Editor's Summary

Awake and Refreshed

A spindle prick on the finger, and Princess Aurora couldn't keep her eyes open; one hundred years later, Sleeping Beauty was awakened with a kiss. But persistent daytime sleepiness ––hypersomnolence––is no fairy tale, and neither the cause nor a cure is apparent. Now, Rye *et al.* begin to illuminate, in patients with primary hypersomnias, the neurobiology that underlies sleepiness of unknown etiology.

A disabling condition, primary hypersomnia is characterized by lethargy, trance-like states, and "sleep drunkenness" even after prolonged, deep, nonrestorative sleep. The authors showed that cerebrospinal fluid (CSF) from these hypersomnolent subjects contains a small (500 to 3000 daltons) trypsin-sensitive substance that stimulates the in vitro function of selected γ-aminobutyric acid (GABA) receptors only in the presence of GABA––an inhibitory neurotransmitter that stimulates GABA receptors, quells consciousness, and induces sleep.

GABA receptors are known to bind a class of psychoactive sedating drugs called benzodiazepines (BZDs). Hypersomnolent CSF samples mimicked the effects of BZD on GABA receptors but did not compete with BZD binding in human brain tissue, suggesting that the newly identified substance functions by a distinct mechanism. Furthermore, the BZD receptor antagonist flumazenil reversed hypersomnolent-CSF activation of GABA signaling, even though the drug is known to be a competitive antagonist of BZD and blocks BZD action by binding to the classical BZD-binding domain of GABA receptors. Most importantly, flumazenil restored vigilance in some hypersomnolent subjects. Together, these mechanistic studies pinpoint a potential new neuropharmacological pathway for a 25-year-old drug.

The current study suggests that one of the "spindle pricks" that puts hypersomnolent subjects to sleep is a substance in CSF that augments inhibitory GABA signaling. A deeper understanding of the neurobiology of primary hypersomnia should help scientists discover new "kisses" that restore wakefulness––in fewer than 100 years.

SLEEP

Modulation of Vigilance in the Primary Hypersomnias by Endogenous Enhancement of GABA_A Receptors

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The biology underlying excessive daytime sleepiness (hypersomnolence) is incompletely understood. After excluding known causes of sleepiness in 32 hypersomnolent patients, we showed that, in the presence of 10 μM γ-aminobutyric acid (GABA), cerebrospinal fluid (CSF) from these subjects stimulated GABA_A receptor function in vitro by $84.0 \pm 40.7\%$ (SD) relative to the $35.8 \pm 7.5\%$ (SD) stimulation obtained with CSF from control subjects (Student's t test, $t = 6.47$, $P < 0.0001$); CSF alone had no effect on GABA_A signaling. The bioactive CSF component had a mass of 500 to 3000 daltons and was neutralized by trypsin. Enhancement was greater for α2 subunit– versus α1 subunit–containing GABA_A receptors and negligible for α4 subunit–containing ones. CSF samples from hypersomnolent patients also modestly enhanced benzodiazepine (BZD)–insensitive GABA_A receptors and did not competitively displace BZDs from human brain tissue. Flumazenil—a drug that is generally believed to antagonize the sedative-hypnotic actions of BZDs only at the classical BZD-binding domain in GABA_A receptors and to lack intrinsic activity—nevertheless reversed enhancement of GABA_A signaling by hypersomnolent CSF in vitro. Furthermore, flumazenil normalized vigilance in seven hypersomnolent patients. We conclude that a naturally occurring substance in CSF augments inhibitory GABA signaling, thus revealing a new pathophysiology associated with excessive daytime sleepiness.

INTRODUCTION

Consciousness states, including the extremes of sleep and wake, are orchestrated by distributed neural networks and a diverse set of signaling molecules (1, 2). Principal among these molecules is γ-aminobutyric acid (GABA), which affects both tonic (slow) and phasic (fast) inhibition by binding to and stimulating GABA_A receptors (GABA_ARs). Because of their ubiquitous presence throughout the central nervous system (CNS), these receptors can inactivate widespread regions of the brain and thereby dampen consciousness (3) and regulate sleep (4). Therefore, drugs designed to enhance GABA_AR function have a long history of clinical use for anesthesia and promoting sleep (5, 6).

Discovery of the GABA_ARs and synthetic modulators has fueled speculation about the existence of endogenous ligands that could affect receptor function and contribute to human disease (7, 8). Pharmacological antagonism of GABA_ARs—for example, with the drug flumazenil—improves vigilance in some patients with hepatic encephalopathy (an impaired state of consciousness that occurs in the setting of a decline in liver function) (9), sleep deprivation (10–12), and idiopathic recurrent stupor (13); however, these studies lack mechanistic detail, and the conferred benefits are short-lived. Effects of GABA_AR antagonism in healthy controls remain ill defined (8, 14). Hence, flumazenil use remains restricted to reversal of overdose of benzodiazepines (BZDs) (a class of sedative-hypnotic and anti-anxiety drugs) and prolonged recovery from anesthesia.

Several naturally occurring molecules such as the diazepam-binding inhibitor (DBI) (15), oleamides (16), and endozepines (17) interact with GABA_ARs, but their endogenous influences on vigilance and states of consciousness in human health and disease have not been convincingly demonstrated. Lethargy, "trance-like states," and "sleep drunkenness" after prolonged, deep, nonrestorative sleep (18–21) distinguish the primary hypersomnias [for example, narcolepsy without loss of muscle tone (cataplexy) and idiopathic (nonnarcoleptic) hypersomnia] from hypocretin-deficient narcolepsy with cataplexy. The neuropeptide hypocretin is required for normal wakefulness and appetite and is decreased in the cerebrospinal fluid (CSF) of some narcoleptic patients relative to controls. These diminished states of consciousness are often unresponsive to drugs that facilitate wakefulness by increasing the concentrations of excitatory monoamines (for example, dopamine, serotonin, and norepinephrine) in the brain (22–25), which suggested to us a pathogenesis rooted in a naturally occurring excess in GABAergic signaling. Therefore, after comprehensively ruling out factors that might account for impaired vigilance common to the non–hypocretin-deficient primary hypersomnias, we addressed whether endogenous enhancement of GABAergic neurotransmission might be responsible, and if so, what mechanisms might underlie it.

RESULTS

Clinico-epidemiological features of hypersomnolent subjects

We recruited 10 men and 22 women who presented for evaluation of a suspected primary hypersomnia. The mean age of the subjects was 34.3 ± 2.3 (SEM) years; their mean body mass index (BMI) was 24.5 ± 0.8; and they complained of sleepiness [with a mean value on the Epworth sleepiness scale (ESS) of 16.9 ± 0.7] despite 75.4 ± 1.7 hours of reported sleep each week. Sleepiness began at a young age (19.8 ± 1.7 years), was chronic (duration of 17.1 ± 1.9 years), and had previously

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been evaluated in 24 of the 32 subjects. Average length of continuous care in our clinic was 5.5 ± 0.9 years. Eight subjects were lost to follow-up, and three of them experienced spontaneous resolution of their sleepiness. A first-degree family member was documented ($n = 5$) or suspected by history ($n = 5$) to satisfy International Classification of Sleep Disorders version 2 (ICSD-2) criteria for a primary hypersomnia (26). Sleepiness was severe enough that four subjects applied for disability and four others required extended leaves of absence from school or work. Daytime sleepiness was confirmed with the multiple sleep latency (MSL) test (27), and the results conformed to ICSD-2 diagnoses of narcolepsy without cataplexy ($n = 11$) and idiopathic hypersomnia with ($n = 6$) and without ($n = 6$) long sleep time. Nine additional subjects with mean MSLs that exceeded the 8-min threshold required for these diagnoses had severe sleepiness despite habitually long sleep (that is, ≥70 hours per week).

The objective psychomotor vigilance task (PVT) (28) in a subgroup of 16 hypersomnolent subjects free of any traditional wake-promoting pharmacological agents corroborated the presence of hypovigilance. The means (± SEM) of median reaction times (RTs) (342.0 ± 21.1 ms), the number of attentional lapses over 10 min (14.3 ± 5.2), and the slowest 10% of 1/RT (1.89 ± 0.47) approximated performance decrements observed in healthy controls kept awake for 20 to 24 continuous hours (29, 30). Patient- and control-group demographics, clinical features, sleep patterns, MSL testing results, and key CSF assay results by diagnostic category are summarized in tables S1 and S2.

