NEURODEGENERATION

Early-onset sleep alterations found in patients with amyotrophic lateral sclerosis are ameliorated by orexin antagonist in mouse models

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Sleep alterations have been described in several neurodegenerative diseases yet are currently poorly characterized in amyotrophic lateral sclerosis (ALS). This study investigates sleep macroarchitecture and related hypothalamic signaling disruptions in ALS. Using polysomnography, we found that both patients with ALS as well as asymptomatic *C9ORF72* and *SOD1* mutation carriers exhibited increased wakefulness and reduced non–rapid eye movement sleep. Increased wakefulness correlated with diminished cognitive performance in both clinical cohorts. Similar changes in sleep macroarchitecture were observed in three ALS mouse models (*Sod1*^{G86R}, *Fus*$^{\Delta NLS/+}$, and *TDP43*^{Q331K}). A single oral administration of a dual-orexin receptor antagonist or intracerebroventricular delivery of melanin-concentrating hormone (MCH) through an osmotic pump over 15 days partially normalized sleep patterns in mouse models. MCH treatment did not extend the survival of *Sod1*^{G86R} mice but did decrease the loss of lumbar motor neurons. These findings suggest MCH and orexin signaling as potential targets to treat sleep alterations that arise in early stages of the disease.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS), the most common adult-onset motor neuron disorder, is a fatal disease, mostly leading to death through progressive paralysis and respiratory insufficiency within 2 to 3 years after the onset of symptoms (*1*). Depending on population, the median age of onset for sporadic ALS is 55 to 65 years of age (*2*). Sporadic ALS (90 to 95%) accounts for the majority of cases, with the remaining 5 to 10% being hereditary, known as familial ALS (fALS) (*3*). More than 30 different genes have been associated with fALS, with mutations in *C9ORF72*, *SOD1*, *TARDBP*, and *FUS* being the most frequent causes of fALS (*4*–*8*).

ALS is clinically defined as the simultaneous degeneration of lower motor neurons in the brainstem and spinal cord and of upper motor neurons in the motor cortex. In recent years, ALS has been demonstrated to broadly affect multiple brain functions and in particular nonmotor brain regions, including the hypothalamus. Hypothalamic atrophy was observed in patients with ALS and presymptomatic risk gene carriers using magnetic resonance imaging (*9*), a finding confirmed by several other groups (*10*–*12*). Furthermore, there were hypothalamic functional abnormalities in the response to drugs or fasting, in both patients with ALS and mouse models (*13*). Recently, we observed prominent neurodegeneration and aggregates of transactive response DNA binding protein 43 (TDP-43) in the lateral hypothalamic area (LHA) (*14*), consistent with previous studies (*15*). In the LHA, the key neuronal populations, melanin-concentrating hormone (MCH) neurons and orexin/hypocretin (ORX) neurons, are critical in sleep regulation (*16*–*18*), and both neuronal populations appear affected in patients with ALS (*14*, *19*, *20*).

Degeneration of MCH and ORX neurons in ALS raises the possibility that sleep could be altered in ALS. Only a few studies have investigated sleep in ALS. Overall, in studies available to date, more than half of patients with ALS report poor subjective sleep quality, and a few polysomnography studies suggest altered sleep architecture among patients with ALS (*21*–*23*). However, previous studies included advanced patients with ALS who may have developed respiratory insufficiency due to disease progression and are therefore not suitable for determining whether sleep alterations occur independently and possibly before motor symptoms. Thus, it remained unknown whether sleep is primarily affected in ALS (*24*).

In this study, we analyzed sleep structure in two cohorts: one comprising 56 patients with early sporadic ALS and 41 healthy individuals and another including 62 first-grade relatives of fALS cases, which we divided by genotyping into 35 presymptomatic risk gene carriers of ALS mutations and 27 control participants. These gene carriers and control participants were each first-degree relatives of patients with confirmed fALS; genetic testing for assignment was performed on a purely scientific basis after the sleep study. We controlled for possible respiratory impairment during sleep by transcutaneous capnometry because nocturnal hypoventilation alters the sleep architecture (*25*) and can be detected earlier by capnometry than by oxygen saturation measurement. We observed early prominent sleep alterations in both cohorts and replicated a major part of these observations in three mouse models. Inhibition of ORX signaling or MCH intracerebroventricular supplementation was sufficient

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to rescue sleep alterations in ALS mouse models. MCH could also preserve motor neurons in an ALS mouse model (*Sod1*^{G86R}). Overall, our results demonstrate that sleep alterations are already prodromal and severe in ALS and are causally related to abnormalities in MCH and ORX signaling.

RESULTS

Patients in the early stages of ALS without respiratory impairment show altered sleep macroarchitecture

To investigate the prevalence of sleep alterations in patients with early symptomatic ALS, we designed a prospective cohort. Three main exclusion criteria were the presence of (i) sleep apnea, (ii) periodic limb movements in sleep, or (iii) nocturnal hypercapnia (see Fig. 1A). In all, 56 patients and 41 age- and sex-matched controls were screened, and 33 patients with ALS and 32 controls met the inclusion criteria (Fig. 1A and Table 1).

Patients with ALS and controls had similar age, sex distribution, and body mass index. The cohort is described in Table 1. In the ALS group, functional status was recorded with ALSFRS-R (ALS functional rating scale revised) at the time of polysomnography (*26*). The resulting mean of 40.51 (± 0.78) indicates that the group of patients was at the early stages of the disease.

Analysis of questionnaires did not reveal any differences in subjective sleep between patients with ALS and controls (fig. S1, A and B). Polysomnography results were analyzed using YASA deep learning algorithm (*27*), and in addition to the manual evaluation of the recordings based on the evaluation criteria of the American Academy of Sleep Medicine, version 3.0 (*28*), each recording was scored over a time window of 6 hours after turning off the lights. Spectral analysis of the polysomnography recordings showed decreased total power and a decreased sigma band along with an increased beta band in patients with ALS (fig. S2). We used eight randomly chosen hypnograms among all recordings and compared the deep learning sleep analysis with manually analyzed hypnograms (Fig. 1B). We found that both analyses were highly concordant (91.6 ± 2.58%; fig. S3). The evaluation of the hypnograms independent of the 6-hour time window, by considering the total night measurement from the time the light was switched off, showed an increased total sleep time and a longer time in bed in patients with ALS (Fig. 1C). In addition, patients with ALS showed an increased sleep onset latency (Fig. 1D) with higher sleep fragmentation compared with controls. The distribution of sleep stages in patients with ALS was severely altered (fig. S4) with an increased percentage of wake (Fig. 1E) and rapid eye movement (REM) sleep (Fig. 1F) and decreased non-REM (NREM) sleep (Fig. 1G). Decreased NREM sleep was mostly due to a strong decrease in NREM3 (deep sleep) and NREM2. NREM1 (light sleep) was preserved (Fig. 1, H to J). Principal components analysis (PCA) using sleep parameters and age showed a complete segregation of patients with ALS from controls (Fig. 1K). Thus, sleep macroarchitecture is altered in patients with ALS, even without respiratory insufficiency as a confounding factor.

Presymptomatic ALS risk gene carriers show altered sleep macroarchitecture

Our initial cohort analysis revealed sleep alterations in patients with early-stage ALS without hypercapnia, yet it could not confirm whether these alterations occur before motor symptoms appear. Therefore, we included a second prospective cohort study that consisted of presymptomatic ALS risk gene carriers using the same inclusion and exclusion criteria (see Fig. 2A). A total of 62 first-degree relatives of patients with fALS were initially screened, of whom 35 individuals had a positive fALS gene test result. Participants were blinded to their test results but could opt to undergo counseling if they wanted to learn their mutation status in accordance with German legislation after the study visits. Of these, 27 individuals met the above inclusion criteria (*SOD1*, $n = 7$; *C9ORF72*, $n = 12$). The first-degree relatives with a negative genetic test constituted the control group of this cohort (fALS controls: 27 screened, 19 meeting inclusion criteria) (Fig. 2A and Table 2). Like in patients with early ALS, sleep questionnaires did not reveal alterations in subjective sleep quality in ALS risk gene carriers (fig. S5, A and B), but the spectral analysis of the polysomnography recordings showed decreased total power in *C9ORF72* mutation carriers, and *SOD1* mutation carriers showed a similar nonsignificant trend ($P = 0.06$). *SOD1* carriers showed increased beta and gamma bands and decreased alpha and sigma bands, and *C9ORF72* carriers showed decreases in delta, theta, alpha, and sigma bands (fig. S6). Consistent with this, presymptomatic ALS risk gene carriers already exhibited macroarchitectural alterations of their sleep pattern with partly varying results, which were observed depending on the mutation. Total sleep time was decreased in *SOD1* gene carriers but unchanged in *C9ORF72* gene carriers (Fig. 2, B and C, and fig. S7). In *SOD1* and *C9ORF72* mutation carriers, there was a notable increase in the proportion of wake epochs after the onset of sleep (Fig. 2E). Only in *C9ORF72* mutation carriers was longer sleep onset latency observed (Fig. 2D). In addition, in *C9ORF72* mutation carriers, we found an increased percentage of wake phases (Fig. 2E) and of REM sleep (Fig. 2F) as well as decreased NREM sleep (Fig. 2G), caused by decreased NREM2 and NREM3 (Fig. 2, H to J). *SOD1* mutation carriers displayed intermediate sleep changes with increased wake and decreased NREM3 but normal REM sleep. Consistent with this, PCA revealed a more defined segregation of *C9ORF72* mutation carriers from controls than *SOD1* mutation carriers (Fig. 2K). These results show that sleep alterations are present in individuals carrying ALS risk genes, presumably many years before expected motor symptom onset.

