin, A. O. Schmitt, P. Adorjan, T. Hildmann, C. Piepenbrock, Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates. *Bioinformatics* **20**, 3005-3012 (2004).
2. J. M. Gaspar, NGmerge: merging paired-end reads via novel empirically-derived models of sequencing errors. *BMC Bioinformatics* **19**, 536 (2018).
3. R. Lüthy, I. Xenarios, P. Bucher, Improving the sensitivity of the sequence profile method. *Protein Sci* **3**, 139-146 (1994).
4. T. Matsuki *et al.*, Selective loss of GABA(B) receptors in orexin-producing neurons results in disrupted sleep/wakefulness architecture. *Proc Natl Acad Sci U S A* **106**, 4459-4464 (2009).
5. C. B. Kimmel, W. W. Ballard, S. R. Kimmel, B. Ullmann, T. F. Schilling, Stages of embryonic development of the zebrafish. *Dev Dyn* **203**, 253-310 (1995).
6. A. Seifinejad *et al.*, Molecular codes and in vitro generation of hypocretin and melanin concentrating hormone neurons. *Proc Natl Acad Sci U S A* **116**, 17061-17070 (2019).