Ruling out of conventional factors as causes of sleepiness

All urine toxicology screens were negative for sedative agents and BZDs (see Supplementary Materials and Methods and Supplementary Results). Metabolic profiling did not reveal a uniform pattern of anemia, iron deficiency, or hypothyroidism. The three primary hypersomnia subgroups [narcolepsy without cataplexy ($n = 11$) and idiopathic hypersomnia with and without long sleep time ($n = 11$)], habitually long sleepers ($n = 9$), and controls ($n = 16$) exhibited no differences in CSF hypocretin [one-way analysis of variance (ANOVA), $F_{4,47} = 0.46$, $P = 0.77$] (table S1). The concentrations of GABA in CSF from a subset of 14 hypersomnolent patients and 13 controls were also not different (one-way ANOVA, $F_{4,26} = 1.48$, $P = 0.24$) (table S2). The amino acid contents of CSF from these 14 hypersomnolent subjects also failed to reveal a common disorder of amino acid metabolism (table S2).

Activation of GABA$_A$Rs by hypersomnolent CSF

Because of previous suggestions that vigilance might be affected by endogenous GABA$_A$R modulators, we used patch clamping to test CSF effects on inhibitory chloride currents evoked by 10 μM GABA on human embryonic kidney (HEK) 293 cells transfected with genes that encoded the human α1, β2, and γ2 subunits of the GABA$_A$R family (Fig. 1A; control, 10 μM GABA alone, trace a); the α1β2γ2-containing version is the most abundant GABA$_A$R subtype in the human brain (31). Application of CSF from a representative hypersomnolent subject (case 2) with habitually long, unrefreshing sleep and sleepiness elicited a negligible chloride current commensurate with what would be expected from normal CSF GABA concentrations (for example, several hundred nanomolar) (Fig. 1A, trace b). However, chloride currents evoked by 10 μM GABA were enhanced by 82.0 ± 4.7% (±SEM; $n = 24$ individual current measurements derived from 10 unique cells) when applied in combination with this CSF, consist-

ent with the presence of a positive allosteric modulator (Fig. 1A, trace c). Such a near doubling of the current amplitude (that is, 100% potentiation) is equivalent to the action of midazolam (13 ng/ml) (a BZD) in the CSF (32). This midazolam concentration typically arises 60 min after an intravenous dose of 0.05 mg/kg (for example, ~4 mg administered to a 70-kg patient) and is associated with a level of sedation in which a patient responds only after their name is called loudly or repeatedly (33, 34). Enhancement by CSF was reversed with flumazenil (Fig. 1A, trace d), a pharmacological agent that is conventionally used as a competitive antagonist at the classical BZD-binding domain of GABA receptors and that lacks activity with respect to currents mediated by GABA alone (Fig. 1A, trace e) (35). Currents evoked by 10 μM GABA in HEK293 cells returned to baseline levels after washout of pharmacological agents that had been co-applied (Fig. 1A, trace f).

The magnitudes of enhancement of GABA by CSFs differed among the four hypersomnolent groups and controls [one-way ANOVA with post hoc pairwise group differences (Tukey's studentized range, $F_{4,47} = 11.22$, $P = 0.0002$)] and were significantly greater in the narcolepsy without cataplexy group ($t = 4.32$, $P = 0.0046$) and in the habitually long sleepers ($t = 4.24$, $P = 0.0054$) versus controls (Fig. 2). The percentages of individual enhancement for CSF samples from the 32 patients and from a subgroup of 28 who were not ingesting rapid eye movement (REM) sleep–suppressant medications at the time of their polysomnographic evaluation did not correlate with any metric of sleep [for example, self-reported hours of sleep, polysomnographically defined sleep efficiency, total sleep time (TST), or REM sleep amount as a percentage of TST]; metric of sleepiness (for example, ESS and MSL); or clinical (age of onset, disease duration, family history, and comorbid restless legs syndrome/periodic leg movements of sleep), biological (CSF hypocretin or GABA concentrations), or demographic (age or gender) features. Potential time-of-day GABA potentiation effects were evaluated by regressing time of day of each lumbar puncture expressed ordinally (that is, ranked) (beginning at 10:00 and ending at 16:30) on the relative percentage of enhancement measured in each patient's CSF. The resulting regression was not statistically significant for time of day (β = 0.00243, $P = 0.7725$).

Specificity of action of affected CSF at GABA$_A$R subunits

To provide additional mechanistic insights into the actions of CSF from hypersomnolent patients on GABA$_A$Rs, we performed voltage-clamp electrophysiological recordings of HEK cells that expressed recombinant human GABA$_A$Rs with different subunit compositions along with classical ligand-binding assays. Querying several different receptor constructs called for relatively large volumes of CSF (for example, 6 to 8 ml versus 1 ml). Given this requirement and the commonality of GABAergic bioactivity observed among hypersomnolent patient CSFs, samples were pooled from four affected subjects (three habitually long sleepers and one idiopathic hypersomnolent with long sleep) and two controls referred for evaluation of suspected narcolepsy with cataplexy, but whose normal subjective (average ESS of 4) and objective (average MSL of 12 min; PVT median RT of 226.3 ms with no lapses in attention) vigilance measures, normal CSF hypocretin levels (average hypocretin level of 330.4 pg/ml), and clinical histories were both consistent with a diagnosis of basilar migraine. The magnitude of enhancement of peak GABA currents at our standard α1β2γ2s receptors by pooled hypersomnolent CSF was roughly one-half of that at α2β2γ2s receptors (71.4 ± 36.4% versus 176.4 ± 41.3%; $t = 4.88$;

$P = 0.0005$, Student's t test), whereas at $\alpha4\beta2\gamma2s$ receptors, responses were undetectable ($-0.20 \pm 14.5\%$; $t = 4.91$; $P = 0.0014$) (Fig. 1B). A second sample of this pooled affected CSF enhanced standard $\alpha1\beta2\gamma2s$ receptors (Fig. 1C) to a significantly greater degree than did pooled control CSF (Fig. 1E) ($186.1 \pm 34.9\%$ versus $31.1 \pm 2.5\%$; $t = 8.84$; $P = 0.0029$, Student's t test). This pooled affected CSF enhancement of chloride currents evoked by co-application of 10 µM GABA was nearly completely (that is, $90 \pm 2.5\%$) reversed with flumazenil (Fig. 1C).

