IGFBP3 Colocalizes with and Regulates Hypocretin (Orexin)

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Abstract

Background: The sleep disorder narcolepsy is caused by a vast reduction in neurons producing the hypocretin (orexin) neuropeptides. Based on the tight association with HLA, narcolepsy is believed to result from an autoimmune attack, but the cause of hypocretin cell loss is still unknown. We performed gene expression profiling in the hypothalamus to identify novel genes dysregulated in narcolepsy, as these may be the target of autoimmune attack or modulate hypocretin gene expression.

Methodology/Principal Findings: We used microarrays to compare the transcriptome in the posterior hypothalamus of (1) narcoleptic versus control postmortem human brains and (2) transgenic mice lacking hypocretin neurons versus wild type mice. Hypocretin was the most downregulated gene in human narcolepsy brains. Among many additional candidates, only one, insulin-like growth factor binding protein 3 (IGFBP3), was downregulated in both human and mouse models and co-expressed in hypocretin neurons. Functional analysis indicated decreased hypocretin messenger RNA and peptide content, and increased sleep in transgenic mice overexpressing human IGFBP3, an effect possibly mediated through decreased hypocretin promoter activity in the presence of excessive IGFBP3. Although we found no IGFBP3 autoantibodies nor a genetic association with IGFBP3 polymorphisms in human narcolepsy, we found that an IGFBP3 polymorphism known to increase serum IGFBP3 levels was associated with lower CSF hypocretin-1 in normal individuals.

Conclusions/Significance: Comparison of the transcriptome in narcolepsy and narcolepsy model mouse brains revealed a novel dysregulated gene which colocalized in hypocretin cells. Functional analysis indicated that the identified IGFBP3 is a new regulator of hypocretin cell physiology that may be involved not only in the pathophysiology of narcolepsy, but also in the regulation of sleep in normal individuals, most notably during adolescence. Further studies are required to address the hypothesis that excessive IGFBP3 expression may initiate hypocretin cell death and cause narcolepsy.

Introduction

Narcolepsy-cataplexy is a common sleep disorder affecting 0.02–0.16% of the general population in the United States, Europe and Asia. Disease onset may be insidious or abrupt, typically occurring around adolescence, and is characterized by excessive daytime sleepiness, cataplexy (sudden loss of muscle tone triggered by emotions) and other manifestations of abnormal Rapid Eye Movement (REM) sleep.

Narcolepsy has characteristic biological markers including Human Leukocyte Antigen (HLA) association and dysfunction of hypocretin (also called orexin) neurotransmission. Almost all patients with narcolepsy-cataplexy share a common HLA allele, DQB1*0602 [1] suggesting an autoimmune basis for the disorder. Over 90% of narcolepsy-cataplexy cases are associated with a dramatic decrease in hypocretin-1 (HCRT1) in the cerebrospinal fluid [2].

Hypocretin-1 is a neuropeptide produced by 50,000–70,000 hypocamal neurons in the human brain. The HCRT peptides are derived from a precursor, preprohypocretin, which is cleaved into two homologous peptides HCRT1 and HCRT2 [3]. These act on target sites through two receptors, HCRT receptor-1 and HCRT receptor-2. Deficient HCRT neurotransmission is suffi-
cient to produce narcolepsy, as animal models with dysregulated HCRT transmission exhibit a narcolepsy-like phenotype [4,5]. Of notable interest is a transgenic mouse model where the HCRT promoter drives a form of ataxin-3 containing a large polyglutamine repeat, resulting in HCRT cell death and a narcolepsy-like phenotype at 2–3 weeks of age [6].

Human neuropathological studies have extended on these results. In situ hybridization (ISH) studies have shown disappearance of HCRT mRNA in the perifornical area of narcoleptic brains. Furthermore, the concentrations of HCRT1 and HCRT2 in the cortex and pons, two areas with HCRT projections, are dramatically decreased [7]. Immunohistochemical studies also revealed more than 90% decrease in HCRT cell counts in the hypothalamus of narcoleptic subjects [8]. The loss of HCRT signal is most likely not a simple failure in producing hypocretin peptides alone. Hypocretin cells contain prodynorphin (PDYN) and neuronal pentraxin II (NPTX2; Neuronal Activity-Regulated Pentraxin) [9,10], and studies have shown that these two proteins are missing in the perifornical area, but not in other regions, of narcoleptic brains [11,12]. This could imply that HCRT producing cells are quiescent and do not produce HCRT, PDYN and NPTX2 or, more likely, that these cells are missing entirely. Some authors have noted residual gliosis in the perifornical region [13], which combined with the strong HLA association, favors the hypothesis of autoimmune mediated destruction of HCRT neurons. However, most attempts to prove the autoimmune hypothesis, for example through the detection of HCRT-cell specific autoantibodies, have been unsuccessful [14].

A deeper understanding of HCRT cell physiology is required, including the identification of genes and proteins that may be the target of an autoimmune attack or may modulate hypocretin expression/metabolism to make this cell population more susceptible to potential apoptosis. In this study, we have used postmortem human brain samples to compare the transcriptome of narcoleptic versus control subjects, with confirmation in animal models of narcolepsy. Our primary goal was to identify other genes and associated proteins that may be dysregulated in the posterior hypothalamus of narcoleptic patients, potentially expressed in HCRT neurons, as such genes are likely to be novel narcolepsy susceptibility genes.

Results

Identification of brain region specific transcripts

A total of 11 control and 6 narcolepsy brains were analyzed by microarray (Table 1). To validate our methods, we first compared transcript abundance across brain regions in control samples and identified brain region specific transcripts (Table S1). Of the 7 identified genes with putative preferential expression in the posterior hypothalamus, three are known to be restricted to this region: prepromelanin concentrating hormone, preprohypocretin, and histidine decarboxylase. Likewise, arginine vasopressin and oxytocin are expressed in the anterior hypothalamus. In the locus coeruleus (LC) where 14 genes were found to be region specific, seven, including dopamine-beta-hydroxylase and tyrosine hydroxylase, are known to be expressed specifically in the LC. These results offered a strong validation of sample selection, dissection, array experiment procedure and the statistical analysis methods used in this study. (Table S1)

Identification of transcripts dysregulated in human narcolepsy

In the comparison of narcolepsy vs control posterior hypothalam, a total of 35 downregulated and 11 upregulated genes were identified by analysis of microarray expression data. Of these, only nine genes were confirmed by Quantitative Reverse Transcriptase-Polymerase Chain Reaction (QRT-PCR), all of which were downregulated in narcolepsy. Hypocretin was the most significantly decreased gene by Significance Analysis of Microarray (SAM) [15] ranking, and was second in terms of mean fold change. QRT-PCR confirmation indicated a dramatic 57.4 fold decrease

| ID | cause of death                        | age | sex | PMI (h) | brain pH | 28S/18S | DQB1  | DQB1  |
|----|--------------------------------------|-----|-----|---------|----------|---------|-------|-------|
| C1 | not available                         | 77  | F    | 6.0     | 6.76     | 0.62    | 0302  | 0502  |
| C2 | colon cancer                          | 61  | M    | 48.0    | 6.93     | 0.9     | 0301  | 0301  |
| C5 | prostate cancer                       | 77  | M    | 48.0    | 6.72     | 0.52    | 0201  | 0604  |
| C7 | cerebellum cells cancer meningitis    | 74  | M    | 4.5     | 6.62     | 0.86    | 0301  | 0501  |
| C8 | kidney cancer; metabolic encephalopathy | 75  | F    | 8.0     | 6.74     | 0.77    | 0201  | 0303  |
| C10 | breast cancer                         | 48  | F    | 12.5    | 6.64     | 0.64    | 0503  | 0603  |
| C12 | chronic obstructive pulmonary disease | 82  | M    | 5.0     | 6.78     | 0.88    | 0602  | 0201  |
| C13 | dementia                              | 92  | M    | 7.0     | 6.91     | 0.65    | 0602  | 0602  |
| C14 | heart failure                         | 90  | M    | 6.0     | 6.6      | 0.53    | 0602  | 0602  |
| C15 | heart failure                         | 61  | F    | 8.2     | 6.66     | 0.5     | 0602  | 0301  |
| C16 | not available                         | 77  | M    | 2.0     | 6.63     | 0.54    | 0201  | 0301  |
| N4 | not available                         | na  | F    | na      | 6.63     | 0.95    | 0602  | 0201  |
| N5 | lung cancer                           | 68  | F    | 2.5     | 6.76     | 1.48    | 0602  | 0603  |
| N6 | dementia                              | 58  | F    | 42.0    | 6.55     | 0.87    | 0602  | 0602  |
| N7 | dementia                              | 89  | F    | 20.0    | 6.53     | 1.12    | 0602  | 0201  |
| N8 | not available                         | 60  | M    | 3.5     | 6.7      | 1.21    | 0602  | 0303  |
| N101 | epidural hemorrhage                   | 69  | M    | 10.5    | 6.75     | 0.76    | 0602  | 0502  |