Sleep defects correlate with cognitive deficits

Both patients with ALS and presymptomatic risk gene carriers underwent cognitive screening using the Edinburgh Cognitive and Behavioural ALS Screen (ECAS) during their clinical follow-up to determine whether the observed sleep alterations could be related to cognitive performance. To this aim, we performed a correlation analysis between ECAS subscores and sleep parameters in the whole population of affected or at-risk individuals (Fig. 3A). After correction for multiple comparisons, we observed a negative correlation between the percentage of wake and the verbal fluency subscore as well as the total ECAS score (Fig. 3, A to C). This negative correlation was also observed in patients with ALS (fig. S8A), *SOD1* (fig. S8B), or *C9ORF72* (fig. S8C) mutation carriers when analyzed separately. Thus, sleep disturbances are related to cognitive performance in ALS, especially verbal fluency.

Three mouse models of ALS show altered sleep macroarchitecture

The presence of altered sleep macroarchitecture in patients with ALS and presymptomatic risk gene carriers prompted us to investigate sleep patterns in transgenic ALS mouse models. We used three models with different ALS-causing genes and vastly divergent disease progression.

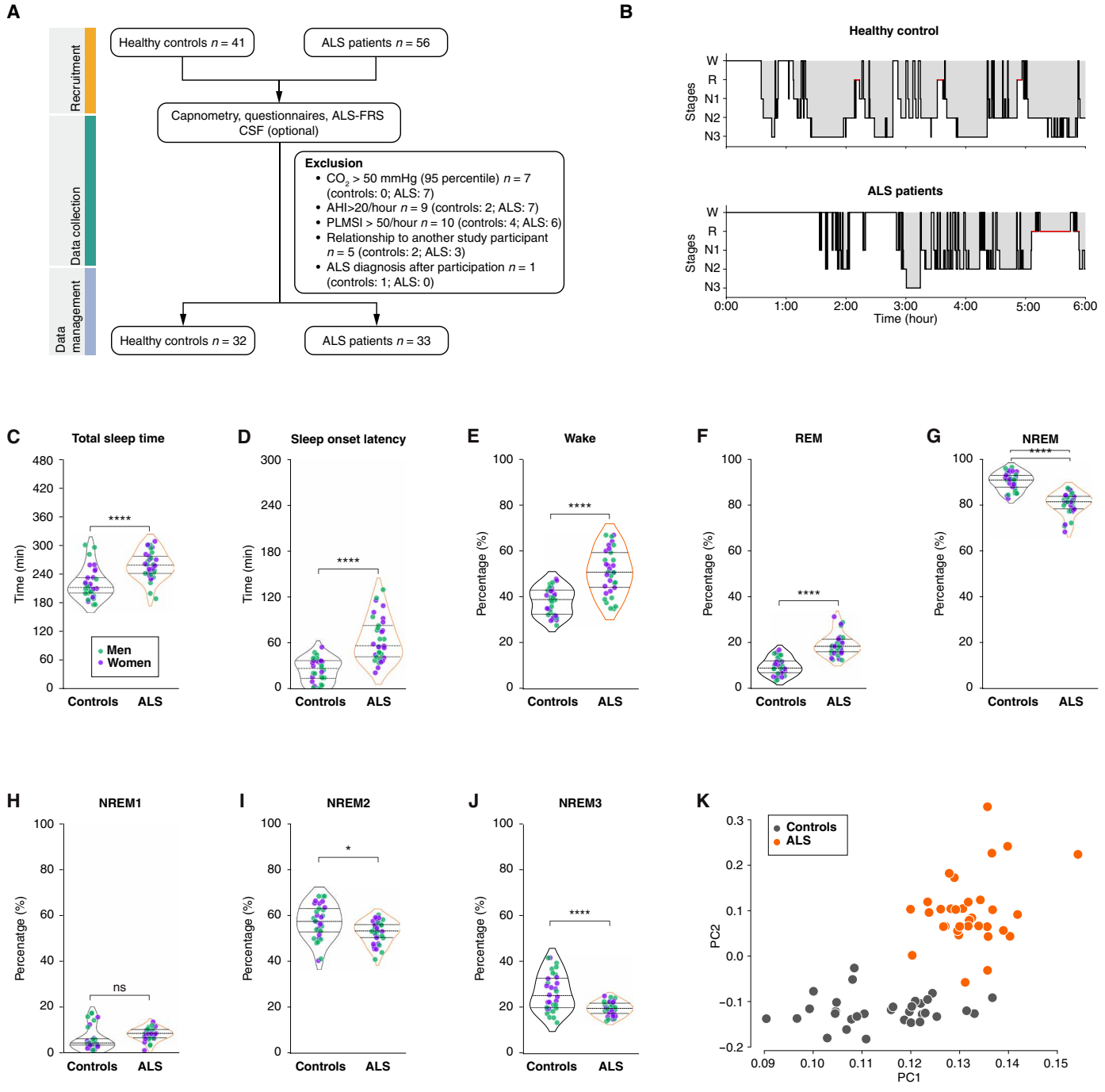

Fig. 1. Sleep alterations in patients with early ALS. (**A**) Flow chart of the study. (**B**) Representative hypnograms of a healthy control and one patient with sporadic ALS. Monitoring was performed over a 6-hour period (1 epoch = 30 s). (**C**) Total sleep time (total duration of REM, NREM1, NREM2, and NREM3 in the sleep period time). (**D**) Sleep onset latency (latency to the first epoch of any sleep). (**E** to **J**) Percentage of wake (E), REM (F), NREM (G), NREM1 (H), NREM2 (I), and NREM3 (J). (**K**) PCA of patients with ALS versus healthy controls using sleep parameters and age. In all panels, men are shown in green and women in purple. Corrected *P* values are shown. Percentages of REM and NREM were calculated over the total sleep time, excluding wake episodes. Percentage of wake was calculated over the whole recording period. ****$P < 0.0001$, independent Student's *t* test with Welch's *t* test correction; sex effect $P = 0.4296$. Data are presented as median and interquartile ranges.

Transgenic expression of G86R mutation in *Sod1* leads to severe, fast-progressing motor symptoms around 90 days old and a death around 120 days (*29*, *30*). Knock-in expression of a C terminally truncated FUS protein or prion-promoter driven expression of Q331K mutant form of TDP-43 leads to mild, late-onset motor neuron disease with a mild symptomatic onset around 4 and 10 months, respectively (*31*–*34*). To characterize sleep patterns, we implanted intracortical electrodes during adulthood and performed electroencephalography (EEG) 5 to 6 days after surgery (Fig. 4A). Quantification of sleep states through manual analysis, or automated sleep analysis using NeuroScore, showed

Table 1. Descriptive statistics of the study population of patients with ALS and healthy controls. SEM, standard error of means; BMI, body mass index; ALSFRS-R, amyotrophic lateral sclerosis functional rating scale revised; ns, $P > 0.05$, nonparametric Kruskal-Wallis test. —, not applicable.

	Patients with ALS	Healthy controls	*P* value
Women (%)	14 (42.4)	10 (31.3)	—
Men (%)	19 (57.6)	22 (68.7)	—
BMI (mean ± SEM)	26.38 (±0.78)	26.63 (±0.77)	0.885 (ns)
Age (mean ± SEM)	58.56 (±1.74)	56.22 (±2.81)	0.652 (ns)
ALSFRS-R (mean ± SEM)	40.51 (±0.78)	—	—

an overall concordance of 94.49 ± 2.27% ($n = 6$; fig. S9), and hypnograms obtained were highly similar in a pilot experiment. We thus relied on automated sleep analysis for further experiments. Hypnograms of *Sod1*^{G86R} mice showed increased wake, decreased NREM, and decreased REM sleep (Fig. 4, B to E) at 75 days of age, before the onset of motor defects. In contrast, there were no alterations in sleep patterns at 3 months of age in *Fus*$^{\Delta NLS/+}$ mice, an asymptomatic age in this strain (Fig. 4, F to I). At the symptomatic age of 10 months old, *Fus*$^{\Delta NLS/+}$ mice showed increased wake, decreased NREM, and decreased REM sleep (Fig. 4, J to M). Spectral analysis further confirmed these results with decreased total power as well as decreased delta and alpha bands along with increased beta and gamma bands, consistent with our recent report (fig. S10) (*35*). In contrast with human patients with ALS, mouse models showed decreased power in the theta band (fig. S10). A similar phenotype was observed in transgenic *TDP43*^{Q331K} mice (figs. S11 and S12). In all three mouse models, the sleep phenotype was consistently observed in both male and female mice. Thus, ALS mouse models recapitulate the early sleep alterations observed in humans, except for the percentage of REM sleep (and theta band power), which is increased in humans and decreased in mice.