At BZD-insensitive $\alpha1(H102R)\beta2\gamma2s$ receptors, enhancement by pooled affected CSF was partially retained ($72.1 \pm 12.2\%$ versus $186.1 \pm 34.9\%$; $t = 6.89$; $P = 0.0002$, Student's t test) (Fig. 1D), and the percent reversal with flumazenil was also less ($27.84 \pm 5.8\%$ versus $90 \pm 2.5\%$; $t = 8.96$; $P < 0.001$, Student's t test). Although pooled CSF from the two control subjects enhanced current responses to GABA at standard $\alpha1\beta2\gamma2s$ receptors (Fig. 1E) that was slightly greater than at BZD-insensitive receptors (Fig. 1F) ($31.1 \pm 2.5\%$ versus $22.9 \pm 4.0\%$; $t = 3.45$;
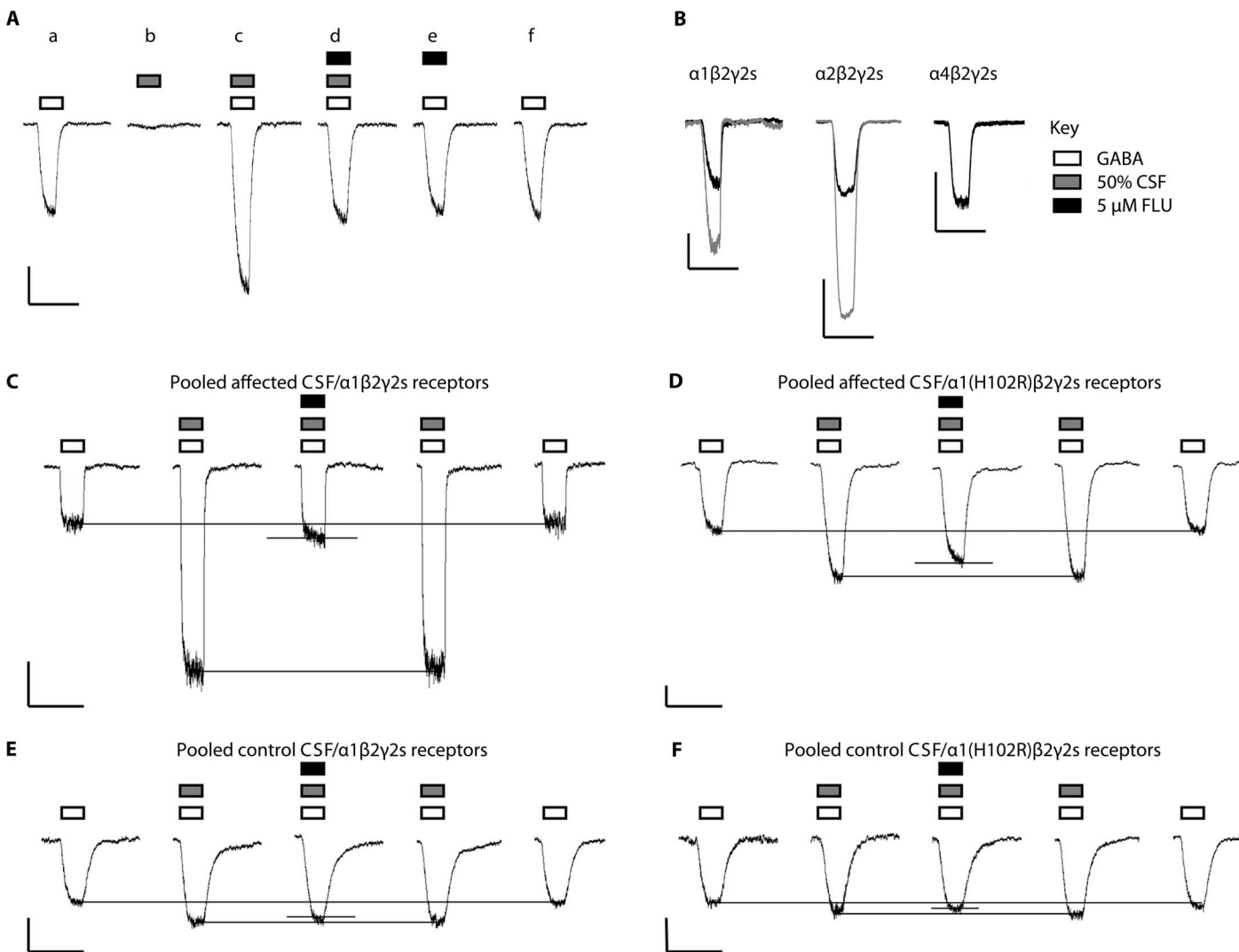
Fig. 1. Effect of hypersomnolent and control CSF on GABA$_A$R-mediated chloride currents in vitro. Chloride current traces were captured by patch-clamping HEK293 cells that expressed recombinant pentameric human GABA$_A$Rs with various subunit compositions. Cells were superfused with 10 µM GABA (white bars), affected or control CSF diluted 1:1 with artificial CSF (gray bars), 5 µM of the $\alpha1$ GABA$_A$R antagonist flumazenil (black bars), or a combination of these. (**A**) GABA alone (trace a); CSF from subject 2 (see Table 1) alone (trace b) or in combination with GABA (trace c) and GABA plus flumazenil (trace d); flumazenil with GABA (trace e); and after drug washout (trace f). Vertical (current) calibration bars denote 200 pA. (**B**) Hypersomnolent CSF effects on GABA$_A$Rs with different α subunit compositions. Currents observed with GABA alone (black traces) or in combination with a pooled sample of hypersomnolent CSFs (gray traces) at $\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2s$, and $\alpha4\beta2\gamma2s$

receptors. Vertical calibration bars denote 40, 100, and 45 pA, respectively. (**C** and **D**) The allosteric enhancement by pooled affected CSFs from four hypersomnolent patients was greater at wild-type $\alpha1\beta2\gamma2$ (C) than at BZD-insensitive $\alpha1(H102R)\beta2\gamma2s$ (D) GABA$_A$Rs. The magnitude of reversal with flumazenil (FLU) was also greater at wild-type $\alpha1\beta2\gamma2$ than at $\alpha1(H102R)\beta2\gamma2s$ GABA$_A$Rs [that is, comparing the magnitude of change between the third and fourth traces in (C) versus (D)]. (**E** and **F**) Pooled control CSFs from two patients with basilar migraine and normal subjective and objective measures of vigilance modestly enhanced wild-type $\alpha1\beta2\gamma2s$ (E) and BZD-insensitive $\alpha1(H102R)\beta2\gamma2s$ (F) GABA$_A$Rs, but the reversibility with flumazenil was less than that observed with pooled affected CSF and not different between receptor types. See the text for details. Vertical (current) calibration bars in (C) to (F) equal 100 pA. Horizontal calibration bars in (A) to (F) equal 5 s.
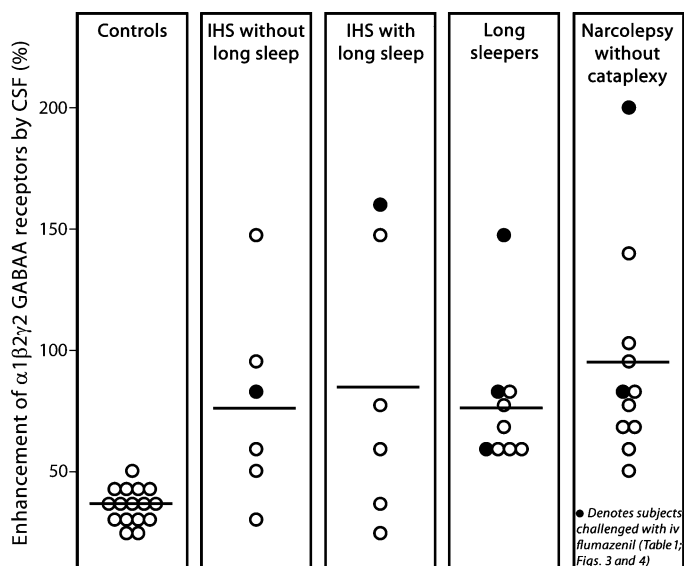
Fig. 2. Modulation of α1β2γ2s GABA$_A$Rs by CSF of control subjects (n = 16) and hypersomnolent subjects (n = 32). Magnitudes of enhancement of GABA-evoked chloride currents [as demonstrated in Fig. 1 (for example, compare trace c versus trace a in Fig. 1A)] determined for CSF samples from controls or hypersomnolent subjects. One-way ANOVA revealed statistically significant differences among groups ($F_{4,47}$ = 11.22, P = 0.0002) with pairwise group differences (Tukey's studentized range), demonstrating statistically greater values for long sleepers (group 4) (P = 0.0046) and narcolepsy without cataplexy subjects (group 5) (P = 0.0054) versus control subjects (group 1). Filled circles represent seven subjects who were studied in greater detail relative to ones represented by open circles (see Table 1) inclusive of an intravenous (iv) flumazenil challenge (see Figs. 3 and 4). IHS, idiopathic hypersomnia.

P = 0.014, Student's t test), the percent reversals with flumazenil were modest at both of these receptor types and indistinguishable from one another (18.3 ± 6.5% versus 26.4 ± 19.5%; t = 0.39; P = 0.71). Conventional ligand-binding assays (fig. S1) of individual CSFs from four unique hypersomnolent patients confirmed that classical BZDs and BZD-like compounds were not responsible for the observed enhancement of GABA-mediated currents.

Stability and reproducibility of GABA$_A$R enhancement by CSF from hypersomnolent patients

To control for potential effects of psychoactive medications and any differences that CSF procurement and handling might have had on measurements of GABA$_A$R function, we performed assays on samples from affected patients and control subjects free of wake-promoting and sedative-hypnotic agents that were collected and stored over comparable time frames [January 2001 to August 2010 (affected) versus April 1998 to December 2009 (controls)]. We controlled for any delays in aliquoting, freezing, or physiological assessments by demonstrating that GABA$_A$R potentiation elicited by four CSF samples from hypersomnolent patients measured immediately after thawing was comparable to values obtained after the same CSF samples remained at room temperature for 72 hours after thawing (76.4 ± 8% versus 73.9 ± 6.5%; 84.3 ± 12.9% versus 85.2 ± 5.4%; 64.9 ± 4.3% versus 91.9 ± 3.3%; 84.8 ± 8.6 versus 118.9 ± 11.1%, respectively). To explore intrasubject variability and medication effects, we measured

GABA$_A$R potentiation in three CSF samples collected from a single case at various time points (subject 2 in Table 1). The percent GABA$_A$R potentiations observed in the two samples collected 27 months apart, which were free of psychoactive medications, were indistinguishable from one another (77 ± 5.8% versus 75.6 ± 5.7%) as well as from the potentiation elicited by a CSF sample from the same patient collected when modafinil and dextroamphetamine were being ingested (79.4 ± 3.8%) [one-way ANOVA with post hoc pairwise group differences (Tukey's studentized range), $F_{2,15}$ = 0.11, P = 0.893]. Interassay variability was similarly negligible in this case. Two electrophysiologists determined GABA$_A$R potentiations to be 86.0 ± 5.5% and 84.3 ± 12.9%, respectively, when additional unique pairs of CSF aliquots collected 27 months apart were retested independently.