Age and gender distribution, postmortem interval (PMI) and brain pH are not significantly different between narcoleptic subjects (N4–N101) and controls (C1–C16). doi:10.1371/journal.pone.0004254.t001
in preprohypocretin transcript abundance, identifying the known central feature of narcolepsy. The eight other confirmed candidates, leiomodin 1 (LMOD1), cold shock domain protein A (CSDA), G protein-coupled receptor 4 (GPR4), endothelin 1 (EDN1), neuropeptide Y (NPY), growth arrest and DNA-damage-inducible, beta (GADD45B), interleukin 1 receptor-like 1 (IL1RL1) and insulin-like growth factor binding protein 3 (IGFBP3) were downregulated 1.6 to 6.1 fold (Table 2).

Previously known HCRT co-expressed genes were not reported in our analysis either because they were not listed within the top 100 SAM ranked candidates (NPTX2, GAL, galanin), and CART (cocaïne and amphetamine regulated transcript), or expression was classified as “absent” (PDLX) using the microarray suite software (MAS) 5.0 algorithm. The array signals for these genes were decreased (PDLX: 2.4 fold, p = 0.005; NPTX2: 1.3 fold, p = 0.5; GAL: 2.3 fold, p = 0.06; CART: 1.2 fold, p = 0.36) as were the QRT-PCR comparisons (PDLX: 1.8 fold, p = 0.03; NPTX2: 1.6 fold, p = 0.07; GAL: 1.9 fold, p = 0.09; CART: 1.3 fold, p = 0.24). These results further validated our method.

IGFBP3 is co-localized in hypocretin producing cells in mouse brains

In situ hybridization (ISH) of all human putative downregulated genes was performed in mice (C57/BL/6j) (Table 2). Of the 8 candidates confirmed by QRT-PCR as downregulated in narcolepsy brains, only one gene, Insulin-like Growth Factor Binding Protein-3 (Igfbp3), was clearly enriched in the perifornical region where HCRT neurons are located (Table 2, Fig. 1). Among the other genes, many did not appear to be expressed at all in the studied brain regions or the expression was restricted to regions other than the perifornical area (Table 2).

To determine if IGFBP3 is selectively expressed by HCRT neurons in the perifornical area, we used Hert-ataxin-3 hemizygous (HZ) transgenic [6], and Hert knock-out (KO) mice [4]. When we performed ISH with 35S-probes and exposed X-ray film to these slides, signal in the perifornical area was visible after 4 day exposure in the littermate wild type (WT) mice, whereas this region remained invisible after 40 days of exposure in ataxin-3 mice (Fig. 1). Loss of HCRT peptide in ataxin-3 mice could potentially induce secondary changes in gene expression in neighboring cells. Therefore we studied Hert KO mice and found that Igfbp3 expression was similar to that of WT mice (Fig. 1) indicating that loss of HCRT peptide itself did not induce changes in Igfbp3 expression.

To investigate coexpression of HCRT and Igfbp3, we performed Igfbp3 ISH followed by HCRT immunostaining. Colocalization of both signals was seen in a majority (~80%) of hypocretin neurons (Fig. 1). Using this technique, we also surveyed Igfbp3 expression throughout the mouse brain. Significant Igfbp3 expression was restricted to a few brain areas including posterior hypothalamus, cerebellar Purkinje neurons, and a group of cells in the pons localized in or close to the pedunculopontine nucleus. Weaker signal was also seen in the ventromedial hypothalamus, granular and pyramidal layers of the hippocampus, and endopiriform nucleus. Expression in all regions other than posterior hypothalamic area was unaffected in the Hert-ataxin-3 hemizygous (HZ) mice.

IGFBP3 is localized in human hypocretin producing cells

IGFBP3 immunoreactivity was surveyed using various antibodies (after screening selectivity by western blot) through an entire human hypothalamic block. Blood vessels gave strong signal with IGFBP3 antibody, possibly due to residual blood which has very high IGFBP3 content. Other cells, mostly of non-neuronal origin and negative for the NeuN neuronal marker were also stained. Cellular IGFBP3 staining of NeuN positive large neurons was only noted in the perifornical area. IGFBP3 and HCRT double immunostaining detected colocalization in 10-20% of HCRT neurons in the perifornical area (Fig. 2). All neurons showing colocalization of IGFBP3 and HCRT appeared to have a reduced hypocretin signal. Double-stained fibers were also observed in multiple other hypothalamic areas. This colocalization was also seen in some of the few remaining HCRT neurons in brains from narcolepsy patients.

Identification of transcripts downregulated in the hypocretin-ataxin-3 hemizygous transgenic mouse model

Gene expression in perifornical posterior hypothalamus was compared in Hert-ataxin-3 transgenic mice lacking most hypocretin neurons versus wild type animals (two pools of 30 mice). Transcripts with the highest fold changes are reported in Table 3. Genes with known colocalization, such as NPTX2 and dynorphin, were downregulated, validating the model. Other transcripts were also found (Table 3), only one of which, IGFBP3, was downregulated in human narcolepsy hypothalami. For this reason, functional studies involving IGFBP3 and hypocretin were next carried out.

Evaluation of IGFBP3 levels, genotype, and IGFBP3 antibodies in blood and CSF of narcolepsy patients

We investigated whether human narcolepsy is associated with dysregulated IGFBP3 levels in the blood and CSF. Mean levels of IGFBP3 in age and sex matched narcolepsy patients (all with low CSF hypocretin-1) versus controls were 59.4±3.4 ng/ml (n = 11) and 58.0±4.5 (n = 11) ng/ml in serum and 31.2±2.1 ng/ml (n = 27) and 29.0±1.9 ng/ml (n = 35) in CSF respectively. As previously reported [16], levels of IGFBP3 correlated with age but not sex in adults. After controlling for these factors, there was no significant difference in age of onset.

A single base polymorphism in the IGFBP3 promoter region (rs 2854744) strongly correlates with plasma IGFBP3 [17] levels. We tested whether this IGFBP3 polymorphism is associated with human narcolepsy-cataplexy by testing 130 trios using the transmission disequilibrium test (TDT). No difference in transmission was observed (53.5% versus 46.5% for the A allele; Chi sq = 0.77, p = 0.38).

We also explored the possibility that autoantibodies directed against IGFBP3 could be identified. We expressed human IGFBP3 in COS cells, and extracted proteins were western blotted. The resulting membrane was incubated with sera from 22 human narcoleptic and 20 control subjects and revealed with anti human IgG. None of the patients (some with disease onset less than a year prior to blood sampling) or controls had specific reactivity to expressed IGFBP3.