Sleep alterations in mouse models are partially rescued by MCH

Because MCH neurons degenerate in ALS (*14*) and MCH promotes sleep, particularly REM sleep (*17*, *36*, *37*), we hypothesized that MCH administration could rescue sleep alterations. To test this, we implanted an intracerebroventricular osmotic pump in parallel with intracortical electrodes for EEG (Fig. 5A). Pumps were filled with either vehicle or MCH (*14*). Vehicle-treated *Sod1*^{G86R} mice showed sleep alterations similar to those of untreated *Sod1*^{G86R} mice. MCH treatment increased REM sleep in *Sod1*^{G86R} mice and decreased wake duration (Fig. 5, B to E). However, MCH infusion exacerbated the NREM sleep deficit (Fig. 5, B to E). Similarly, partial normalizing effects of MCH on sleep architecture were also observed in *Fus*$^{\Delta NLS/+}$ mice (Fig. 5, F to I). Although MCH did not modulate wake duration in wild-type (WT) mice, it did increase REM sleep and decrease NREM sleep (Fig. 5, B to I). Overall, MCH supplementation could ameliorate REM sleep alterations as well as NREM and wake alterations in at least two of the studied ALS mouse models.

Sleep alterations in mouse models are fully rescued by a dual orexin receptor antagonist

ORX and MCH neurons play partially antagonistic roles in sleep/wake regulation. We previously showed that *hcrt* mRNA expression is decreased in *Sod1*^{G86R} mice with the onset of symptoms (*16*), and orexin expression was showed to be lost in postmortem tissue in patients (*20*). We thus hypothesized that ORX neurons could also be involved in the observed sleep alterations, through abnormal orexin signaling changes. We first characterized ORX neuronal counts in presymptomatic *Sod1*^{G86R} (75 days of age) and *Fus*$^{\Delta NLS/+}$ (10 months of age) mice but did not observe a loss of this neuronal population in these mouse models (fig. S13, A to D). Neither ORX nor MCH neurons showed prominent p62 or ubiquitin pathology before end stage in *Sod1*^{G86R} mice (fig. S14, A to D), arguing against the sleep phenotype being caused by loss of ORX neurons or neuronal dysfunction caused by pathological aggregates. Orexin and MCH can induce gene expression changes in neurons. For instance, orexin receptor stimulation leads to increased expression of *Sgk1* and *Junb* (*38*) in hypothalamic neurons, and MCH stimulation increases *Foxo1* and *Sirt1* expression in the hypothalamus (*39*). We observed that *Sgk1* and *Junb* expression were increased in *Sod1*^{G86R} (75 days of age) and *Fus*$^{\Delta NLS/+}$ (4 months of age) mouse hypothalami (fig. S15), and *Foxo1* and *Sirt1* expressions were decreased (fig. S15, A and B). These results suggest increased orexinergic and decreased MCHergic signaling in ALS mouse hypothalami.

Dual orexin receptor antagonists (DORAs), such as the US Food and Drug Administration–approved drug suvorexant, can acutely inhibit orexin signaling (*40*, *41*). We thus administered either suvorexant or its vehicle in mouse models equipped with EEG cortical electrodes (Fig. 6A). Because mice are nocturnal animals, they are at rest/sleep during the day ("light phase") and active during the night ("dark phase"). Acute administration of suvorexant at the onset of the light phase was able to rescue sleep alterations in *Sod1*^{G86R} mice. In particular, it normalized wake, REM, and NREM sleep similarly to WT untreated animals (WT vehicle versus *Sod1*^{G86R} with suvorexant, $P = 0.195$; Fig. 6, B to E); similar results were obtained in 10-month-old *Fus*$^{\Delta NLS/+}$ mice (Fig. 6, F to I) and *TDP43*^{Q331K} mice (fig. S16, A to D). This effect was observed in both male and female mice. Suvorexant did also decrease wake duration, increase REM sleep, and decrease NREM sleep in WT mice (Fig. 6, B to I).

MCH rescues motor neurons in *Sod1*^{G86R} mice

We hypothesized that rescuing sleep defects could mitigate disease progression in an ALS mouse model. To test this hypothesis, we sought to modulate lateral hypothalamus neuropeptide signaling chronically. Chronic intracerebroventricular delivery of MCH was possible using osmotic mini-pumps, using a similar protocol as in Fig. 5A. This protocol only allows MCH delivery for 2 weeks and

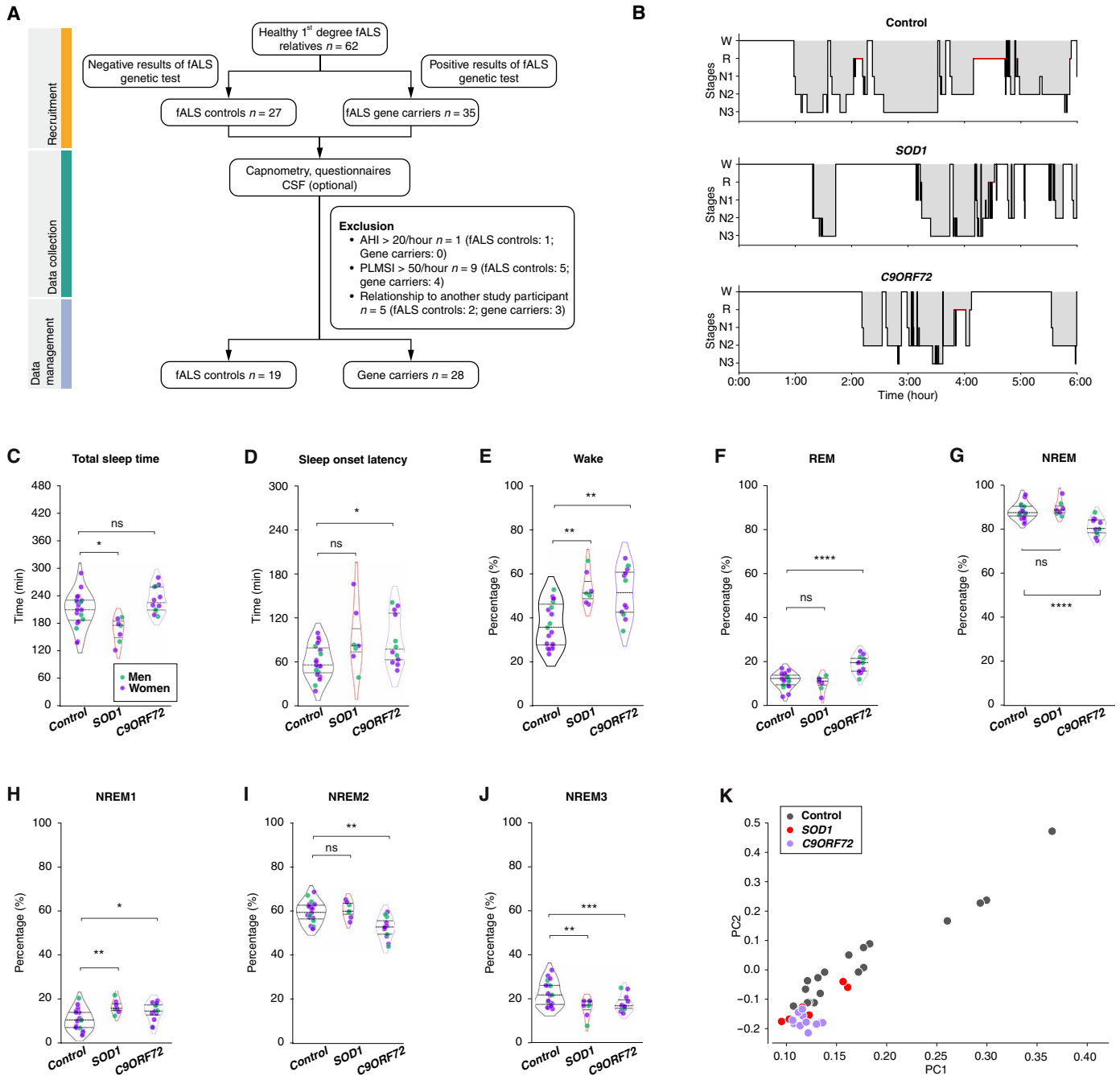

Fig. 2. Sleep alterations in presymptomatic ALS risk gene carriers. (**A**) Flow chart of the study. (**B**) Representative hypnograms of a fALS control, one *SOD1* ALS gene carrier, and one *C9ORF72* risk gene carrier over a 6-hour period (1 epoch = 30 s). (**C**) Total sleep time (total duration of REM, NREM1, NREM2, and NREM3 in the sleep period time). (**D**) Sleep onset latency (latency to the first epoch of any sleep). (**E** to **J**) Percentage of wake (E), REM (F), NREM (G), NREM1 (H), NREM2 (I), and NREM3 (J). (**K**) PCA analysis of presymptomatic *SOD1*, *C9ORF72* gene carriers, and fALS controls using sleep parameters. In all panels, men are shown in green and women in purple. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$; one-way ANOVA with one-step Bonferroni correction; sex effect $P = 0.4096$. Data are presented as median and interquartile ranges. Corrected *P* values are shown.

was limited by the osmotic mini-pump delivery period (Fig. 7A). After intracerebroventricular cannulation, $Sod1^{G86R}$ mice were longitudinally followed until reaching humane end points for survival. In these experiments, MCH supplementation did not increase the survival of $Sod1^{G86R}$ mice (Fig. 7A). To determine whether chronic intracerebroventricular delivery affected motor neuron degeneration, we repeated the experiment and euthanized treated mice at 90 days of age, i.e., at the end of the osmotic mini-pump delivery period (Fig. 7B). MCH treatment allowed the preservation of lumbar motor neurons at this age (Fig. 7, B and C). These results indicate that