Characteristics of entity responsible for the bioactivity at GABA$_A$Rs

In a molecular sizing experiment, the 500- to 3000-dalton fractions of two pools of CSF, each derived from seven hypersomnolent patients, enhanced GABA$_A$R chloride currents in excess of the activity retained in the >3-kD fraction [exact Wilcoxon rank sum test, W = 21.0, 76.08 ± 19.26% versus 2.31 ± 1.29%, P = 0.036 (pool A); exact Wilcoxon rank sum test, W = 53.0, 70.83 ± 16.1% versus 2.6 ± 3.55%, P = 0.006 (pool B)]. In a second sizing and then neutralization experiment, enhancement of GABA$_A$R function by the 500- to 3000-dalton fractions of CSF from these same two pools was neutralized by trypsin digestion to a level equivalent to that observed in the pooled CSF from nine nonsleepy controls (Table 2).

Normalization of vigilance with flumazenil

Seven subjects whose ages, gender, and diagnoses were representative of the larger group of 32 underwent further analyses (Table 2). Five were women and two were men, and diagnoses of idiopathic hypersomnia (n = 2), narcolepsy without cataplexy (n = 2), and habitually long sleep (n = 3) were represented (Fig. 2, filled circles). Six subjects (subjects 1 to 6) had persistent, disabling sleepiness that was poorly responsive to conventional psychostimulants alone or in combination with newer wake-promoting agents. One additional subject (subject 7) had two witnessed episodes of Kleine-Levin–like behavioral disinhibition that occurred on a backdrop of lifelong sleep requirements of 84 hours each week, during which an MSL test exhibited the electrodiagnostic features of narcolepsy lacking cataplexy. Average duration of follow-up in our clinic for these seven subjects was 5.7 ± 1.6 years. Hypocretin CSF concentrations varied substantially in two of four subjects (subjects 3 and 7). There was a large range in the magnitudes of enhancement of GABA-evoked currents by their CSF samples [n = 7; mean enhancement of 116.2 ± 53.8% (SD); range, 58.5 to 200%], which distinguished these patients from controls [Student's t test, t = 3.99, n = 16, 35.8 ± 7.5% (SD), P = 0.0069]. The magnitude of enhancement by CSF exceeded that of plasma by 1.5 to 3.5 times in all but subject 6. Quantitative plasma amino acid (table S3) and acylcarnitine profiles were not suggestive of any common disorders of amino acid or fatty acid metabolism. BZDs, their metabolites, and newer sedative-hypnotics selective for α1-containing GABA$_A$Rs were undetectable in plasma by ultrasensitive, high-performance liquid chromatography followed by tandem mass spectrometry (Supplementary Results).

Given the in vitro evidence that CSFs from these seven hypersomnolent patients enhanced GABA$_A$R function by a flumazenil-reversible

Table 1. Demographic, clinical, and biological features of seven subjects who were assessed for reversal of hypersomnolence with flumazenil. Multiple rows of data for individual subjects represent values obtained from separate evaluations. SE, sleep efficiency (%); RDI, respiratory disturbance index; PLMI, periodic leg movement index; MSL, mean sleep latency; SOREMps, sleep-onset REM sleep periods; NA, not available; NP, not performed; IHS, idiopathic hypersomnia; LS, long sleeper; N-C, narcolepsy lacking cataplexy; RLS, restless legs syndrome; OSA, obstructive sleep apnea; PLMD, periodic leg movement disorder.

Sample	Sex	Age	BMI	Age at onset	Weekly sleep (hours)	ESS	TST (min)	SE%	RDI	PLMI	MS (min)	SOREMps	CSF-hypocretin (pg/ml)	GABA receptor enhancement		Primary sleep diagnosis	Additional diagnoses
														CSF	Plasma		
1	F	34	20.9	19	85	20	443	96	7.6	30.6	2.6	0	401	160 ± 9.2	52.5 ± 5	IHS + LS	RLS
							684	91	3.7	0	10	0					
2	F	25	22.6	18	≥70	20	330	91	2.2	0	9.4	3	350	82 ± 4.7	53 ± 13	LS	
							304	71	0	0	13.5	0	407				
3	M	26	18.8	14	90	22	372	82	2.3	9.4	11	3	231	149 ± 20.4	48 ± 13	LS	
							720	98	4.3	0	13.8	1	82				
													154				
4	F	58	27.1	14	76	19	428.5	93	14.3	44.5	NP	NP	359	200 ± 21.7	56 ± 3	N-C + LS	OSA
							375	97	5.5	3.5	9.9	0					PLMD
							NA	NA	1.2	10.3	4.1	2					
5	F	60	19.8	53	60	20	449	88	11.9	0	4.6	1	366	83.3 ± 10.9	60 ± 10	IHS − LS	OSA
							412	92	13.5	0	NP	NP	402				
							NP	NP	NP	NP	5.2	1					
							NP	NP	NP	NP	3.2	1					
6	F	23	19.9	19	94	13	51	95	0.1	0	14.8	0	392	58.5 ± 3.5	57 ± 13	LS	
7	M	21	27.5	8	84	14	463	89	0.8	0	5.3	3	307	80.6 ± 4.0	44.5 ± 1.8	N-C + LS	Kleine-Levin syndrome
							531	88.3	1.6	0	NP	NP	172				

mechanism, and lacking any alternative explanation for their medically refractory hypersomnolence, we reasoned that flumazenil would afford symptomatic relief. We performed a preliminary, single-blind, placebo-controlled, fixed-order challenge with intravenous injections of physiological (0.9%) saline (placebo) followed by low (0.35 to 0.5 mg) and high (1.5 to 2.0 mg) flumazenil doses to test its potential beneficial effects on objective and subjective measures of vigilance. Flumazenil improved psychomotor vigilance (Figs. 3 and 4, A and B) and subjective alertness (Fig. 4C).

Improvements in vigilance sustained in one hypersomnolent subject treated for 4 years with new flumazenil formulations

Significant first-pass metabolism renders ingestion of oral flumazenil impractical (14, 35); therefore, we derived new sublingual and transdermal formulations for chronic and ongoing use in one individual with idiopathic hypersomnia (subject 1 in Table 2). Although her nightly sleep duration remained at 9 to 10 hours, she nearly always awakened refreshed without an alarm, and daytime sleepiness was markedly reduced, with efficacy maintained for 4 years (table S4). To rule out the possibility that this experience reflected a spontaneous remission expected to occur in ~10% of subjects with idiopathic hypersomnia (23, 36, 37), she was reassessed in the sleep laboratory both while taking her regular dose of flumazenil and after it was withdrawn under continuous clinical and electrophysiological monitoring with frequent assessments of vigilance and mood. A diary kept while she adhered to her regular medication regimen (Supplementary Results) revealed an average of 11.1 hours of total sleep for each of the five nights before medication withdrawal. After the fifth night, sublingual flumazenil was administered at 08:50 (12 mg), 12:05 (12 mg), 16:08 (6 mg), and 18:53 (6 mg), and her usual transdermal flumazenil was not applied that evening. Sleep commenced at 22:39 and ended at 11:59 the following day [709.5 min (11.8 hours) of TST]. Despite interruptions for blood draws ($n = 5$) and urination ($n = 1$), sleep efficiency was 88.6%, with 4% stage N1, 47% stage N2, 9% stage N3, and 40% REM sleep. Flumazenil was withheld the following day, and a short latency to sleep onset of 4.5 min was observed at 16:39, when she was asked to lie quietly and try to go to sleep. She slept for 15 min, during which no REM sleep was observed before she was awakened. Two 6-mg sublingual flumazenil doses were then administered. Assessments ($n = 3$) of vigilance over the next 2 hours when compared to the three medication-free hours before flumazenil dosing ($n = 5$) demonstrated subjective (Stanford Sleepiness Scale) and objective (slowest 10% of the reciprocal of RT) improvements in vigilance (exact Wilcoxon rank sum test, $W = 6$ and $W = 21$, respectively, both $P < 0.05$). These improvements were comparable to those noted when she received 2.0 mg of intravenous flumazenil 4 years earlier (Fig. 4). Increased power in the α (8 to 12 Hz) frequency band of the electroencephalogram was observed coincident with improved vigilance and provided further objective evidence of the alerting affects of sublingual flumazenil (fig. S2).