IGFBP3 inhibits hypocretin production in vivo

To investigate whether IGFBP3 regulates hypocretin cell physiology and sleep in vivo, we studied IGFBP3 knockout mice (mIgfbp3 KO; C57BL/6j strain) [18] and two human IGFBP3 transgenic lines (CD-1 strain) [19]: a transgenic mouse strain overexpressing human IGFBP3 (hIGFBP3 transgenic), and a transgenic strain overexpressing a mutated form of hIGFBP3 that does not bind IGF (hmultIGFBP3 transgenic) [20]. These two lines allowed us to distinguish IGF dependent and independent effects of IGFBP3. Quantitative PCR analysis of human IGFBP3 transcripts in the hypothalamus of these models indicated that
Table 2. Human narcolepsy susceptibility candidate genes analyzed by microarray, quantitative RT-PCR and distribution shown by in situ hybridization in mice.

| Affymetrix Probe ID | Gene Name (Gene Symbol) | SAM # | 1/fold change | P (u-test) | Taqman probe ID | judgement 1/fc | P (u-test) | clone ID | Localization in hypocretin area |
|---------------------|-------------------------|-------|---------------|-----------|----------------|----------------|-----------|---------|--------------------------------|
| 206211_at           | selectin E (SELE)       | A 3   | 9.1           | 0.020     | HS00174057_m1  | 3.3            | 0.167     | n/a     | n.t.                                          |
| 207642_at           | hypocretin (HCRT)       | A 1   | 8.7           | 0.001     | HS00533664_m1  | 57.4           | 0.002     | 6974695 | expressed                                   |
| 206346_at           | prolactin receptor (PRLR) | A 2  | 6.1           | 0.013     | HS00168739_m1  | 1.0            | 1.000     | 5055400 | n.c.                                         |
| 203766_s_at         | leiomodin1 (smooth muscle) (LMOD1) | A 4  | 4.5           | 0.013     | HS00201704_m1  | 2.2            | 0.014     | 3810437 | n.e.                                         |
| 238018_at           | hypothetical protein LOC285016 | B 4  | 4.4           | 0.010     | HS01050040_g1  | 1.3            | 0.366     | 5060814 | n.e.                                         |
| 235852_at           | storn 2 (STN2)          | B 7   | 4.0           | 0.010     | HS00263333_m1  | 1.0            | 0.584     | 6475895 | n.c.                                         |
| 201161_s_at         | cold shock domain protein A (CSDA) | A 7  | 3.7           | 0.081     | HS01124963_m1  | validated      | 2.0       | 0.014  | 6581631 | n.c.                                         |
| 231044_at           | RPS-1O65J22.5 (LOC127003) | B 8   | 3.5           | 0.010     | n/a            | n.t.           | 1746040  | n.e.                                           |
| 241682_at           | kelch-like 23 (KLHL23)  | B 77  | 2.68          | 0.9       | HS00826289_m1  | 0.9            | 0.519     | 6817852 | n.e.                                         |
| 222507_s_at         | TMEM9 domain family, member B (TMEM9B) | B 15  | 3.5           | 0.106     | HS00221018_m1  | 0.8            | 0.465     | 5347159 | n.e.                                         |
| 223333_s_at         | angiopeitin-like 4 (ANGPTL4) | B 1  | 3.0           | 0.030     | HS00211522_m1  | 2.6            | 0.201     | 5148869 | n.e.                                         |
| 226814_at           | ADAM metalloepitide with thrombospondin type 1 motif, 9 (ADAMTS9) | B 6  | 3.4           | 0.030     | HS00172025_m1  | 1.7            | 0.302     | 3999494 | n.e.                                         |
| 206236_at           | G protein-coupled receptor 4 (GPR4) | A 5   | 3.4           | 0.001     | HS00270999_s1  | validated      | 1.6       | 0.053  | 4038700 | n.c.                                         |
| 227697_at           | suppressor of cytokine signaling 3 (SOCS3) | B 5  | 3.2           | 0.030     | HS00269575_s1  | 2.1            | 0.121     | 6830087 | n.c.                                         |
| 217414_x_at         | hemoglobin, alpha 2 (HBA2) | A 16  | 3.2           | 0.043     | HS00361191_gl  | 3.0            | 0.068     | 5053650 | n.e.                                         |
| 206512_at           | U2 small nuclear RNA auxiliary factor 1-like 1 (U2AF1L1) | A 100 | 3.2           | 0.282     | HS00428253_g1  | 0.9            | 0.465     | 6822265 | n.c.                                         |
| 230828_at           | GRAM domain containing 2 (GRAMD2/LOC196996) | B 13  | 3.1           | 0.005     | HS01584657_m1  | 1.8            | 0.197     | 890859  | n.e.                                         |
| 241534_at           | ATPase, class I, type 8B, member 1 (ATPB1) | B 91  | 3.1           | 0.149     | HS00194444_m1  | 1.4            | 0.121     | 6308651 | n.e.                                         |
| 228766_at           | CD36 molecule (thrombospondin receptor) (CD36) | B 47  | 3.0           | 0.106     | HS00169627_m1  | 2.1            | 1.000     | 3481681 | n.e.                                         |
| 211699_x_at         | chromosome 11 open reading frame 70 (C11orf70/MGC13040) | B 2   | 3.0           | 0.048     | HS00262911_m1  | 1.6            | 0.366     | 6334520 | n.e.                                         |
| 222802_at           | endothelin 1 (EDN1)     | B 25  | 2.8           | 0.005     | HS00174961_m1  | validated      | 3.0       | 0.010  | 6824438 | n.e.                                         |
| 206001_at           | neuropeptide Y (NPY)   | A 15  | 2.7           | 0.020     | HS00173470_m1  | validated      | 3.5       | 0.359  | 5683102 | n.e.                                         |
| 207896_s_at         | deleted in lung and esophageal cancer 1 (DLEC1) | A 70  | 2.7           | 0.181     | HS00201098_m1  | 1.7            | 0.366     | 872087  | n.c.                                         |
| 231830_x_at         | RAB11 family interacting protein 1 (class I) (RAB11FIP1) | B 19  | 2.7           | 0.048     | HS00368787_m1  | 1.0            | 0.914     | 4945175 | n.e.                                         |
| 209304_x_at         | growth arrest DNA-damage-inducible, beta (GADD45B) | A 21  | 2.6           | 0.005     | HS00169587_m1  | validated      | 2.2       | 0.020  | 5032648 | n.c.                                         |
| 236894_at           | LINE-1 type transposase domain containing 1 (LITD1/ECAT11) | B 11  | 2.6           | 0.034     | HS00219458_m1  | undet          | 962927   | n.e.                                           |
| 236034_at           | microcephaly, primary autosomal recessive 1 (MCPH1) | B 83  | 2.6           | 0.030     | HS00226253_m1  | 0.8            | 0.361     | 6416651 | n.e.                                         |
| 203548_s_at         | lipoprotein lipase (LPL) | A 68  | 2.6           | 0.081     | HS00173425_m1  | 1.1            | 0.584     | 6315514 | n.e.                                         |
| 211775_x_at         | hypothetical MGC13053  | A 41  | 2.6           | 0.043     | HS00706966_s1  | 1.5            | 0.273     | 2937689 | n.c.                                         |
| 206091_at           | matrilin 3 (MATN3)      | A 19  | 2.5           | 0.020     | HS00159081_m1  | 4.7            | 0.302     | 6306229 | n.e.                                         |
IGFBP3 is overexpressed approximately twice in hIGFBP3 transgenic animals and 10 times in hmutIGFBP3 transgenic animals, but is absent in IGFBP3 knockout animals (data not shown), validating these models. Immunocytochemistry did not reveal hypocretin cell defects nor decrease in cell number in Igfbp3 knockout or transgenic animals (data not shown). We next measured hypocretin-1 peptide content and hypothalamic preprohypocretin expression (Fig. 3A–C) and found that preprohypocretin mRNA expression was significantly decreased in hIGFBP3 transgenic animals compared to controls, but unaltered in the other models (including hmutIGFBP3 transgenic and Igfbp3 KO mice)(Fig 3A, C). Hypocretin-1 peptide contents were significantly decreased primarily in hIGFBP3 transgenic and less so in hmutIGFBP3 transgenic mice compared to controls, indicating both IGF dependent and less prominently independent properties of IGFBP3 affect hypocretin production (Fig 3B). Hypocretin cell