Table 2. Descriptive statistics of the study population of fALS risk gene carriers. ns, $P > 0.05$, nonparametric Kruskal-Wallis test. —, not applicable.

	fALS gene carriers		fALS controls	*P* value	
	SOD1	*C9ORF72*		*SOD1*	*C9ORF72*
Women (%)	5 (28.5)	9 (75)	14 (73.7)	—	—
Men (%)	2 (71.5)	3 (25)	5 (26.3)	—	—
BMI (mean ± SEM)	27.31 (±1.44)	24.91 (±1.36)	26.06 (±1.59)	0.420 (ns)	0.582 (ns)
Age (mean ± SEM)	47.33 (±6.51)	37.92 (±3.24)	41.84 (±2.67)	0.858 (ns)	0.081 (ns)

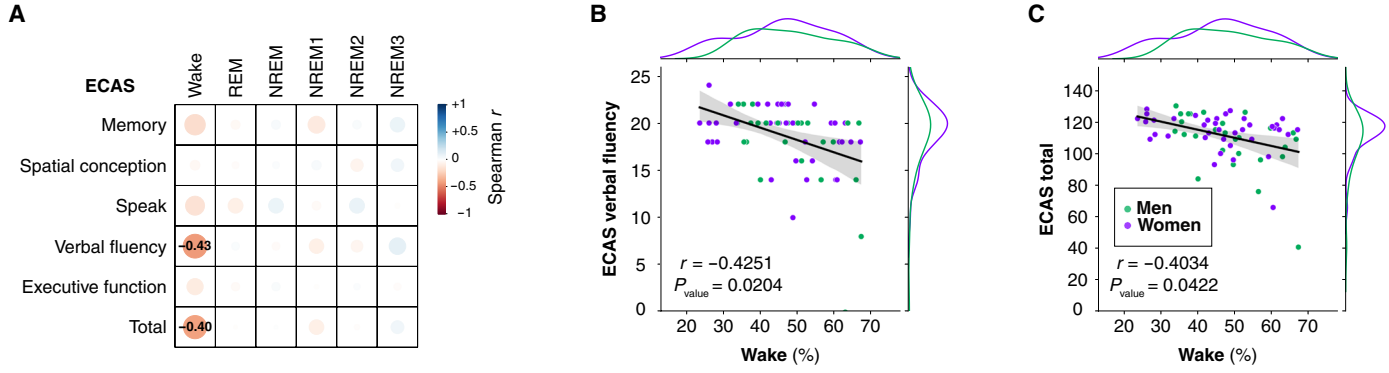

Fig. 3. Correlation analysis between sleep parameters and cognitive function. (**A**) Correlation matrix showing Spearman correlation coefficient *r* for each of the corresponding correlations performed. The six indicated sleep parameters (wake, REM, NREM, NREM1, NREM2, and NREM3) were correlated with ECAS subscores and total score for all patients with ALS and presymptomatic risk gene carriers. (**B** and **C**) Correlation between wake percentage and ECAS verbal fluency subscore (B) or total ECAS score (C). In all panels, men are shown in green and women in purple. Spearman *P* value was adjusted with Holm correction. Spearman correlation coefficient *r* and corrected *P* value are indicated. Side distribution represents sex distribution across both variables (men in green, women in purple).

modulation of LH sleep regulatory peptides could preserve motor neurons, at least in this mouse model.

DISCUSSION

Alterations in sleep are a hallmark of multiple neurodegenerative disorders and of normal aging (*42*), and ALS is not an exception. Several previous studies have shown the occurrence of sleep alterations in patients with ALS (*23*, *43*).

However, available studies to date included patients with manifest disease, thus with possibly ongoing progressive respiratory impairment as an effect of disease progression (*44*). Sleep alterations were usually correlated to disease progression in these studies. Thus, these studies could not determine whether sleep alterations were secondary to disease progression or preexisting motor defects. Our current study provides complementary evidence, both in humans and in mouse models, that sleep alterations are an early phenomenon in ALS that precedes respiratory impairment and even motor symptoms.

To ascertain that sleep alterations are not dependent on other possible respiratory conditions, we performed two clinical cohort studies in different populations. First, we prospectively included early patients with ALS and defined our exclusion criteria to allow us to rule out other sleep disorders that may alter sleep architecture, including nocturnal hypercapnia. This ensured that all included participants had normal respiratory function. We uncovered alterations in the sleep macroarchitecture of patients with ALS, particularly increased sleep onset latency, decreased deep sleep (NREM3), and increased wake after sleep onset, none of which were self-reported by the participants. This first study showed the early occurrence of sleep alterations in patients with manifest disease, and it was impossible to exclude that neurological deficits including ongoing motor symptoms might have influenced sleep architecture.

To circumvent this limitation, we performed an analogous prospective cohort study in presymptomatic ALS risk gene carriers. We observed a similar, yet milder, sleep phenotype in this population of presymptomatic carriers. *C9ORF72* mutation carriers showed alterations in the same direction as patients with ALS for sleep onset latency, deep sleep (NREM2 and NREM3), REM sleep, and wake after sleep onset. *SOD1* mutation gene carriers only showed decreased deep sleep and increased wake after sleep onset, consistent with a milder clinical picture in *SOD1* as compared with *C9ORF72* patients with ALS. The EEG power spectral analysis supports the polysomnographic findings of altered sleep architecture with a reduced delta band in patients with ALS and gene carriers, which is consistent with the reduced deep sleep component and increased or tending to increased beta frequencies consistent with an increased percentage of wake.

Consistent with the two cohort studies, we observed similar sleep alterations in three transgenic mouse models of ALS. In mice expressing mutant SOD1, which are characterized by rapid disease progression and appearance of symptoms at about 3 months of age, we observed increased wake and decreased NREM at 75 days of age. At this age, no muscle denervation and no detectable motor