DISCUSSION

We have discovered a naturally occurring constituent in CSF that is unique to patients suffering from nonnarcoleptic primary hypersomnias and that acts as a positive allosteric modulator of synaptic GABA_ARs. These data point to a new shared pathophysiology for a family of sleepiness disorders whose classification has heretofore proved difficult and for which evidence-based standards of care are not well established (22).

There is a long history of searches for endogenous sleep-inducing factors and exploration of the biochemical and neural mechanisms by which they influence natural sleep (38, 39). A variety of hormones (for example, melatonin and vasoactive-intestinal peptide), cytokines (for example, interleukin-1 and tumor necrosis factor), lipids (for example,

prostaglandin D2 and oleamides), peptides (for example, delta sleep–inducing peptide and the DBI), and endozepines have been implicated in the induction or maintenance of sleep, with interest often traceable to their accumulation in the CSF of sleep-deprived animals. Some of these factors produce sleep when exogenously delivered to animals not deprived of sleep. Remarkably, the relevance of these substances to humans and human disease has never been established.

Genuine endogenous effects of delta sleep–inducing peptide (40), DBI (15), oleamides (16), or endozepines (17) on physiological fluctuations in vigilance in humans remain unclear. Although each modulates GABA signaling, these agents are pharmacologically, structurally, and behaviorally distinct from the GABAergic bioactivity we describe here in CSF from hypersomnolent patients. DBI, as its name implies, displaces diazepam from the BZD recognition site on GABA_ARs, and DBI binding is reversible by flumazenil, whereas the bioactivity in hypersomnolent CSF interacts with a flumazenil-sensitive site near to, but distinct from, the traditional BZD-binding domain. The 9000-dalton mass of DBI also exceeds the 3000-dalton upper limit for the presumably peptidergic bioactivity that we describe. Moreover, DBI acts as a negative rather than a positive allosteric modulator and therefore has a postulated role in evoking anxiety and, by inference, heightened arousal, as opposed to somnolence (15).

In contrast to the neutral effect of our hypersomnolent CSF-based activity, oleamide and its derivatives enhance BZD binding in the brain (41), and although they potentiate $\alpha 1\beta 2\gamma 2s$ GABA_AR-mediated chloride currents, this physiology is not reversed with flumazenil (42, 43). These compounds are also small (~300 daltons) fatty acid amides and thus incompatible with our data on sizing and neutralization of the CSF bioactivity with trypsin. Endozepines, a class of small (<500 daltons) nonpeptidergic compounds of unknown chemical structure (13), are present in mammalian brain and CSF, act as positive allosteric modulators at $\alpha 1\beta 2\gamma 2s$ GABA_ARs, and compete for

Table 2. GABA_AR enhancement by the pooled CSFs of hypersomnolent patients is concentrated in a 500- to 3000-dalton fraction and neutralized by trypsin digestion. The 500- to 3000-dalton fractions of pooled CSFs from two nonoverlapping sets of seven hypersomnolent patients (pools A and B) enhanced GABA_AR chloride currents in excess of a similarly derived fraction of CSF pooled from nine nonsleepy controls (pool C) ($t = 4.03$, $P < 0.0014$). Trypsin digestion neutralized the enhancement in pool A ($P < 0.0022$) and pool B ($P < 0.026$) but did not affect pool C's modulation of GABA_ARs. n = number of measurements.

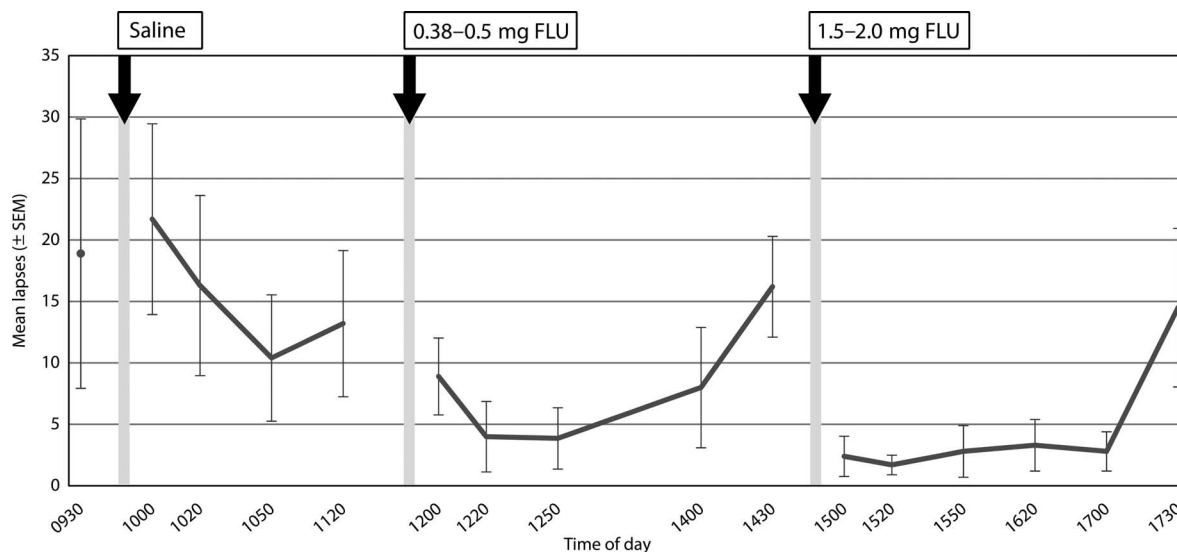| Sample	CSF fraction (500 to 3000 daltons)	
	No trypsin digestion	Trypsin digestion
Pool A (hypersomnolent)	92.85 ± 11.14 ($n = 6$)	20.27 ± 9.09 ($n = 6$)
Pool B (hypersomnolent)	116.10 ± 32.84 ($n = 6$)	33.32 ± 6.55 ($n = 6$)
Pool C (control)	32.63 ± 5.67 ($n = 6$)	30.74 ± 4.76 ($n = 5$)