IGFBP3 Regulates Hypocretin

**Table 2. cont.**

| Microarray data | Quantitative RT-PCR data | in situ hybridization data |
|-----------------|--------------------------|---------------------------|
| **Affymetrix Probe ID** | **Gene Name (Gene Symbol)** | **SAM Chip #** | **1/ fold change** | **P (u-test)** | **TaQman probe ID** | **P (u-test)** | **Clone ID** | **Localization in hypocretin area** |
| 239151_at | hypothetical protein LOC255326 | B | 41 | 2.5 | 0.015 | n/a | n.t. | n/a | n.t. |
| 212143_s_at | insulin-like growth factor binding protein 3 (IGFBP3) | A | 11 | 2.5 | 0.001 | Hs00181211_m1 | validated | 2.0 | 0.010 | 6437611 | expressed |
| 207526_s_at | interleukin 1 receptor-like 1 (IL1R1) | A | 40 | 2.5 | 0.081 | Hs0045093_m1 | validated | 6.1 | 0.028 | MGC 3007621 | n.e. |
| 218775_s_at | WW, C2 and coiled-coil domain containing 2 (WWC2/BOMB) | A | 65 | 2.5 | 0.181 | Hs00227904_m1 | validated | 1.2 | 0.273 | 5142576 | n.c. |
| 231728_at | calciphosine (CAPS) | B | 65 | 2.5 | 0.268 | Hs00362033_g1 | validated | 1.1 | 1.000 | n.t. | not expr. in rodents |

n.t.; not tested, n.e.; no expression, n.c.; no coexpression in perifornical hypocretin area.

probe set ID is from Affymetrix, QRT-PCR probe ID is from Applied Biosystems, and All clones for in situ hybridization are IMAGE clones except where noted.

Gene expression was compared between 6 narcolepsy and 8 control postmortem posterior hypothalami (A and B Genechip). Narcolepsy candidate genes selected by statistical analysis of microarray data are listed at left, quantitative RT-PCR results are in the center, and results of **in situ** hybridization in mouse hypothalamus are at the right. As only downregulated genes were confirmed by RT-PCR studies, upregulated candidates are not shown. Genes labeled with “validated” were confirmed as narcolepsy related genes. Note that IGFBP3 is the only gene validated by QRT-PCR and showing a hypocretin-like distribution pattern. Some candidates showed uniform signal over the whole brain section, potentially indicating ubiquitous expression, but we regarded the staining as background and classified the gene as not expressed if no anatomical variation in staining level was observed.

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Figure 1. IGFBP3 signals in wild type, ataxin-3 hemizygous, and hypocretin KO mice. The upper panel shows IGFBP3 ISH staining in wild type (A: WT), ataxin-3 hemizygous (B:HZ) and HCRT knockout (C: KO) mice. HCRT staining in neurons (arrowheads) is markedly reduced or absent in the ataxin-3 mouse. The lower panel shows IGFBP3 ISH signal (D: purple; digoxigenin staining with BCIP/NBT), HCRT fluorescence (E: red; Alexa Fluor) immunostaining, and a composite picture (F), indicating that many hypocretin neurons (asterisks) are positive for IGFBP3 in a WT mouse. Scale bar 20 μm.

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counts were nonetheless normal in the hIGFBP3 transgenic model (3269±69 in HZ versus 3230±140 in WT, n = 4 each, age 8 weeks, mean±SEM). As a control, Melanin Concentrating Hormone (MCH) expression was also studied and found to be unaffected by hIGFBP3 overexpression or Igfbp3 absence (Fig. 3D).

IGFBP3 overexpression increases sleep at the end of the active period

To assess whether IGFBP3 overexpression could modulate cell death, hIGFBP3 transgenic mice were crossed with Hcrt-ataxin-3 transgenic mice known to develop hypocretin cell loss at 3–6 weeks of age. Hypocretin cell counts were carried out at 8 weeks of age, and no effect of the hIGFBP3 transgene was noted (595±36 in double HZ (hemizygous) versus 610±67 in Hcrt-ataxin-3/hIGFBP3 HZ/WT, n = 5 each). We next characterized whether overexpression of IGFBP3 and resulting effects on hypocretin transmission affect sleep and wakefulness by conducting sleep studies on hIGFBP3 transgenic mice and their littermates. Although no overall significant difference in sleep amounts were observed (Table S2), we found that these animals exhibit more sleep at the end of the dark period. Sleep deprivation was also performed, and revealed a very similar recovery profile, although more sleep was again observed in transgenic animals prior to light onset (Fig. 4).

IGFBP3 reduces promoter activity of hypocretin in neural cell lines

As IGFBP3 is known to have modulatory effects on transcription, for example through its binding to nur 77 [21], a possible explanation for IGFBP3 effects on sleep could be transcriptional modulation of the preprohypocretin gene. To test this hypothesis, prepro-hypocretin promoter activity was examined in several cell lines of various origins in the presence of a transfected IGFBP3 construct (Fig. 3E). We found that IGFBP3 reduced promoter activity of preprohypocretin in vitro. This effect was only observed in the neural (neuroblastoma-derived) cell line SH-SY5Y, suggesting the need for neural-specific cofactors.

An IGFBP3 polymorphism known to increase IGFBP3 serum levels is associated with reduced CSF hypocretin levels

To test whether IGFBP3 activity regulates hypocretin levels in vivo, DNA samples of 262 Caucasian subjects with normal CSF hypocretin-1 levels (>200 pg/ml), either drawn from healthy control subjects or from subjects with a complaint of sleepiness unexplained by hypocretin deficiency, were typed for rs2854744 (−2202A/C IGFBP3 promoter polymorphism). As previously reported, no relationship between sleepiness/disease status, age or sex and hypocretin levels were found. Interestingly however, a significant dose dependent increase in hypocretin-1 level was found with increasing doses of rs2854744 C, the allele associated with decreased IGFBP3 levels (Fig. 3F). These results suggest that this IGFBP3 polymorphism modulates hypocretin production in vivo in humans.

Discussion

Gene expression profiling in postmortem human brain samples using microarrays is a difficult and controversial area [22]. To validate our technique, we compared transcript abundance in regions known to contain specific neurotransmitters of importance in sleep regulation. After filtering out genes with inconsistent expression, a combination of a permutation method (SAM) and
descriptive quantitative ranking of fold changes was found to be the most appropriate statistical analysis. The anterior and posterior hypothalami are known to promote sleep and wake respectively. The LC area was selected as a prototypical HCRT receptor-1 bearing adrenergic cell group, while the diagonal band was selected as an HCRT receptor-2 rich area containing cholinergic neurons.