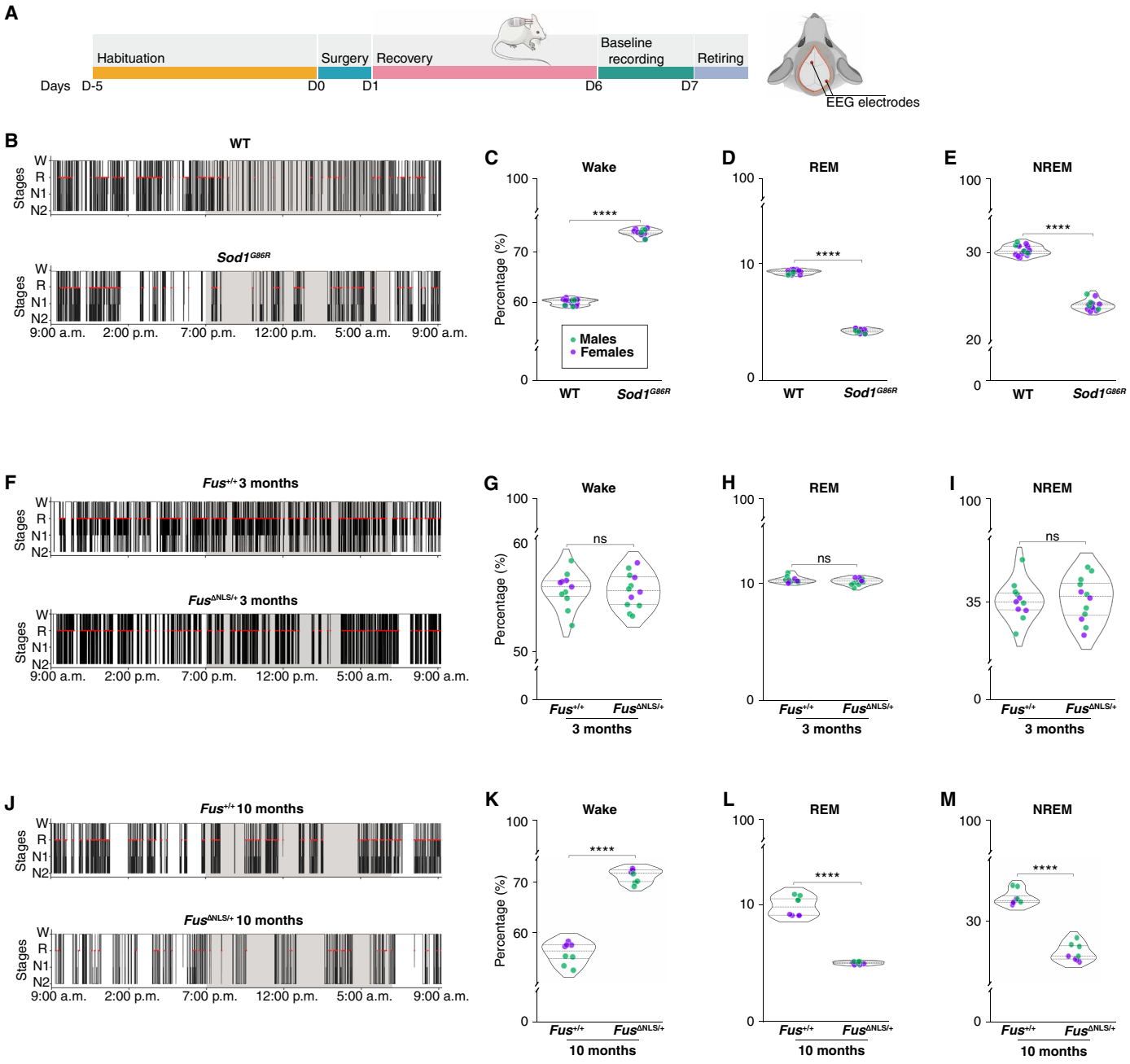

Fig. 4. Sleep alterations in *Sod1*^{G86R} and *Fus*$^{\Delta NLS/+}$ mice. (**A**) Experimental design. (**B** to **E**) Representative hypnograms of *Sod1*^{G86R} mice and their nontransgenic wild-type (WT) littermates at 75 days of age (before motor symptom onset) (B) and quantification of wake (C), REM (D), and NREM (E) *Sod1*^{G86R} mice $n = 14$; 10 females and 4 males; WT: $n = 18$; 14 females and 4 males. (**F** to **I**) Representative hypnograms of *Fus*$^{\Delta NLS/+}$ mice and their WT littermates (*Fus*$^{+/+}$) at 3 months of age (F) and quantification of wake (G), REM (H), and NREM (I) *Fus*$^{\Delta NLS/+}$ mice: $n = 12$; four females and four males; *Fus*$^{+/+}$: $n = 11$; four females and seven males. (**J** to **M**) Representative hypnograms of *Fus*$^{\Delta NLS/+}$ mice and their WT littermates (*Fus*$^{+/+}$) at 10 months of age (J) and quantification of wake (K), REM (L), and NREM (M) *Fus*$^{\Delta NLS/+}$ mice: $n = 8$; four females and four males; *Fus*$^{+/+}$: $n = 8$; four females and four males. Recordings were performed over a 24-hour period (1 epoch = 5 s). Grayed areas correspond to the active phase, when lights were off, whereas white areas indicate the nonactive phase, where lights were on. ****$P < 0.0001$, independent Student's *t* test with Welch's *t* test correction; sex effect: *Sod1*^{G86R}, $P = 0.6302$; *Fus*$^{\Delta NLS/+}$ 3 months, $P = 0.6708$; *Fus*$^{\Delta NLS/+}$ 10 months, $P = 0.0953$. Data are presented as median and interquartile ranges. Corrected *P* values are shown. ns, not significant.

impairment or weight loss are observed. In mice carrying a heterozygous knock-in mutation of *Fus* or transgenic expression of mutant *Tdp-43* , both with very slow disease progression and no progression to overt paralysis and manifest ALS symptoms, we observed the same sleep alterations at 10 months of age. No alterations were observed in *Fus* knock-in mice at 3 months of age. Our results are consistent with recent results in flies, in which the expression of human TDP-43 profoundly disrupted sleep (*45*). Similarly, Milioto

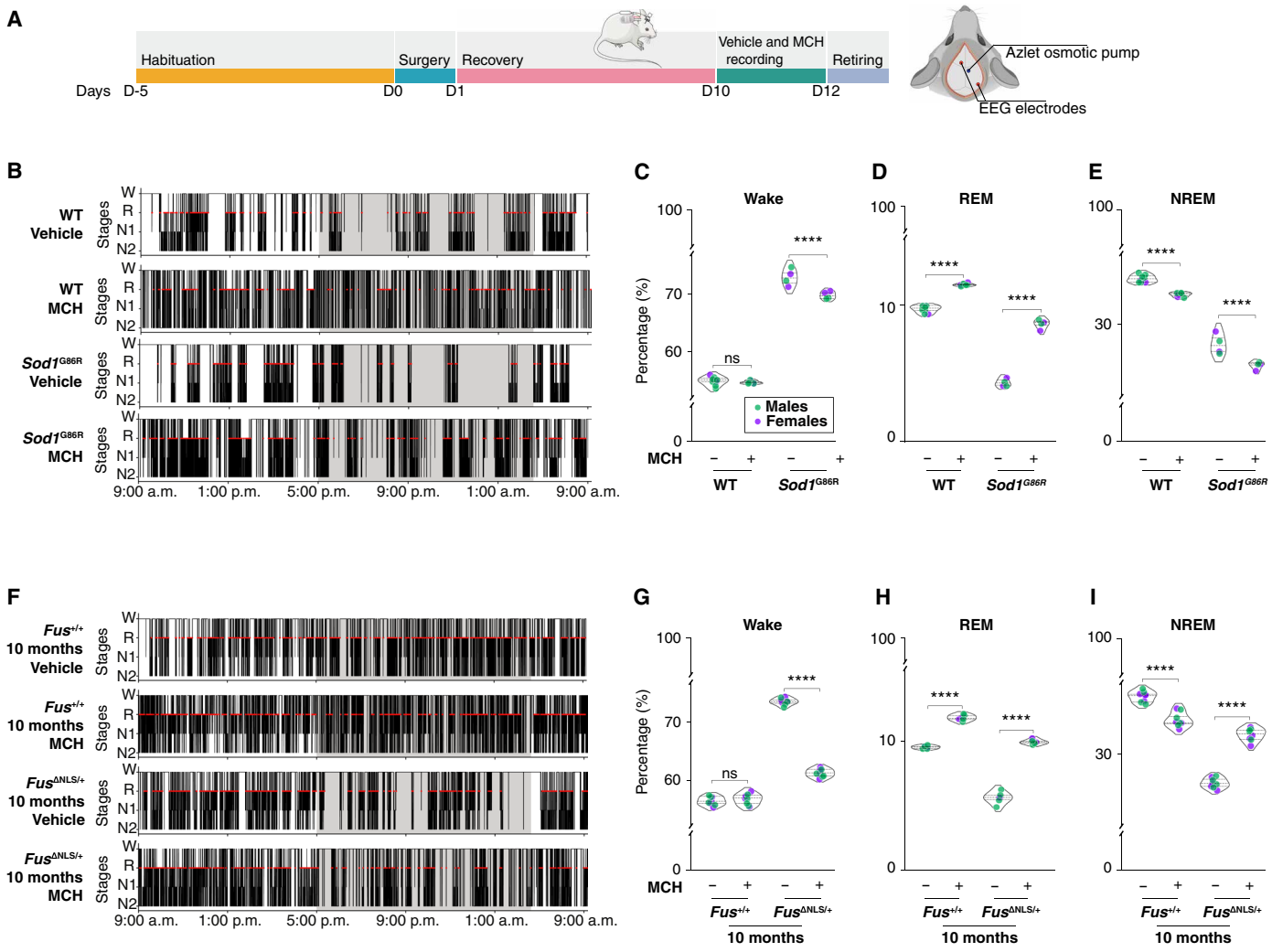

Fig. 5. MCH partially rescued sleep alterations in *Sod1*^{G86R} and *Fus*$^{\Delta NLS/+}$ mice. (**A**) Experimental design. (**B** to **E**) Representative hypnograms of *Sod1*^{G86R} mice and their nontransgenic WT littermates treated with vehicle or MCH from 75 days of age onward (before motor symptom onset) (B) and quantification of wake (C), REM (D), and NREM (E) *Sod1*^{G86R} mice: $n = 8$; four females and four males; WT: $n = 8$; two females and six males. (**F** to **I**) Representative hypnograms of *Fus*$^{\Delta NLS/+}$ mice and their WT littermates (*Fus*$^{+/+}$) at 10 months of age (F) and quantification of wake (G), REM (H), and NREM (I) *Fus*$^{\Delta NLS/+}$ mice: $n = 8$; four females and four males; *Fus*$^{+/+}$: $n = 8$; four females and four males. Recordings were performed over a 24-hour period (1 epoch = 5 s). Grayed areas correspond to the active phase, when lights were off, whereas white areas indicate the nonactive phase, where lights were on. ****$P < 0.0001$, two-way ANOVA with one-step Bonferroni correction; genotype effect: *Sod1*^{G86R}, $P < 0.0001$; *Fus*$^{\Delta NLS/+}$ 10 months, $P < 0.0001$; sex effect: *Sod1*^{G86R}, $P = 0.9187$; *Fus*$^{\Delta NLS/+}$ 10 months, $P = 0.1442$. Data are presented as median and interquartile ranges. Corrected P values are shown.

and collaborators performed neuropixel recordings in lightly anesthetized mice expressing poly(GR) from the endogenous *C9ORF72* locus and observed decreased slow oscillations and increased gamma band activity (*46*). These data do not directly assess sleep patterns in lightly anesthetized animals, and they nevertheless are consistent with our findings of increased wake in multiple models of ALS. Thus, despite the different species and the vastly different circadian rhythms and sleep patterns, patients with ALS, ALS risk gene carriers, and ALS mouse models showed increased wake and decreased NREM sleep compared with their respective controls. The only notable species difference was that REM sleep was increased in humans and decreased in mice.