Fig. 3. Normalization of attentional lapses by intravenous flumazenil in seven hypersomnolent subjects whose CSFs enhanced GABA-mediated currents in excess of controls. Graph depicts means (± SEM) of attentional lapses on the PVT (y axis) by time of day (x axis). Administration of intravenous saline and low (0.38 to 0.50 mg) and high (1.5 to 2.0 mg) doses of flumazenil infusions is indicated by black arrows. Reduction in attentional lapses occurs over the 2 hours after both low and high flumazenil doses. Reemergence of lapses at 2.5 hours approaches baseline (saline) performance levels. The initial number of lapses under high flumazenil conditions was significantly lower than that under saline conditions [4.58 (± 1.7, SEM) versus 15.1 (± 5.3, SEM), Wilcoxon $Z = 70.0$, two-tailed $P = 0.049$]. As a normative comparison, the mean (± SEM) number of lapses in 29 nonsleepy (ESS < 10) outpatient controls was 1.034 ± 0.265.
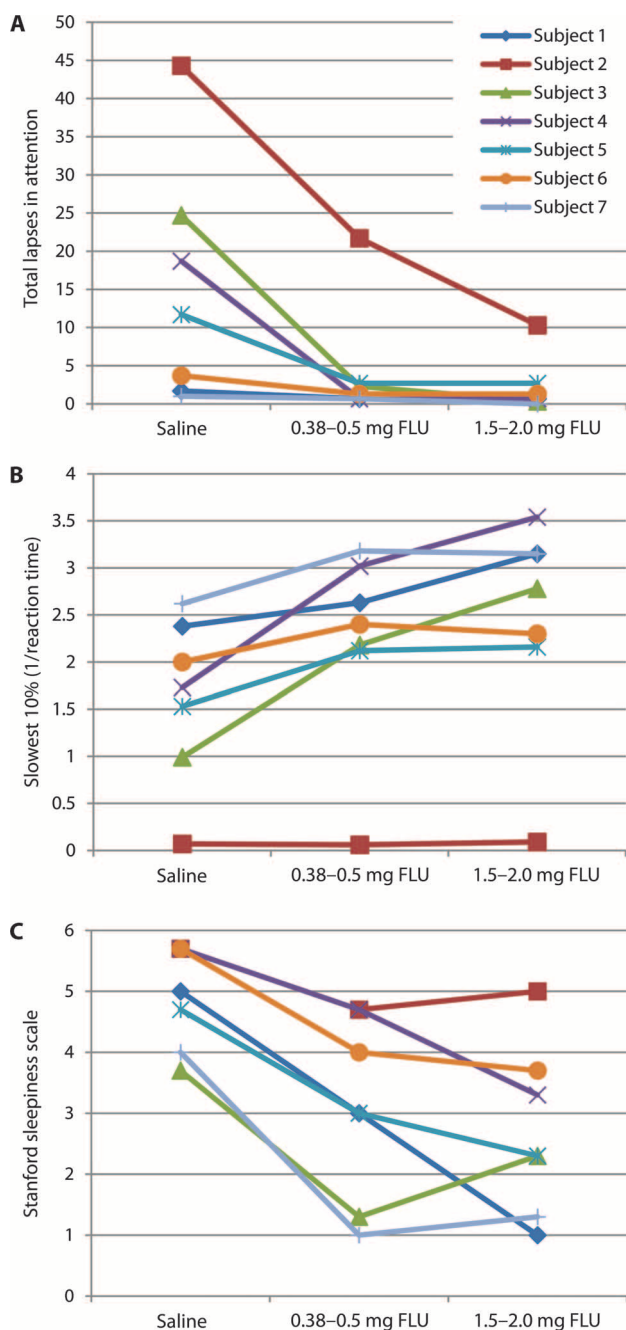
Fig. 4. Individual behavioral responses to flumazenil challenge. (**A** to **C**) Vertical axes represent (A) attentional lapses, (B) reciprocal of the slowest 10% of RTs, or (C) subjective alertness (7, most sleepy; 1, most alert). Horizontal axes represent averages of measurements at 10, 30, and 60 min after intravenous infusion of saline and two flumazenil dosages (low flumazenil and high flumazenil). Repeated-measures ANOVA indicated statistically significant improvements in attentional lapses ($F_{2,12} = 6.97$, $P = 0.0098$), reciprocal of the slowest 10% of RTs ($F_{2,12} = 9.71$, $P = 0.0031$), and subjective alertness ($F_{2,12} = 21.66$, $P < 0.0001$) relative to baseline. Post hoc contrasts revealed significant ($P < 0.01$) improvement in subjective alertness for lower-dose flumazenil versus saline and in attentional lapses and subjective alertness for higher-dose flumazenil when compared to saline. Individual differences in all measures emphasize the importance of evaluating each subject's flumazenil response relative to their own unique neurobehavioral baseline.

binding to the classical BZD domain (44); in contrast, the CSF bioactivity in our hypersomnolent patients has a greater mass, is likely peptidergic, and did not affect such binding. Although elevations in endozepines correlate with episodes of apparent idiopathic recurrent stupor in human subjects (13, 45), the young age, female preponderance, and typically stable, chronic clinical course of our patients distinguish them from the predominantly middle-aged males afflicted by paroxysmal stupor putatively mediated by endozepines. Moreover, lorazepam ingestion can mimic apparent idiopathic recurrent stupor, and its covert administration resulted in erroneous diagnoses from the failure of routine urine radioimmunoassay to detect many BZDs and their metabolites (46, 47). In our study, the highest-sensitivity chromatographic methods combined with tandem mass spectrometric methods did not detect, in plasma from our patients, lorazepam, other BZDs, their metabolites, or any of the newer sedative-hypnotics selective for α1-containing GABA$_A$Rs.

The chemical nature of the CSF constituent underlying our findings and the site within the GABA$_A$R complex at which it acts remain to be determined. Because we tested different receptor constructs with pooled versus individual CSFs, enhancement at both α1- and α2-containing receptors might have resulted from more than one constituent. Because ionotropic GABA receptors exhibit sequence homologies with other members of the nicotinicoid superfamily of ligand-gated ion channels (48), the variability in responsiveness to flumazenil (Fig. 4, A to C) may reflect additional actions of affected CSF samples on nicotinic acetylcholine, glycine, or serotonin [5-hydroxytryptamine type 3 (5-HT$_3$)] receptors. Diversity in the severity of sleepiness and its degree of reversibility with flumazenil (Fig. 4) may imply that vigilance is a complex trait influenced by multiple factors such as hypocretin and the excitatory neurotransmitter histamine, which may be reduced in many patients with pathological sleepiness (49).

Flumazenil

Our findings show that much remains to be learned concerning the molecular, pharmacological, and therapeutic properties of flumazenil beyond its intended use in reversal of BZD overdose and anesthesia. Flumazenil is generally believed to be a pure, competitive antagonist at the classical BZD-binding domain lacking major intrinsic pharmacological or behavioral activity per se (35). However, in more complex systems such as brain slices (50) and whole organisms (8), flumazenil acts as a partial and an inverse agonist at GABA$_A$Rs (8, 14, 35). Emblematic of this partial agonism, intravenous 0.5-mg doses of flumazenil slightly impair simple and complex RTs and subjective vigilance in healthy controls (51, 52). This modest soporific effect contrasts with the improvements in vigilance and subjective alertness that we observed with the same dose range of flumazenil in our hypersomnolent subjects. Illustrative of its inverse agonism and supportive of the existence of endogenous GABA$_A$R ligands, 2.0 mg of flumazenil can increase anxiety in premenstrual women (8, 53, 54).

Alerting effects of flumazenil of the magnitude and duration that we demonstrate have never been reported in healthy controls (8). Flumazenil's behavioral effects derived from more than 25 years of preclinical, postmarketing, and open-label experience are summarized in a classic pharmacological text as "vague" (14). Flumazenil confers some short-term (~1 hour) and very modest (several minutes) dose-dependent reductions in sleep amounts to individuals with a predisposition to sleepiness under a unique experimental paradigm of a

single night's sleep deprivation (10, 11). In the situation of sleepiness that emerges after 41 hours of continuous wake, even 20 mg of intravenous flumazenil does not salvage vigilance (55). In apparent idiopathic recurrent stupor, flumazenil is similarly devoid of substantive benefits to vigilance. The therapeutic benefits were described simply as short-lived (that is, on the order of minutes) (45, 56). Our experience contrasts with these results. We document nearly 2 hours of subjective and objective benefit after administration of 1.5 to 2.0 mg of intravenous flumazenil (Fig. 3), which was sustained in one subject treated for 4 years with sublingual and transdermal formulations (table S4). Because flumazenil's metabolites are devoid of activity and the typical 0.5-mg intravenous dose occupies ≥50% of brain $GABA_A Rs$ with a half-life of about 30 min, the enhancement of vigilance observed beyond 1 hour might appear aberrant (14, 35). However, the temporal course of the observed behavioral benefits is entirely consistent with the known pharmacokinetics and pharmacodynamics of flumazenil in reversing BZD-related sedation: Actions are rapid in onset (within 40 to 90 s), and magnitude and duration of action vary (15 to 140 min) by dose and route of administration (35). The persistence of behavioral benefits beyond 1 hour might alternatively reflect positive feedback within a distributed, $GABA_A R$-sensitive neural network loop that has been posited to govern transitions to and from the extremes of consciousness states (57, 58). Thus, the wake-promoting effects of flumazenil demonstrated here appear new and putatively unique to a proportion of primary hypersomnics and long sleepers whose CSFs share common evidence of an endogenous, positive allosteric ligand of $GABA_A Rs$.

Flumazenil-related improvements in vigilance in this unique patient population appear to be clinically meaningful. Results obtained with comparable objective and subjective assessment tools for intra-individual differences and pharmacological mechanisms that affect vigilance support this conclusion, albeit differences in methodologies and the etiologies of sleepiness may impart some limitations. We used the PVT because it is one of the most widely used objective metrics for assessing sleepiness, given its reliability and convergent, ecological, and theoretical validity (28). Failure to respond rapidly to a visual stimulus that one is expecting on the PVT (that is, a lapse in attention) is a useful primary outcome for alertness because its conceptual and statistical properties are superior to mean and median RTs (59). There is a minimal learning effect with the PVT when administered for 10 min at 2-hour intervals throughout the day. Control subjects who sleep 8 hours per night exhibit an average of 2.67 ± 0.29 SEM lapses in attention with no significant time-of-day effects (60). Extension of the time on task to 20 min at 2-hour intervals negatively affects PVT performance when conducted in the setting of cumulative sleep deprivation (28).