The comparison of regions showed a remarkably high expression of genes characteristic for these regions (Table S1). In the LC, for example, enriched genes included the presynaptically

| Affymetrix Probe ID | Gene name (Gene symbol) | 1/Fold change |
|---------------------|-------------------------|---------------|
| Decreased in Hz     |                         |               |
| 448821_at           | Tyrosinase (Tyr)         | 34.3          |
| 452022_at           | BAALC isoform 1-6-8 (Baalc) | 27.9        |
| 422411_s_at         | Eosinophil-associated ribonuclease 3 (Ear3) | 16.0        |
| 418353_at           | CD5 antigen (CD5)        | 9.2           |
| 416128_at           | Tubulin, alpha 6 (Tuba6) | 9.2           |
| 426003_at           | Neurotrophin-3 receptor non-catalytic isoform 1 (trkB) | 8.6        |
| 451014_at           | Receptor tyrosine kinase-like orphan receptor 1 (Ror1) | 8.0        |
| 450772_at           | Wingless-related MMTV integration site 11 (Wnt11) | 7.5        |
| 416266_at           | Prodynorphin (Pdyn)      | 5.7           |
| 418035_a_at         | DNA primase, p58 subunit (Prim2) | 5.3        |
| 420471_at           | Hypocretin (Hcrt)        | 5.3           |
| 439199_at           | Protein phosphatase 2α, catalytic subunit, alpha isoform (Ppp2ca) | 5.3    |
| 437618_x_at         | G protein-coupled receptor 85 (Gpr85) | 4.9        |
| 421767_at           | Adenosine kinase (Adk)   | 4.6           |
| 450091_at           | Immunoglobulin mu binding protein 2 (Ighmbp2) | 4.0        |
| 427168_a_at         | Collagen type XIV (Col14a1) | 4.0        |
| 437502_x_at         | CD24a antigen (Cd24a)    | 3.7           |
| 432129_a_at         | Paired related homeobox 1 (Prx1) | 3.7        |
| 450315_at           | Pheromone receptor V3R8 (V3R8) | 3.5        |
| 455639_at           | Rap2 interacting protein (Rap2ip) | 3.5        |
| 423062_at           | **Insulin-like growth factor binding protein 3 (Igfbp3)** | 3.2        |
| 435950_at           | Periplakin (Pplp)        | 3.2           |
| 421516_at           | Orphan receptor RTR (Nr6a1) | 3.2        |
| 419628_at           | Ceh-10 homeo domain containing homolog (Chx10) | 3.0        |
| 450533_a_at         | Zinc finger protein regulator of apoptosis and cell cycle arrest (Zac1) | 3.0    |
| 427835_at           | Transcription factor Oct-1 isoform 7 (Pou2f1) | 2.8        |
| 423026_at           | Rad51 homolog c (Rad51c) | 2.8           |
| 425175_at           | Glialcolin (C1ql3)       | 2.8           |
| 425866_at           | ETS-domain transcription factor (Fev) | 2.6        |
| 418494_at           | Early B-cell factor 2 (Ebf2) | 2.6        |
| 426180_a_at         | MSG2 alpha salivary protein (Vcs2) | 2.6        |
| 452380_at           | Ephrin receptor A7 (Epha7) | 2.6        |
| 419221_a_at         | Regulator of G-protein signaling 14 (Rgs14) | 2.5        |
| 449960_at           | Neuronal pentraxin 2 (Nptx2) | 2.5        |
| 421109_at           | Camello-like 2 (Cml2)    | 2.5           |
| 460354_a_at         | Mitochondrial ribosomal protein L13 (L13mt) | 2.5        |
| 427832_at           | Testicular alpha tubulin (Tuba-rs1) | 2.5        |
| Increased in Hz     | Toll-like receptor 4 (Tlr4) | 19.7       |

The perifornical area of 30 wild type and 30 ataxin-3 transgenic mice lacking hypocretin cells were punched (see Fig. S1) and samples pooled to perform microarray experiments. Hcrt-ataxin-3 transgenic mouse dysregulated genes are listed by order of transcript abundance (fold change). Most transcripts were decreased in Hcrt-ataxin-3 mice, some of which are known to be colocalized with hypocretin (NPTX2, dynorphin). Note that IGFBP3, which was identified by expression profiling using human hypothalami, was also decreased in this experiment.

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Table 3. Mouse transcripts most differentially dysregulated in the perifornical area of ataxin-3 transgenic animals versus control mice.
Figure 3. IGFBP3 inhibits HCRT production in vivo and in vitro. (A, B) Hypocretin-1 peptide content is significantly decreased in both hypothalamus and brainstem of IGFBP3 overexpressing transgenic mice (hIGFBP3 TG). In mutant IGFBP3 overexpressing mice (hmutIGFBP3 TG), the hypocretin-1 peptide shows slight but significant decrease only in brainstem, not in hypothalamus (A, B). Hypocretin mRNA is also significantly decreased in IGFBP3 (hIGFBP3 TG) mice but not in hmutIGFBP3 TG mice. (C) MCH mRNA level is not affected in hIGFBP3 TG or hmutIGFBP3 TG TG mice. (D) IGFBP3 expression reduces preprohypocretin promoter activity in the SH-SY5Y neuroblastoma cell line, but not in non-neural cell lines (HeLa, HEK, SF126, Becker). (F). In human subjects, rs2854744, −202 C, a promoter polymorphism allele known to be associated with reduced IGFBP3
neurobiology of these regions. It is notable that our study compared only four regions but was sufficient to effectively identify a large number of region-specific genes.

These promising results led us to compare narcolepsy and control posterior hypothalamic, the primary site of the biochemical defect in narcolepsy (Table 2). In this comparison we used slightly less stringent criteria with inclusion of the top 100 SAM dysregulated genes followed by QRT-PCR validation using the same brain sample sets. No genes were confirmed to be upregulated, whereas 9 of 35 candidates were confirmed as downregulated in the posterior hypothalamus of narcolepsy patients. As expected, preprohypocretin was the top candidate in this analysis, displaying a 9-fold change in expression through the array comparison and a more than 50 fold change when verified by RT-PCR (Table 2). This striking result suggests that if this precise neuroanatomical region had been targeted, it would have been possible to discover the central feature of narcolepsy - HCRT deficiency- without a preconceived hypothesis. A recent microarray analysis in Parkinson's disease also yielded excellent results after careful dissection of the substantia nigra [24]. Our work thus validates the use of postmortem samples for finding the cause of certain neuropsychiatric disorders, a nascent field, provided that careful selection of neuroanatomical regions is performed, as in these studies.

The posterior hypothalamic candidates were also subjected to neuroanatomical screening in mouse brain. The primary goal of this analysis was to identify genes that are coexpressed with HCRT and therefore lost in narcoleptic brains in association with death of these cells. Very few such candidates are currently known, and none is specific for HCRT neurons. Known coexpressed genes in various species include NPTX2, GAL, PD1X, ENTPD3 (extronucleo-side triphosphate diphosphohydrolase 3) and CART. These transcripts were not identified in our human array analysis as they either were expressed in other areas of the brain, leading to a low SAM ranking (NPTX2, GAL, CART) or because the expression was classified as absent in most samples (PD1X, ENTPD3), although the QRT-PCR analysis indicated moderately decreased expression of these genes in narcolepsy (see results).

A combination of QRT-PCR confirmation in human hypothalamus and neuroanatomical screening in the mouse brain identified a single factor, IGFBP3 that is coexpressed with HCRT in both humans and mice and present in only a few other brain areas. Confirmation of the colocalization was performed using multiple techniques in both mouse (Fig. 1) and human (Fig. 2). A combination of ISH and immunostaining showed Igfbp3 expression in a majority (~80%) of HCRT neurons in mice. One of the most striking findings was the observation that Igfbp3 signal decreased dramatically in the perifornical hypocretin cell region of Hct-ataxin-3 transgenic mouse lacking most hypocretin producing cells.

Although the other 7 QRT-PCR confirmed genes in the human study were not found to be expressed in HCRT neurons, additional work will be needed to explore the importance of these genes in the pathophysiology of narcolepsy. In some cases, expression was not detected by in situ hybridization in mice but may still be present at low levels. Further, hypocretin cell loss in narcolepsy might be triggered by yet unknown changes in surrounding cells or structures. These factors would not be identified in animal models with disrupted hypocretin neurotrans-
mission but would be downregulated in human brains. In this respect, *H1R* may be of special interest as we found a 6.1 fold difference by QRT-PCR, and this orphan receptor has a role in immune regulation [25]. The other downregulated genes that were found, or previously known to express elsewhere (eg *NP*, *GPR4*), may reflect physiologically important network remodeling in narcolepsy.