Overall, we show here a consistent pattern of ALS-associated sleep alterations, which occur before motor impairment and before respiratory deficits. Sleep alterations were also observed in other neurodegenerative diseases such as Alzheimer's disease (AD) (*47*) or Parkinson's disease (PD) and more recently behavioral variants of frontotemporal dementia (bvFTD) (*48*, *49*). In their study of the day-night rhythm in patients with bvFTD measured by actimetry, Filardi *et al.* (*49*) found both increased time in bed and increased total sleep time. These results are in line with our findings in patients with ALS. In contrast with that, patients with AD and patients with PD show reductions in REM sleep, which is not observed in early patients with ALS, a reduction in the proportion of REM sleep in patients with ALS develops as soon as nocturnal hypoventilation sets in. Another notable difference between ALS and other neurodegenerative diseases was that, in the absence of respiratory impairment, patients with ALS have been found to report normal subjective

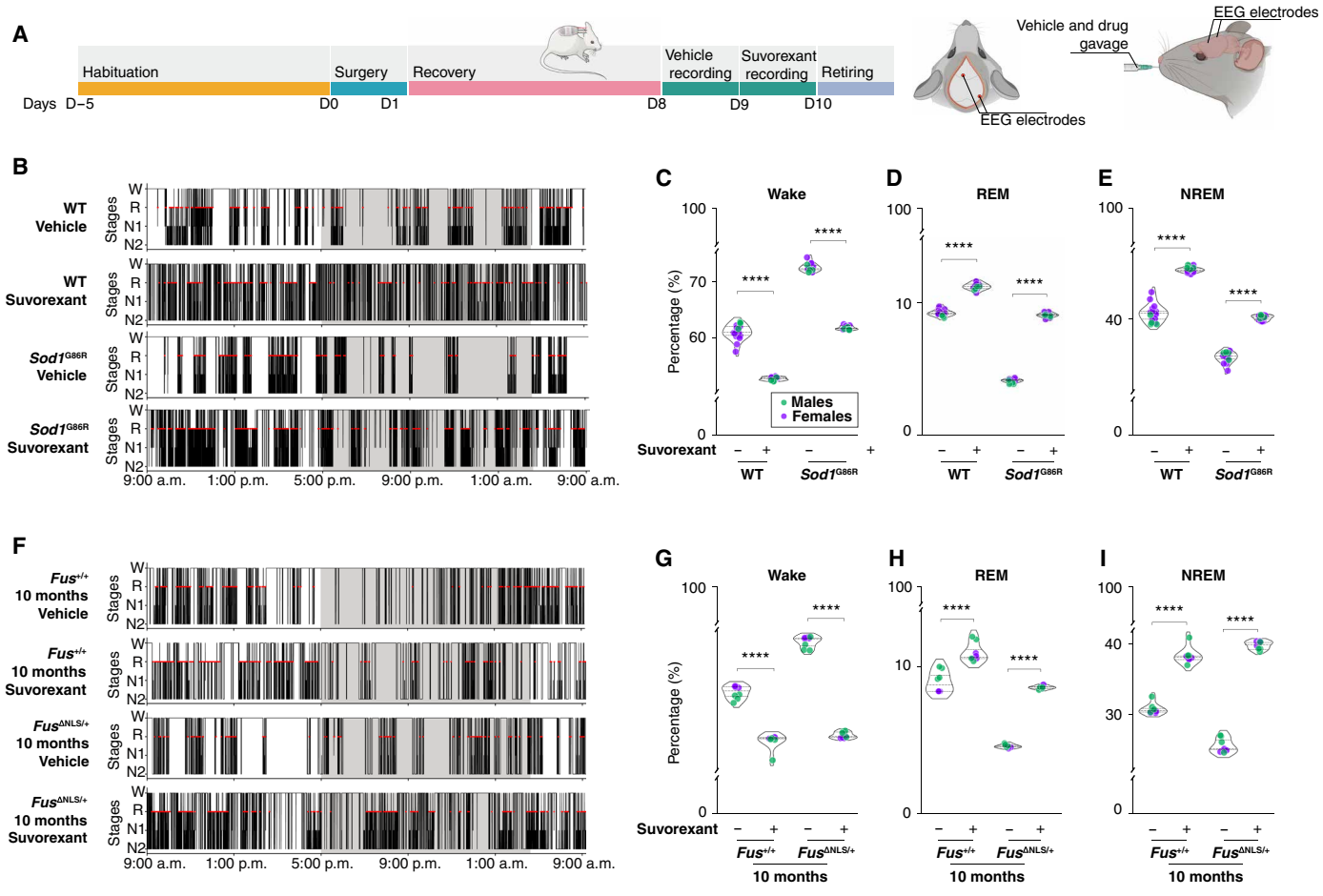

Fig. 6. Rescued sleep alterations by repurposing suvorexant in *Sod1*^{G86R} and *Fus*$^{\Delta NLS/+}$ mice. (**A**) Experimental design. (**B** to **E**) Representative hypnograms of *Sod1*^{G86R} mice and their nontransgenic WT littermates after administration of the vehicle solution or suvorexant at 75 days of age (before motor symptom onset) (B) and quantification of wake (C), REM (D), and NREM (E) *Sod1*^{G86R} mice: $n = 14$; 10 females and 4 males; WT: $n = 18$; 14 females and 4 males. (**F** to **I**) Representative hypnograms of *Fus*$^{\Delta NLS/+}$ mice and their WT littermates (*Fus*$^{+/+}$) at 10 months of age after administration of the vehicle solution or suvorexant (F) and quantification of wake (G), REM (H), and NREM (I) *Fus*$^{\Delta NLS/+}$ mice: $n = 8$; four females and four males; *Fus*$^{+/+}$: $n = 8$; four females and four males. Recordings were performed over a 24-hour period (1 epoch = 5 s). Grayed areas correspond to the active phase, when lights were off, whereas white areas indicate the nonactive phase, where lights were on. ****$P < 0.0001$, two-way ANOVA with one-step Bonferroni correction; genotype effect: *Sod1*^{G86R}, $P < 0.0001$; *Fus*$^{\Delta NLS/+}$ 3 months, $P = 0.0116$; *Fus*$^{\Delta NLS/+}$ 10 months, $P < 0.0001$; sex effect: *Sod1*^{G86R}, $P = 0.4722$; *Fus*$^{\Delta NLS/+}$ 3 months, $P = 0.7640$; *Fus*$^{\Delta NLS/+}$ 10 months, $P = 0.2218$. Data are presented as median and interquartile ranges. Corrected *P* values are shown.

sleep quality when using standardized questionnaires. In AD and PD, subjective sleep is affected, suggesting that patients with ALS or gene carriers are not aware of their sleep alterations or that the sleep changes are too subtle to be realized or have been present for a long time such that they are not noticed by those affected. It is also possible that the von Economo neurons in the anterior cingulate cortex—which are known to be affected by ALS and play an important role in subjective judgment and emotion—compromise the awareness of these alterations (*50*, *51*). The increased wake percentage and the sleep fragmentation we observe here are comparable to sleep changes observed in normal aging and could be consistent with accelerated aging in patients with ALS (*52*). Further studies on sleep macro- and microarchitecture in patients with ALS are warranted to characterize similarities and differences among aging, ALS, and other neurodegenerative diseases with respect to sleep alterations.

Our preclinical data suggest that alterations in key LHA signaling underlie ALS-associated sleep alterations. We previously showed that the hypothalamus is atrophied early in the disease process of ALS, even in presymptomatic risk gene carriers, and that ALS pathology was mostly found in the lateral hypothalamus (*10*, *14*, *53*). Our recent studies have shown that MCH neurons are lost in ALS mouse models and patients, whereas ORX neurons are preserved in animal models but lost in patients (*20*). We further show that MCH or ORX neurons do not exhibit major ALS-related pathology until a late stage of disease in *Sod1*^{G86R} mice, suggesting that dysfunction of sleep circuits, rather than individual degeneration of their cellular components, underlies the observed sleep defect.

MCH and ORX neurons play an important role in sleep control, with MCH neurons promoting REM sleep (*54*, *55*) and ORX neurons promoting arousal (*56*). ORX neurons and MCH neurons are generally thought to antagonize each other during the different stages of sleep (*57*, *58*). Given that MCH neurons are lost in ALS and that ORX neurons remain largely intact, we hypothesized that the observed sleep alterations stem from an imbalance in orexinergic tone, favoring orexinergic output. Consistent with this hypothesis, intracerebroventricular MCH supplementation partially rescued