The cumulative time on task during our single-blind, placebo-controlled, fixed-order challenge with saline and low and high doses of flumazenil was much greater than what is traditionally accrued by subjects in typical experimental protocols because PVTs were performed at 20- to 30-min intervals throughout the entire day (a total of 13 to 15 individual PVTs per subject). Although one might have expected to observe performance fatigue in hypersomnolent patients when challenged with such a burden (130 to 150 min of testing over a 480- to 520-min day), improvements in vigilance were observed and persisted to the end of the testing day. As manifest in the change in mean of mean lapses in attention with higher-dose flumazenil [15.1, under baseline saline conditions, to 2.3 ± 1.4 (SEM), an 85% reduction (Figs. 3 and 4)], performance improved to a level approaching that

of nonsleepy controls [1.034 ± 0.265 (SEM); see Fig. 3 legend]. This degree of improvement exceeds that reported for modafinil (12.5 to 10.25 lapses; 18% reduction over a more sensitive 20-min PVT session length) when used for shift-work sleepiness (61)—a condition in which comparatively less is known about the molecular pharmacology underlying sleepiness and modafinil's mechanism of action. Patient-estimated sleepiness levels on the seven-point Stanford Sleepiness Scale were also reduced for the hour after administration of 1.5- to 2.0-mg flumazenil [baseline mean of means of 4.9 decreasing to 2.7; change of −2.2 ± 0.4 (SEM), a 45% reduction; Fig. 4C], and these exceed modafinil's performance on the more sensitive 10-point Karolinska Sleepiness Scale in shift work–related sleepiness (change of −1.5 ± 0.2, a 21% reduction). Modafinil is a common treatment for primary hypersomnia, and although comparable efficacy data are unavailable, it is generally acknowledged to be suboptimal in about one-third of patients (25).

Intravenous flumazenil improved vigilance in our hypersomnolent patients irrespective of the conventional diagnostic category into which patients fell. Because the magnitudes of enhancement of GABA by CSFs also did not fall neatly into current nosological schemes, the three traditional hypersomnolent subgroups that make up the primary hypersomnias might more appropriately be considered as belonging to a single entity whose spectrum extends to habitually long sleepers. This view is supported by some opinions (21, 26) and recent clinical and genetic data (62–64) suggesting that these conditions share much in common and describe a continuum as opposed to being distinct pathological entities.

Limitations
One limitation of the current study is the lack of identification of the molecule(s) responsible for the physiological effects to which we ascribe behavioral relevance. However, the identification of many of what are now accepted to be classical neurotransmitters and neuropeptides began with bioassays that predated delineation of their chemical structures. For GABA, several years passed between Florey's seminal work on its inhibitory effects at the crayfish stretch receptor and delineation of the inhibitor's chemical structure (65), and the identities of endozepines remain unknown nearly 20 years after they were posited as a cause of idiopathic recurrent stupor.

Second, an individual patient's level of $GABA_A R$ potentiation did not correlate with any clinical variable of sleepiness or flumazenil-related variable in vigilance. This tempers the conclusion that CSF GABAergic bioactivity is the sole determinant of sleepiness and is a reminder of the diversity of alternate factors that influence this complex trait (66, 67). Individual differences in CSF concentrations of the compensatory wake-promoting substances hypocretin and histamine reported in the primary hypersomnias and the inadequacy of metrics to capture verbal reports of sleepiness are a few of many factors that could account for our inability to correlate the level of an individual's GABA potentiation with vigilance metrics. Alternatively, an absence of correlation may simply reflect that CSF sampling and vigilance assessments were performed on individual visits that were typically separated by months.

Third, the clinical interventions with flumazenil were single-blinded rather than double-blinded. An A-B-A-B-A-B (repeated withdrawal) design within a single subject lends substantial confidence and provides proof of concept for our conclusion that flumazenil can reverse daytime sleepiness in the primary hypersomnias. The alternate explanation—

that fluctuations in arousal state documented with subjective and several objective measures coincided with treatment condition by chance on three of three occasions—is unlikely.

Finally, the patient sample size was small and could be prone to selection bias; for example, most of the patients were refractory to conventional wake-promoting drugs and presented to a tertiary referral center highly motivated and able to participate in time-intensive, taxing, invasive, and repetitive diagnostic and experimental protocols. Larger, meticulously controlled trials are necessary to establish the extent of flumazenil's efficacy in the broader, general population of primary hypersomnias.

New therapeutic target

The fact that the pathological basis of the primary hypersomnias is unknown has clouded diagnostic acumen and thereby limited epidemiological investigations and the development of standards of care. Given the severity and chronicity of sleepiness, the unmet clinical need is substantial. Traditional psychostimulants and newer wake-promoting agents that enhance vigilance by increasing concentrations of excitatory monoamines (for example, dopamine, serotonin, and norepinephrine) in the brain remain the default mainstays of treatment but are variably effective and fraught with side effects (22–25). Their routine use in clinical practice reinforces heuristic constructs that hold loss of function in the brain's wake-promoting networks as central considerations when faced with patients who manifest impairments in consciousness inclusive of excessive daytime sleepiness (EDS). The scope of drug development efforts for newer therapeutics to promote wake has therefore been trained on neuroexcitatory compounds such as hypocretin receptor agonists and agents that promote downstream monoaminergic systems (histamine, dopamine, and norepinephrine) (68). That impaired consciousness inclusive of somnolence reflects a gain in function in endogenous $GABA_AR$ signaling is a departure from this prevailing view and points to disinhibition at $GABA_ARs$ as an alternative therapeutic target. Because the ionotropic $GABA_ARs$ are one of the most intensely studied targets for pharmacological compounds that affect vigilance (5, 6), the diversity of natural and synthetic GABAergic ligands (69) promises new treatments for the primary hypersomnias.

MATERIALS AND METHODS

Subjects

Patients (22 women and 10 men) who presented between January 2001 and August 2010 for evaluation of a suspected primary hypersomnia were recruited for participation and provided informed consent for lumbar punctures to determine an etiology for their sleepiness. Twenty-eight of the 32 subjects were free of any prescribed wake-promoting agents for at least 5 days before undergoing this procedure. All denied ingestion of over-the-counter or prescribed sedative-hypnotic agents that may have confounded the results. Subjects (11 women and 5 men) requiring lumbar punctures for headache ($n = 6$) or reduction of venous pressure during aortic aneurysmal repair ($n = 1$) or who had conversion disorder ($n = 1$), were familial control for an amyotrophic lateral sclerosis biomarker study ($n = 1$), or had positive syphilis serology ($n = 1$), Tolosa-Hunt syndrome ($n = 1$), seizure ($n = 1$), optic neuritis ($n = 1$), polyneuropathy ($n = 1$), familial spastic paraparesis ($n = 1$), or delirium in the setting of parkinsonism ($n = 1$), but without complaints of EDS, served as controls. Control lumbar punctures were

performed between April 1998 and December 2009. All lumbar punctures were performed between 10:00 and 16:30, and all CSF samples were aliquoted within 1 hour and stored in 1-ml volumes in 1.5-ml Fisherbrand, polypropylene, siliconized, low-retention tubes at −80°C. Conventional analyses of 12 of the 16 control samples revealed normal protein (40.2 ± 4.8 mg/dl) and glucose (62.3 ± 4.1 mg/dl) values, one control sample was contaminated by excess red blood cells (1370 red blood cells/μl), and two control samples exhibited a mild pleocytosis (13 and 21 nucleated cells/μl). All subjects consented to participation in research protocols and use of their clinical records with approval from the Emory Institutional Review Board.

Ascertainment

Each patient had detailed physical and neurological examinations and was evaluated for EDS, habitual sleep patterns, and complaints, and their medical histories were reviewed. Propensity to fall asleep was assessed with the ESS, which rates one's chance of dozing as 0 (none) to 3 (high) in eight soporific situations (70). Objective metrics of vigilance were obtained with the 10-min PVT, which is a widely used tool with excellent psychometric properties for assessing sleepiness (28). The PVT is not confounded by learning effects and is sensitive to experimentally induced, acute (29, 30), and chronic (71) sleep restriction and the beneficial effects of psychostimulants and wake-promoting agents on vigilance (61, 72). We used the PVT-192 monitor (Ambulatory Monitoring Inc.) with instructions to subjects to monitor a rectangular box on the device for appearance of a red-stimulus counter, and then to press a response button with their dominant hand to stop the counter for display of the RT in milliseconds. Interstimulus intervals varied randomly from 2 to 10 s for the 100 stimuli presented over each testing session. Subjects were encouraged to press the button as soon as each stimulus appeared to attain the lowest possible RT, but to avoid anticipatory button presses that resulted in a false start warning on the display. Performance data were downloaded and reduced with the accompanying React software to derive means and medians of RTs, lapses of attention (RT >500 ms), mean slowest 10% of 1/RTs (that is, response times in the lapse domain), and slope of 1/mean RTs (that is, time on task decrement). Although we used all of these dependent metrics in statistical analyses (see below), we focused on lapses in attention and mean slowest 10% of 1/RTs as primary outcomes for alertness because their conceptual and statistical properties are superior to the more widely used mean and median RT metrics (59).