Double immunostaining also revealed additional IGFBP3 immunoreactive, hypocretin negative cells in human hypothalamus (both neurons and glial cells). The IGFBP3 protein is primarily produced and secreted by the liver and is the major carrier of insulin-like growth factors 1 and 2 (*IGF1, IGF2*) in the blood. The resulting IGFBP3-IGF complex is typically taken up by cells through IGF receptor 1 to produce intracellular effects [26]. In this context, uptake of IGFBP3 by brain cells could render them immunoreactive without accompanying gene expression. We found that CSF levels of IGFBP3 were high, suggesting significant translocation from serum into brain extracellular fluid. Other explanations could involve antibody specificity issues and relative differences in abundance between protein and mRNA in different cells. Induction of *IGFBP3* expression can occur in neurons and glial cells under various conditions, for example hypoxia [27], and this might also cause differences between humans and mice.

What function could IGFBP3 have in hypocretin producing cells with regard to narcolepsy? As the protein is co-localized with HCRT, IGFBP3 could have been an autoantigen involved in the hypothesized autoimmune attack directed against the HCRT neurons. We found no evidence for such autoantibodies in human sera and CSF, however. IGFBP3 levels were similar in CSF and serum of narcolepsy versus controls, and it is abundant in various human brain cells, possibly reflecting cellular uptake of circulating IGFBP3. These make it an unlikely candidate in directing an autoimmune process specifically toward HCRT neurons.

Even if not directly involved as an autoantigen in causing narcolepsy, IGFBP3 is still an interesting candidate as it plays key roles in regulating cell proliferation and apoptosis [28]. The interaction of IGFBP3 with IGFs is generally believed to have primarily pro-growth effects by favoring target availability of IGF, but growth-inhibiting effects have also been noted. IGFBP3 is also reported to have proapoptotic properties independent of IGF-binding. For example, IGFBP3 expression in cancer cells and in the circulation is associated with less malignant growth and decreased cancer risk in breast [29], prostate [30] and other cancers [31]. Of note, the proapoptotic effects commence with IGFBP3 translocation into the nucleus and binding of the retinoid-X-receptor-α (RXRα), with subsequent mobilization of the RXRα binding partner Nur77 from the nucleus to mitochondria, an event followed by caspase activation and apoptosis [21]. We found no intranuclear IGFBP3 staining in human hypocretin cells (Fig 2 D,E), suggesting no proapoptotic effects in surviving hypocretin cells.

Recently however, proapoptotic effects of IGFBP3 independent of nuclear translocation and protein secretion have been demonstrated, suggesting additional cytoplasmic pathways also promote apoptosis [30]. It is thus possible that IGFBP3 produced within hypocretin cells would not be bound to IGFs, potentially increasing vulnerability to proapoptotic processes. Indeed, hypocretin neurons in slice cultures are more sensitive than neighboring cells to NMDA receptor-mediated injury [32]. To test the hypothesis that excessive IGFBP3 in hypocretin cells contributes to this cell death, we crossed *Hcrt*-ataxin-3 transgenic mice (animals with targeted cell death 2–4 weeks after birth) with transgenic mice overexpressing human IGFBP3 (*hIGFBP3*), but found no effects on the speed of hypocretin cell death. The lack of effects of IGFBP3 on cell death in this model might be due to the differences in circulating vs hypocretin cell specific IGFBP3 overexpression, or to lack of an IGFBP3 effect in the context of ataxin-induced cell degeneration, but is consistent with the notion that IGFBP3 may be proapoptotic only in specific circumstances, such as cancerous cells.

The results above do not support the involvement of IGFBP3 in causing hypocretin cell death in narcolepsy. We therefore next explored whether IGFBP3 modulates hypocretin transmission, with complementary *in vitro* and *in vivo* functional studies using *hIGFBP3* transgenic mice. We found that increased IGFBP3 decreases both hypocretin mRNA and hypocretin peptide content in hypothalamus and target areas (Fig 3A–C). Although these effects could be indirectly mediated by minor endocrine abnormalities in these animals, for example hyperglycemia [33], the *in vitro* findings that IGFBP3 expression suppresses HCRT promoter activity (Fig 3E) and that the functional IGFBP3 polymorphism rs2854744 is associated with reduced hypocretin transmission, observed as lower levels of Hcrt-1 in human CSF (Fig 3F), makes this hypothesis unlikely. Overexpression of *hmutIGFBP3*, a mutant form that does not bind IGF, also reduces hypocretin peptide content in brainstem but not in hypothalamus suggesting both IGF dependent and independent effects on hypocretin transmission. Hypocretin transmission was normal in *Igfbp3* knockout mice, potentially reflecting functional redundancy among the 7 known IGFBP family members.

Increased IGFBP3 expression (which was stable across the 24 hrs) was shown to have functional effects on sleep, as *hIGFBP3* transgenic mice slept significantly more prior to light onset. It is notable that the effect was primarily observed at the end of the active period. As hypocretin release is highest at this time of the day (equivalent to the evening in humans), higher IGFBP3 levels may affect hypocretin transmission only at times of highest demand, through the reduction of releasable peptide stores in terminals.

IGFBP3 and IGF serum levels are highest around puberty, and drop thereafter [16,34]. It is thus interesting to speculate that increased sleepiness during puberty could coincide with peak IGFBP3 levels. Most work to date has focused on the studies of Growth Hormone, the primary determinant of IGF, while the effects of IGF on sleep seem complex [35,36]. Additional investigations of the chronic effects of IGFs and IGFBP3 on sleep changes around puberty are needed [34]. IGFBP3, together with IGF, is to be added to the growing list of metabolic indicators that have been reported to regulate hypocretin activity.

In summary, this study exemplifies the successful use of human postmortem brain for microarray analysis of human neuropathology. The analysis not only confirmed known genes colocalized with hypocretin cells but also identified a new candidate with functional relevance to hypocretin cell physiology and sleep regulation. This factor, IGFBP3, is only expressed at high levels in a few neuronal cell groups besides hypocretin cells, and regulates hypocretin transcription. We hypothesize that increased IGFBP3 amount in HCRT cells decreases HCRT production and reduces wake under physiological conditions. When expression exceeds a specific threshold however, it may initiate hypocretin cell death and cause narcolepsy.

**Materials and Methods**

**Human studies**

**Human samples.** All blood and DNA samples were of Caucasian origin. Narcolepsy and control brain donors were primarily recruited through the Stanford narcolepsy brain...
donation program and the Stanford Brain Bank. Samples from 9 narcoleptic patients (89% Caucasian) and 14 Caucasian controls were dissected. Six narcolepsy and 11 control samples were analyzed after passing quality control (Table 1). Patients were all HLA DQB1*0602 positive with cataplexy. Sera from 11 narcolepsy and 11 controls, CSF from 27 narcolepsy and 35 controls were also used for IGFBP3 measurements. DNA samples from 130 parent-child trios (proband and parents), and 252 individuals with available CSF hypocretin level values in the control range, were obtained and used. Informed consent was obtained in accordance with Stanford human subjects policy and the principles of the Declaration of Helsinki.

Brain dissection. Four brain regions were dissected: posterior and anterior hypothalamus, LC and diagonal band of Broca. Coronal sections (0.9 mm) of hypothalamus and diagonal band, and transaxial brainstem sections were cut from frozen blocks, mounted, and stored at −80°C. Digital photographs of the blocks were used for orientation and identification of target regions. The location of hypothalamic structures and the diagonal band were determined using human atlas coordinates [37]. The LC was identified by atlas location [38] and coloration.

Posterior hypothalamic samples were collected from the mamillary body (atlas fig.32, optic chiasm +13 mm) to the level where the fornix enters the hypothalamus (fig. 25, optic chiasm +4 mm). Anterior hypothalamic samples were collected from this level (fig. 24, optic chiasm +3 mm) to the optic chiasm (fig. 19, optic chiasm −2 mm). Diagonal band areas closely surrounded the anterior hypothalamus in the same planes. Hypothalamic and diagonal band samples were dissected by scalpel, LC samples were collected with a 1.2 mm Palkovits punch (Stoeling Co., Wood Dale, IL).