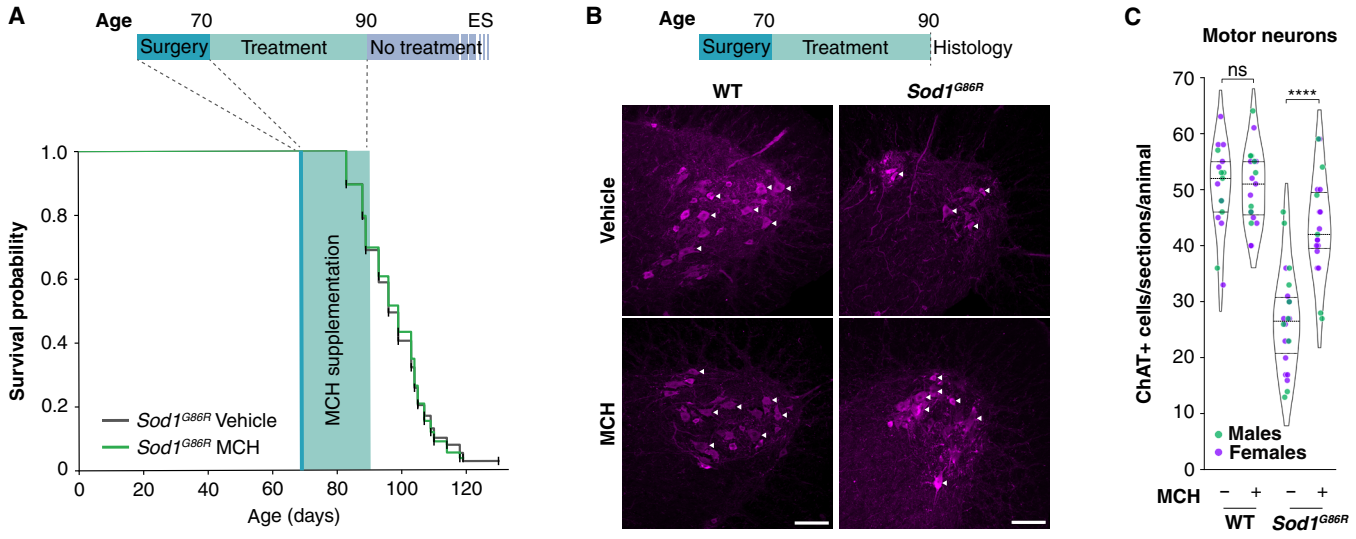

Fig. 7. MCH protects motor neurons in *Sod1*^{G86R} mice. (**A**) Survival curves of *Sod1*^{G86R} mice implanted with an osmotic mini-pump intracerebroventricularly delivering either vehicle or MCH; *Sod1*^{G86R} vehicle: $n = 17$; 6 females and 11 males; *Sod1*^{G86R} MCH: $n = 12$; 5 females and 7 males. No sex difference was observed. ns, $P > 0.05$, Kaplan-Meier model with a log-rank test with Wilcoxon weighting. The experimental design is presented above the survival curve. Osmotic mini-pumps delivered the drug for 15 days after implantation. Animals were euthanized when reaching the humane end point, which was determined by the paralysis of the hindlimbs. (**B**) Representative confocal images of ChAT immunostaining in the spinal cords of (L1 to L6) *Sod1*^{G86R} and WT littermates with either vehicle or MCH supplementation at 90 days of age. Scale bar, 100 μm. (**C**) Motor neuron counts in *Sod1*^{G86R} or WT littermates treated with either vehicle or MCH. Mice in (B) and (C) were euthanized at the end of the delivery period by the osmotic mini-pumps (90 days of age). WT vehicle: $n = 17$; 10 females and 7 males; WT MCH: $n = 19$; 11 females and 8 males; *Sod1*^{G86R} vehicle: $n = 22$; 12 females and 10 males; *Sod1*^{G86R} MCH: $n = 19$; 13 females and 6 males. Males are shown in green and females in purple. $^{***}P < 0.001$, Two-way ANOVA with one-step FDR-BKY correction; genotype effect: *Sod1*^{G86R}, $P < 0.0001$; sex effect: WT, $P = 0.6241$; *Sod1*^{G86R}, $P = 0.9925$. Data are presented as median and interquartile ranges. Corrected *P* values are shown.

sleep alterations in ALS mice. Notably, MCH's effect on wild-type animals makes this effect non–disease specific. In all studied ALS mouse models, ORX antagonist normalized sleep alterations to a greater extend. This difference in efficacy between MCH supplementation and ORX antagonist could be due to a more prominent role of ORX neurons in sleep modulation. Yet, differences in mode of administration (intracerebroventricular cannulation and continuous delivery versus acute oral administration) or pharmacokinetics could also underlie such a difference. MCH treatment also demonstrates a beneficial effect on lumbar motor neurons. However, no effect on *Sod1*^{G86R} mouse model survival was observed, possibly because of technical limitations and the limited pump bioavailability of MCH or possible receptor desensitization. Futher experiments would need to determine the molecular and cellular mechanism of MCH action in ALS. We also noticed that in *Sod1*^{G86R} (90 days old) and *Fus*$^{\Delta NLS/+}$ (4 months) mice, hypothalamic gene expression of *Junb*, *Sgk1*, *Foxo1*, and *Sirt1* were altered, consistent with profound changes within the neurons' signaling.

Our preclinical study suggests that LH neuropeptides play a critical role in early sleep alterations; however, we do not show that these alterations originate specifically in ORX or MCH neurons, nor do we rule out the involvement of other cell types or structures. Recent studies have observed the involvement of sleep-controlling long pathways in ALS, including cholinergic pathways (*59*, *60*), noradrenaline neurons (*61*), serotonin neurons (*62*), or more indirectly glymphatic dysfunction (*63*). Future work should perform cell-specific interventions to characterize the circuit dysfunctions and their proximal causes.

This translational study has several limitations. The human study includes a limited number of patients with ALS from a single center, and the gene-carrier cohort is restricted to a small population in Germany, thus implying a possible selection bias. In our preclinical mouse study, key limitations are the use of acute orexin antagonist administration, the impossibility of performing motor function assessment, and the lack of direct measurements or MCH and orexin concentrations in the brain. Although MCH supplementation shows effects on lumbar motoneurons, its application as a translational approach remains challenging at this stage.

In our study, we provide a characterization of the possible causes and consequences of sleep changes in ALS that precede motor symptoms. These changes in sleep are initially clinically subtle, so that people before motor onset and even manifest patients do not subjectively notice these sleep changes. Our results suggest that these sleep changes are an expression of hypothalamic dysregulation, which also manifests itself in other changes in patients with ALS, such as altered energy supply, which also precedes the motor manifestation phase of the disease given that patients already show a drop in body mass index years before onset (*64*, *65*). We therefore consider the sleep changes to be part of a prodromal phase that precedes the motor manifestation phase of ALS (*14*, *63*, *66*) that was not identified in the neuroanatomical studies by Brettschneider *et al.* (*67*) and Braak *et al.* (*68*). In addition, it has already been shown that functions classically associated with sleep—cognitive functions and memory—are altered in the preclinical phase of ALS, so far without establishing the link to sleep changes (*69*, *70*). This would be consistent with the observed correlation of a higher proportion

of wakefulness and higher sleep fragmentation with reduced cognitive performance and a selective impairment of verbal fluency in presymptomatic risk gene carriers (*71*). It is plausible that sleep alterations occurring several years before the onset of motor symptoms could contribute to motor deficits or disease progression in patients with ALS. Our observation of the protection of motor neurons through chronic administration of MCH in rodents suggests a possible detrimental role of sleep-related defects in the progression of motor symptoms, because MCH is involved in the regulation of sleep and sleep stages. Further preclinical and clinical research is warranted to investigate the impact of defects in sleep and sleep regulatory neuropeptides on cognitive deficits, weight loss, or motor symptom progression associated with ALS.

MATERIALS AND METHODS

Study design

The aims of this study were (i) to characterize sleep stage alterations in patients with ALS and presymptomatic risk gene carriers and (ii) to investigate sleep deficits and the role of hypothalamic signaling disruptions in mouse models. For human studies, polysomnography was used as the principal tool to analyze sleep macroarchitecture and the wake-sleep distribution in these cohorts. The study was designed as a prospective case-control investigation, focusing on individuals without respiratory symptoms.

The cohort of patients with ALS was approved by the Ethics Committee of the University of Ulm (reference 391/18) and the study in the presymptomatic carriers, which was also approved by the Ethics Committee of the University of Ulm (reference 68/19), in compliance with the ethical standards of the current version of the revised Declaration of Helsinki. In the context of the study, all participants were informed about the content, procedure, and objective of the study. Furthermore, they were granted sufficient time to reconsider their participation. All participants gave written informed consent before enrollment. The study adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for observational research throughout implementation and analysis (*72*). Our sample size in humans was based on the designs of previous polysomnography studies detailed below.

For mouse studies, EEG analysis was used to investigate sleep disturbances in three ALS mouse models (*Sod1*^{G86R}, *Fus*$^{\Delta NLS/+}$, and *TDP43*^{Q331K}). Per os administration of a dual-orexin receptor antagonist and intracerebroventricular delivery of MCH over 15-day treatment were used to recover sleep deficits.

Sample size was estimated on the basis of former studies in rodents (*14*). All experiments were performed in strict compliance with directive 2010/63/EU and regulation (EU) 2019/1010. The projects (2021092415348513 and 2019040815594294) were reviewed and approved by the Ethics Committee of the University of Strasbourg and the French Ministry of Higher Education, Research and Innovation (decree no. 2013-118, 1 February 2013). Animal care occurred in accordance with the *Guide for the Care and Use of Laboratory Animals*. Strict compliance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2.0 guidelines has been ensured. All experiments were analyzed blindly for different treatment and groups.

Patients/participants

Patients with ALS were recruited from the inpatient and outpatient clinics of the Neurologic Department of the University Hospital of Ulm, Germany, from October 2018 to January 2022. The inclusion criteria for patients with ALS included a diagnosis of definite ALS based on the revised El Escorial criteria (*73*). The control participants were recruited from the general population and from patients in the department of neurology during the same period. They were matched with the patients with ALS on the basis of age, sex, and geographical location; the requirement for this group was the exclusion of neurodegenerative diseases. All individuals in the control group were unrelated to ALS or fALS. Presymptomatic carriers of fALS genes were recruited through the study center of the Neurological University Hospital, through which first-degree relatives of confirmed familial patients with ALS received longitudinal follow-up and counseling in the period from October 2019 to November 2022. In accordance with the study protocol, the scheduled investigations were conducted on two consecutive days, with polysomnography performed on the intervening night. A follow-up examination was not included in the study. A report on age and sex for all study participants can be found in the Supplementary Materials. Controls were recruited from the general population at the neurology clinic and matched to patients with ALS on the basis of age, sex, and geographical location; the requirement for this group was the exclusion of neurodegenerative diseases. All individuals in the control group were unrelated to ALS or fALS.