Nocturnal polysomnogram and MSL test interpretations from outside facilities were reviewed ($n = 12$). Initial ($n = 19$) or repeat ($n = 8$) studies were performed as previously described (73). We adhered to ICSD-2 criteria of an MSL of <8 min for any primary hypersomnia to be diagnosed (26). For the five of eight subjects in whom one of two daytime MSLs were >8 min, the single MSL <8 min was used as the default testing session from which a diagnosis was derived. A determination of long sleep (>70 hours per week per ICSD-2 criteria) required a history of >10-hour sleep periods on most nights; repeated tardiness for school, work, or social obligations; and the absence of any polysomnographic features, shift work, and medication or drug use that could have accounted for hypersomnia.

CSF and plasma assays

Hypocretin-1 was measured in crude CSF by radioimmunoassay according to published methods (74). High-performance liquid chromatography with tandem mass spectrometry was used to determine CSF

GABA concentrations at a commercial laboratory (Brains On-Line) (75) and plasma acylcarnitine profiles (76), and free amino acids were quantified by ion exchange chromatography (see Supplementary Materials and Methods for details).

Analytical toxicology of urine and plasma

Urine was collected during MSL tests, and plasma was collected at outpatient visits for toxicological screening of BZDs, BZD-like sedative-hypnotics, opioids, psychostimulants, and cannabinoids (see Supplementary Materials and Methods for details).

In vitro electrophysiological assessments of bioactivity at GABA_ARs

Crude CSFs and plasma were assessed for putative GABAergic activity at recombinant GABA_ARs. Human GABA_AR complementary DNAs (cDNAs) subcloned into the pCIS2 or pcDNA 3.1 expression vectors were transfected into HEK293 cells with standard methods (77, 78), and receptor function was determined with whole-cell patch-clamp electrophysiology (79). Mutations were generated with the QuikChange method (Stratagene). CSF or plasma was diluted 1:1 with extracellular saline such that the final solution contained 10 µM GABA alone or plus 5 µM flumazenil. Cells were voltage-clamped at −60 mV and superfused at 1 ml/min with extracellular saline. Solutions containing GABA, GABA plus CSF, and GABA plus CSF and flumazenil were applied for 2 s with a rapid solution changer (78). The enhancement of GABA_AR function, or percent potentiation, was defined as $100(I_{CSF} - I_{CTRL})/I_{CTRL}$, where I_{CTRL} is the amplitude of the current response to 10 µM GABA alone and I_{CSF} is the response to the 50% CSF solution containing 10 µM GABA. Percent reversal with flumazenil was defined as $R = 100 \times (1 - Pf/Pc)$, where CSF percent enhancement in the absence and presence of flumazenil is denoted by Pc and Pf, respectively. If flumazenil exhibits no effect, $R = 0$, whereas, in the case of complete reversal, $R = 100$. We also tested CSF for (i) bioactivity at BZD-insensitive GABA_ARs [that is, α1 subunit with an arginine for histidine substitution—α1(H102R)] (80, 81); (ii) activity at α2 and α4 subunits; and (iii) competition for traditional BZD-binding sites in human brain homogenates (see Supplementary Materials and Methods for details).

Molecular sizing and neutralization experiments

Pooled CSFs from two unique sets of seven hypersomnic (pools A and B) and nine control (pool C) subjects whose individual CSFs were confirmed individually to exhibit excess or normative in vitro enhancement of GABA_ARs, respectively (all volumes ~20 ml), were passed through a Centricon membrane (3000-dalton cutoff). The >3-kD fractions were reconstituted to their original volumes with standard extracellular saline (see below). The ultrafiltrates (that is, <3-kD fractions) of pools A and B were divided into multiple equal aliquots of 1.5 ml for further analyses and some were stored at −80°C. Some aliquots were digested with sequencing-grade modified porcine trypsin (1 part trypsin to 5 parts protein) for 18 hours at 37°C. Digested and undigested samples were then dialyzed versus deionized water overnight with four changes with a dialysis membrane, allowing passage of molecules of <500 daltons. Samples were then evaporated to dryness with a Savant TurboVap. Lyophylates were reconstituted to 1 ml with standard extracellular saline containing 140 mM NaCl, 3 mM KCl, 6 mM D-glucose, 1.5 mM CaCl$_2$, and 1.0 mM MgCl$_2$; buffered to pH 7.4 with 10 mM Hepes/NaOH; and adjusted to 330 mosmol with sucrose/H$_2$O. All samples were then assayed for bioactivity at GABA_ARs.

In vivo assessment of flumazenil

Twenty milliliters of 0.9% saline or 0.1 mg of flumazenil (Flumazenil Injection, 0.5 mg/5 ml, Sicor Pharmaceuticals), followed by 0.375 to 0.5 mg and then by 1.5 to 2.0 mg of flumazenil, was administered to seven subjects intravenously over 5 min, in six of seven subjects on a single day. The lower range (0.375 to 1.5 mg) applied only to a single patient (subject 6 in Table 1) who complained of very short-lived anxiety during flumazenil administration. Five subjects were free of all wake-promoting agents for ≥3 days before evaluation, whereas two subjects had discontinued treatment the day before evaluation. Reported sleep the night before evaluation was 6.8 ± 0.46 hours. At intervals of 10, 30, 60, 90, 120, and 150 min after infusion, during which subjects were monitored to ensure wakefulness, vigilance was reassessed subjectively with the Stanford Sleepiness Scale, a seven-point self-rating Likert scale sensitive to intraindividual changes in state sleepiness (82). Vigilance was assessed objectively with the PVT. One subject was treated for 4 years with novel sublingual and transdermal flumazenil formulations and effectively underwent an A-B-A-B-A-B withdrawal protocol to titrate dosage with different formulations and to substantiate the persistence of sleepiness and flumazenil efficacy after 4 years of daily medication use (see Results and Supplementary Materials for details).

Statistical analyses

Within- and between-cell differences in peak current responses to different combinations of GABA, CSF, and flumazenil were assessed with parametric (paired and independent sample t tests) or nonparametric (Wilcoxon sign and signed-rank) tests as appropriate. Levels of enhancement of GABA currents by CSF and clinical features were examined for association with Spearman or Pearson correlations for continuous variables and with χ^2 or Wilcoxon rank sum tests for categorical variables. A regression equation was constructed to examine for potential time-of-day effects on enhancement of GABA currents. Ordinal logistic regression analysis was used to develop an equation to determine association of time of day of each lumbar puncture on the relative percentage enhancement of GABA_AR function. Within-group differences between baseline and GABA_AR enhancement after trypsinization measures were assessed with exact Wilcoxon rank sum tests. Between-group measures were analyzed with ANOVAs followed by post hoc within-group comparisons with Tukey's adjustment.

For neurobehavioral measures within patients over time, we used repeated-measures ANOVAs and post hoc within-subject paired comparisons with the Wilcoxon signed-rank test. Objective and subjective assessment of intravenous and sublingual flumazenil in subject 1 was calculated with the exact Wilcoxon rank sum test on the reciprocal of the slowest 10% RTs and Stanford Sleepiness Scale scores, respectively. In instances of unequal variances or sample sizes, appropriate mixed-effects regression models were applied. All statistical analyses were performed with SAS software version 9.2 (SAS Institute).

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/4/161/161ra151/DC1
Materials and Methods
Results
Fig. S1. Radiolabeled ligand-binding assay with crude hypersomnolent CSF.

Fig. S2. EEG spectral power changes in subject 1 associated with sublingual flumazenil treatment.
Table S1. Demographic and clinical features of hypersomnolent patients and controls.
Table S2. Amino acid and GABA contents of CSF.
Table S3. Plasma amino acid contents.
Table S4. Psychometric measures after chronic treatment with sublingual and transdermal flumazenil in subject 1.

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