RNA isolation and Array Hybridization. Biotinylated cDNA synthesized from total RNA was hybridized to microarrays (HG-U133 A and B, Affymetrix, Santa Clara, CA) according to manufacturer protocols (Genechip manual, Affymetrix). Fluorescent array images were scanned (Affymetrix GeneArray 2500 or GeneChip 3000 scanner) and analyzed with global scaling, adjusting mean target intensity to 500 for all probe sets (Affymetrix MAS 5.0 software).

Quality control and sample comparisons. The pH of each sample was measured (homogenate of a 10× dilution of 0.5 g of temporal cortex or striatum in water). Samples with pH<6.5 were excluded [39]. Integrity of extracted RNA was verified by RNA nano LabChips on a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Samples with a 28S/18S ratio below 0.5 were discarded (median ratio 0.81). Microarray data (Affymetrix GeneChip 3000 scanner) and analyzed with geneNorm analysis [40]. After performing QRT-PCR for selected genes in parallel with β-actin and β2-microglobulin, relative expression quantity was calculated. Genes were considered validated when the mean fold change was more than 1.5 and Mann-Whitney U-test indicated statistical significance (p<0.05). Expression differences for CART [41], GAL [42], POMC [9] and NPY [10] were also verified.

Human brain immunohistochemistry. Six hypothalami (4 control, 2 narcolepsy) were fixed in 4% paraformaldehyde (PFA, pH 7.5), cryoprotected and sectioned coronally to obtain a series of 24 sections (40 μm). The following steps were performed at 4°C interspersed with washes. Sections were (I) treated with 0.3% H2O2, (II) post-fixed with 4% PFA, (III) blocked in 1.5% horse serum, (IV) incubated with mouse anti-HCRT monoclonal antibody (1:250) [43] or a monoclonal anti-NeuN antibody (1:50,000; Millipore, Billerica, MA) (V) incubated with biotinylated horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA), (VI) alkaline phosphatase (AP) conjugated ABC (1:100; Vector), and (VII) VectaRed AP-substrate (1:50; Vector) in 0.1 M Tris-HCl (pH 8.4) until satisfactory staining was obtained. Sections were then (VIII) incubated in 1.5% rabbit serum, (IX) goat anti-IGFBP3 antiserum (1:250; AF675 R&D Systems, Minneapolis, MN), (X) biotinylated rabbit anti-goat IgG (1:2000, Vector), (XI) ABC reagent (1:100; Vector), (XII) biotinyl-tyramide (1:500; PerkinElmer) with 0.03% H2O2, and (XIII) Qdot 525 streptavidin conjugate (1:100;Invitrogen) in borate buffer (pH 8.5). Sections were mounted and analyzed under a fluorescence microscope equipped with a CCD camera: images were digitally merged to visualize the colocalization of signals.

Evaluation of hypocretin and IGFBP3 levels, antibodies and IGFBP3 genotyping. CSF and serum IGFBP3 levels were measured in duplicate using a total ELISA kit (DSL-10-6600; Diagnostic Systems Laboratories, Webster, TX) according to the manufacturer’s protocol. Average intra-assay coefficients of variation were 2.5%. CSF hypocretin-1 levels were measured using a radioimmunoassay as reported previously [2]. We tested the original CSF of 27 narcolepsy and 35 matched controls, and serum from 11 narcolepsy and 11 controls.

Full length IGFBP3 cDNA (EcoR1-Apa1 fragment, clone 5287665, Invitrogen) was subcloned into pCMV-Tag3 and transfected into COS-1 cells (Lipofectamine 2000, Invitrogen). Protein was extracted from cells and culture medium by standard methods (RIPA buffer, and protocol Sigma, St. Louis, MO).
Protein extracts were run on 10% SDS-PAGE gels, transferred onto nitrocellulose, and incubated with anti-human IGFBP3 polyclonal antiserum (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase (HRP-conjugated donkey anti-goat IgG (1:4000) and then detected with Supersignal West Pico chemiluminescence reagent (ThermoFisher, Wyman, MA), revealing the 42 kDa IGFBP3 band. Similar membranes were used to detect anti IGFBP3 antibodies in human sera. Membranes were incubated with serum (1:200) followed by HRP-donkey anti-human antiserum (1:5000), and chemiluminescence detection. Twenty two naroceplasy and 20 control samples were tested.

The single base pair polymorphism in the IGFBP3 promoter (rs2854744, −202 A/C) was genotyped in 130 Caucasian naroceplasy-cataleyx trios and 262 adult Caucasian subjects (54% females; mean age 35.8±0.8 years) using established methods [17].

Mouse studies

**Animals and tissue preparation.** Five different mouse lines were used: hypocretin-ataxin-3 transgenic mice lacking hypocretin neurons (Hct-ataxin-3: C57BL/6J background), preprohypocretin knockout mice (Hkt KO: C57BL/6J background), IGFBP3 knockout mice (migfbp3 KO: C57BL/6J background) (courtesy of Dr. JF Pinter, University of Medicine and Dentistry of New Jersey, Picataway, NJ) [18], transgenic mice overexpressing human IGFBP3 cDNA (hIGFBP3 transgenic: CD1 background), transgenic mice overexpressing the Gly56/Gly80/Gly81 mutated form of human IGFBP3 which lacks IGF binding (hmutIGFBP3 transgenic: CD1 background) (courtesy of Dr. L Murphy, University of Manitoba, Winnipeg, Canada) [19]. The latter two mouse strains allow differentiation between IGF-bound and IGF-independent effects of the protein. Two human IGFBP3 genes were driven by the same mouse phosphoglycerate kinase I promoter, and were used to distinguish the effect of IGF binding. In all transgenic comparisons, age matched mice of the corresponding genetic background, usually littermates, were used at all times. Mice were maintained under controlled temperature (21 ± 1°C) and 12 h:12 h light-dark cycle with free access to food and water. The entire study was approved and conducted in accordance with the guidelines of Stanford's Administrative Panel for Laboratory Animal Care.

For neuroanatomical studies, mice were euthanized (pentobarbital) and perfused transcardially with saline followed by 50 ml 10% formalin (pH 7.4). Brains were fixed in 10% formalin, and equilibrated with 20% sucrose/0.5% formalin. Coronal slices (30 μm) containing the whole hypothalamus were sectioned into a 1:5 series and mounted.

**Mouse microarray experiments.** Groups of 30 wild type and 30 ataxin-3 transgenic mice were used. Periformal hypothalamic 0.5 mm Palkovits punches encompassing the hypocretin field (Fig. S1) were collected at ZT 22. Biotinylated hypothalamic 0.5 mm Palkovits punches encompassing the and 30 ataxin-3 transgenic mice were used. Perifornical polymerases (Promega, Madison, WI) and 35S-UTP (Amersham Biosciences, Picataway, NJ) or digoxigenin-UTP (Roche Diagnostics, Indianapolis, IN) by standard methods.

Probes were diluted in standard hybridization buffer to 3×10^6 counts per 125 μl. Sections were pretreated in citrate buffer (pH 6.0), and hybridized with probe at 54°C, followed by RNase A treatment and stringent washes (2×SSC at 50°C; 0.2×SSC, 55°C; 0.2×SSC, 65°C), dehydrated and exposed to films for 1–40 days.

**In situ hybridization of IGFBP3 and HCRT immunostaining.** Digoxigenin-labeled probe (1:500) was used in hybridizations as described. Sections were treated with 3% sheep serum/0.1% Triton X-100, and incubated overnight with alkaline phosphatase-conjugated sheep anti-digoxigenin antibodies (1:500; Roche). Endogenous alkaline phosphatase was blocked (levamisole) and hybridization was visualized by incubation in 0.3 mg/ml NBT/nitroblue tetrazolium and 0.2 mg/ml BCIP/5-bromo-4-chloro-3-indolylphosphate.

Sections with satisfactory IGFBP3 signal were immunostained with highly specific rabbit anti-HCRT-1 antiserum (1:4000; made with human HCRT-1 as immunogen). Slides were washed and incubated with (I) biotinylated goat anti-rabbit IgG (1:500; Jackson Immunoresearch, West Grove, PA), (II) ABC complex (1:1000; Vector), (III) biotinylated tyramide diluted 1:50 in amplification buffer (Perkin Elmer), (IV) Alexa Fluor-conjugated streptavidin (1:200; Invitrogen).

To stain and count hypocretin cell populations, successive sections encompassing the entire hypocretin field were stained using an anti HCRT-1 antiserum as described above. Cells were counted without corrections and blind of genotype status.

**Hypocretin-1 radioimmunoassay.** Frozen brain tissue of animals sacrificed at Zeitgeber time ZT2–ZT3 were extracted with 1 ml of 0.5 M acetic acid and boiled in water bath for 15 minutes. Samples were cooled on ice and centrifugated at 5000×g for 10 minutes. Protein concentration in the supernatant was measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA). The supernatants were dried overnight at 50°C and reconstituted in RIA buffer for radioimmunoassay using a commercially available 125I RIA kit (Phoenix Pharmaceuticals, Belmont, CA) as described [44]. The hypocretin contents were corrected against protein concentrations.

**Preprohypocretin, murine/human IGFBP3 and MCH mRNA quantification.** Total RNA from mice hypothalamic regions with RNA extraction reagents (Qiagen, Valencia, CA) and synthesized cDNA was subjected to TaqMan real time PCR analysis to measure relative proprohypocretin, murine/human IGFBP3, and MCH expression levels in parallel with β-actin, hypoxanthine-guanine phosphoribosyltransferase (HPRT) and GAPDH as internal controls. HPRT was chosen for data normalization due to its stable expression across the genotypes.

**Mouse sleep recording and analysis.** Nine wild type (WT) and 13 transgenic (TG) mice (age 3–6 months) were implanted under isoflurane anesthesia with telemetry transmitters (ETA-C, 3.9 g weight, Data Science International, St. Paul, MN) capable of acquiring and sending electroencephalograph (EEG), temperature, and movement data. The two EEG electrodes were secured with dental cement at the following coordinates: anterior/posterior from bregma (AP) 1.5 mm, lateral (ML) 1.5 mm and AP −3.5 mm, ML −3 mm. An analgesic (5 mg/kg Carprofen) and antibiotics (5 mg/kg/day enrofloxacin) was given subcutaneously. Mice were allowed to fully recover for a minimum of two weeks before the experiments. Animals were recorded for a 48-hour period, with the first 24 h undisturbed, followed by 6 h wake extension by gentle handling, and 18 h undisturbed recovery. EEG was sampled at 250 Hz, and the other parameters were sampled at 50 Hz using DataQuest A.R.T. 3.1 (Data Science International, St. Paul, MN). Recordings were scored manually in
transfection, cells were washed and lysed with 100 
μL luciferase assay reagents (Promega) and PLATE CHAMELEON multilabel 
sequentially twice using the Dual-Luciferase Reporter assay 
reporter plasmids and internal control plasmids were measured 
lysis buffer (Promega). Activities of two luciferases encoded by 
3.2 kb Hcrt/pGL3), 20 ng Renilla luciferase-encoding internal 
reporter plasmids linked to pRL-TK (Promega) were co-transfected with three types of plasmid mixed in the following 
well in 24-well cell culture plate coated with collagen type I (BD 
GIBCO), at 37°C and 5% CO2.

Reporter plasmids. The pGL3-basic plasmid (Promega) encoding 
the firefly luciferase gene was used for the promoter activity 
assessments with introduced sequence and pRL-TK 
plasmid (Promega) encoding Renilla luciferase was used as internal 
control for transfection efficiency. The plasmid 3.2 kb Hcrt/pGL3 
was constructed by cloning the HCRT promoter sequence at 
−3278/−487 [46] into upstream of the firefly luciferase gene in 
pGL3-Basic plasmid; 5′-ccgctcaggGTTGTCTGGCGCTCAG 
GGTG-3′ [corresponds to the first exon sequence just before the 
translation initiator ATG of human prepro-Hcrt gene, and 5′- 
CGAGCGGTGGATCCAGATGCCTCTCAGGAATAG-3′ (−3278) 
were used.

Transient transfection. Cells were seeded at a 250,000 per 
well in 24-well cell culture plate coated with collagen type I (BD Biosciences, Bedford MA) one day before transfection. Cells 
were co-transfected with three types of plasmid mixed in the following 
amount per well with Fugene 6 Transfection Reagent (Roche): 
200 ng firefly luciferase-encoding reporter plasmid (pGL3-basic or 
3.2 kb Hcrt/pGL3), 20 ng Renilla luciferase-encoding internal 
control reporter plasmid (pRL-TK), and 200 ng expression vector 
(pCMV-Tag3 as mock or IGFBP3/pCMV-Tag3).

Luciferase activity measurements. At 24 h after 
transfection, cells were washed and lysed with 100 μL passive 
lysis buffer (Promega). Activities of two luciferases encoded by 
reporter plasmids and internal control plasmids were measured 
sequentially twice using the Dual-Luciferase Reporter assay reagents 
(Promega) and PLATE CHAMELEON multilabel 
platerader (HIDEX, Finland) according to the manufacturer’s 
protocol. Relative luciferase activity (RLA) was determined by 
FLU value divided by RLU value. All RLA values were further 
standardized by the reference RLA value for pGL3-basic plasmid 
with pCMV-Tag3 vector (mock) as 1.0.

Supporting Information

Table S1 Cross-regional comparison of transcript abundance in 
selected human brain regions. Comparison of microarray 
expression data across 4 brain regions in control subjects The 
listed genes have enriched expression in the indicated region, as 
compared to the other regions. For example, PMCH gene 
expression was increased 73.8 fold in the posterior hypothalamus 
when compared to the anterior hypothalamus and ranked #1 
using the SAM analysis. Similarly, PMCH gene expression in the 
posterior hypothalamus ranked first versus the diagonal band 
sample (3.3 fold change), and the LC (275.1 fold change). Only 
genes enriched in one region versus at least two other regions are 
listed (see methods). Found at: doi:10.1371/journal.pone.0004254.s001 (0.13 MB DOC)

Table S2 Overall sleep parameters in mice overexpressing 
human IGFBP3 (TG) versus wild type littermates (WT) Although 
total wake is decreased in TG mice, the difference is not 
statistically significant. Hour by hour analysis revealed that 
decreased wake occurred prior to light onset (see Fig. 4). Found at: doi:10.1371/journal.pone.0004254.s002 (0.06 MB DOC)

Figures S1 Perifornical hypothalamic region dissected for 
microarray analysis in mice (A) and transcript abundance 
distribution plots correlating abundance in wild type versus 
ataxin-3 transgenic mice (B). [A] Immunocytochemistry of hypocretin in wild type (WT, top) and Hcrt-ataxin-3 transgenic 
mouse (bottom). The area collected using punches is outlined by a 
circle in wild type versus transgenic mice lacking most hypocretin 
cells. Transcript abundance distribution (B) in both genotypes is 
highly correlated, and hypocretin is one of the outliers (circled dot). For list of differentially regulated transcripts, see Table 3. Found at: doi:10.1371/journal.pone.0004254.s003 (1.56 MB TIF)

Figure S2 Locomotion (A) and temperature (B) in wild type 
(WT) versus hIGFBP3 transgenic mice (Tg). Note decreased 
temperature and locomotion at the end of the active period in 
hIGFBP3 transgenic mice, mirroring changes in sleep depicted in 
Fig. 4. Found at: doi:10.1371/journal.pone.0004254.s004 (5.70 MB TIF)

Author Contributions
Conceived and designed the experiments: MH EM. Performed the 
experiments: MH KSE SZ LL SEG KT YH EM. Contributed reagents/materials/analysis 
tools: MH AS PEH JM MY TS YH EM. Wrote the paper: MH KSE SZ 
EM.
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