The study in the cohort of patients with ALS was approved by the Ethics Committee of the University of Ulm (reference 391/18) and the study in the presymptomatic carriers, which was also approved by the Ethics Committee of the University of Ulm (reference 68/19), in compliance with the ethical standards of the current version of the revised Declaration of Helsinki. All participants gave informed consent before enrollment.

Medical history was documented. For patients with ALS, the ALSFRS-R and characteristics of disease progression were documented (site of first paresis/atrophy and date of onset). All participants also completed validated daytime sleepiness and sleep quality questionnaires, namely, the Epworth Sleepiness Scale (ESS) (*74*) and the Pittsburgh Sleep Quality Index (PSQI) (*75*).

Patients' inclusion process

The exclusion criteria were designed to eliminate all potential factors that could otherwise alter sleep architecture. Specifically, we aimed to exclude respiratory insufficiency in patients with ALS. Although respiratory insufficiency develops at varying points in the progression of ALS, depending on the individual course, it is typically present in advanced stages and is known to affect sleep architecture. For this reason, patients with ALS underwent transcutaneous capnometry in addition to polysomnography. Furthermore, participants with an apnea-hypopnea index (AHI) above 20 per hour or a periodic limb movement index (PLMSI) above 50 per hour were excluded from the analysis. Detailed inclusion and exclusion criteria are provided in tables S1 and S2.

A power analysis to determine group size was not feasible because of the lack of relevant data on polysomnography in patients with ALS, particularly in asymptomatic risk gene carriers, at the time of the study design. Instead, we based our approach on the designs of previous polysomnography studies (*76*–*78*). The study by Puligheddu *et al.* (*76*) was similarly designed as a case-control study and demonstrated significant differences with group sizes of 28 or 29 participants per group.

We anticipated a 30% dropout rate in the first cohort because of the stringent exclusion criteria. To ensure 30 participants per group

for analysis, a target enrollment of 50 participants per group was established. For the second cohort, it was hypothesized that participants would be divided into two roughly equal groups: gene carriers and family controls based on genotyping results. Because no respiratory insufficiency was expected in this cohort, a dropout rate of 20% was assumed, leading to a planned cohort size of 70 participants.

On the basis of findings from previous studies (*76*–*78*), which highlighted differences in wakefulness and NREM stage distribution, we established the proportion of REM and NREM sleep (including NREM substage distribution) and wakefulness after sleep onset as primary end points for polysomnography analysis. Other polysomnographic parameters, along with the ESS and PSQI questionnaire scores, were defined as exploratory end points.

Blinding was not feasible in the cohort of patients with ALS and healthy controls. The study on the presymptomatic risk gene carriers was blinded, in that the participants were family members of a fALS case and were therefore informed about their risk of being gene carriers. The study on presymptomatic risk gene carriers was conducted in a blinded manner. These individuals were informed about their risk of being gene carriers, yet neither they nor the study personnel were aware of the result of the genetic screening at the time of the study. Randomization was not feasible in this study design for either cohort. Outliers, defined as values exceeding three standard deviations from the mean, were prespecified for exclusion from the analysis. The study adhered to the STROBE guidelines for observational research throughout implementation and analysis.

Neuropsychological assessment

Cognition was measured with the German version of the Edinburgh Cognitive and Behavioral ALS Screen (ECAS) (*79*–*81*) by trained psychologists. The ECAS addresses cognitive domains of language, verbal fluency, executive functions (ALS-specific functions), and memory and visuospatial functions (ALS nonspecific functions). Age- and education-adjusted cutoffs were used (Loose). Behavioral changes were assessed by patients' caregiver/first-degree relative interviews on disinhibition, apathy, loss of sympathy/empathy, perseverative/stereotyped behavior, hyperorality/altered eating behavior, and psychotic symptoms.

Electroencephalography in patients and controls

All participants, patients with ALS, healthy controls, fALS risk gene carriers, and fALS controls underwent a single night of full polysomnography, involving the monitoring of various physiological parameters, including electroencephalography (EEG), surface electromyogram (EMG), electrooculogram, respiratory effort and flow, pulse, and oxygen saturation. All measurements were conducted according to the American Academy of Sleep Medicine (AASM) guidelines (*82*, *83*). The EEG electrodes were placed according to the international 10-20 system, and the following electrodes were used in each participant: midline frontal (Fz), C3, C4, Cz (midline central), P3, P4, midline (Pz), O1, O2, A1, and A2. The sampling rate was 512 Hz in each case. The individually different point in time at which the participant turned off the lights and tried to sleep was marked with a "lights off" marker in each recording.

Sleep analyses in patients and participants

Analyses were performed using available Python packages (only compatible with Python 3.10 or newer, Python Software Foundation, Python Language Reference, version 3.12; available at www.python.org) relying on the MNE package (*84*). EEGs were first deidentified using the open-source Prerau Lab EDF De-identification Tool (version 1.0; 2023) in Python (Prerau Lab EDF de-identification tool; https://sleepeeg.org/edf-de-identification-tool). De-identified EEGs were then notch-filtered to remove the 50-Hz powerline. Independent component analysis was performed to remove all remaining artifacts from the signal (*85*–*89*). Analyses were limited to both sensorimotor cortices (C3 and C4), which are known to be impaired at the onset of the disease, as well as nearby interhemispheric sulci (Fz, Cz, and Pz). Sleep staging was performed on a 6-hour window with 30-s epochs, starting when lights were turned off, using YASA deep learning algorithm (v0.6.4) (*27*) and the spectral analysis. Time in bed and total sleep time were calculated over the whole recording period. The automated sleep staging, hypnograms, and spectrograms were performed using Welch's method (*90*). Sleep pressure was determined using the area under the curve (AUC) of delta power (0.5 to 4 Hz) of the first hour of the 6-hour window. Simpson's rule was used to compute the AUC. REM efficiency was computed by dividing theta power (4 to 8 Hz) by delta power (0.5 to 4 Hz), specifically during REM epochs. Sleep staging and analysis were performed following the AASM's guidelines (*82*, *83*).

EEG analysis in mice

We used NeuroScore software for sleep and seizure analysis 3.4 (Data Science International Inc., St. Paul, MN, USA) to analyze and score the EEG, EMG, and activity count of the animals at baseline, with the vehicle and with suvorexant or MCH. For both vehicle and suvorexant or MCH, the hour after the gavage was removed from the recording to ensure that minimal stress effect would be seen on the recording due to the gavage.

EEG and EMG signals, as well as activity counts, were used to score sleep/wake behavioral states using the NeuroScore software for sleep and seizure analysis 3.4 (Data Science International Inc., St. Paul, MN, USA) Rodent Sleep automated scoring method. We used 5-s epochs for both the EEG and EMG. Then, the automated scoring method classified each epoch into one of the following behavioral states: (0) wake (low-voltage fast EEG, high-voltage EMG, with frequent activity counts); (1) NREM1 (NREM1 sleep: spindling and high-voltage EEG with slow waves, low-voltage EMG and no activity counts); (2) NREM2 (NREM2 sleep: spindling and high-voltage EEG with slow waves, very low voltage EMG) and no activity counts; (4) REM (REM sleep: low-voltage and fast EEG combined with very low-voltage EMG, with occasional short-duration, large-amplitude EMG activity due to muscle twitches and sporadic short-duration activity counts).

The activity count was used as a double check for false-positive EMG activity. Wake, REM, and NREM were analyzed as a percentage over the 24-hour recording period, with epochs of 5 s.

Data were extracted from NeuroScore and used in combination with available Python packages (Python Software Foundation, Python Language Reference, version 3.12; available at www.python.org) to further process the data. YASA toolbox (*27*) was used to generate hypnograms and perform the spectral analysis. Hypnograms were generated following the AASM's guidelines (*82*, *83*).

Statistical analyses

G*Power software (version 3.1.9.6 for macOS, 2023) was used to determine the sufficient sample size needed to reach significant statistical

power using an a priori Student's *t* test coupled with a linear bivariate regression (*91*, *92*). Before any statistical analysis, normality and homoscedasticity were both tested, respectively, with Shapiro-Wilk test (*93*) and Bartlett's test (*94*).

Statistical analysis of two groups was performed using an independent Student's *t* test, from Pingouin (*95*), using the Welch *t* test correction, from SciPy, as recommended by Zimmerman (*96*), and with a large Cauchy scale factor due to the considerate effect size (*97*). When data were heteroscedastic and normality was not met, a Mann-Whitney *U* test was performed using SciPy (*98*).

Follow-up analysis was performed using paired *t* test from SciPy (*98*) or a Wilcoxon–Mann-Whitney rank-sum test from statsmodels (*99*) when normality was not met. *P* values were then adjusted using false discovery rate Benjamini-Krieger-Yekutieli (FDR-BKY) correction.

For statistical analysis of three or four groups, a one-way analysis of variance (ANOVA) or two-way ANOVA was performed using Pingouin (*95*) toolbox. For both one-way ANOVA and two-way ANOVA, a one-step Bonferroni correction was applied. When data were heteroscedastic, and normality was not met, a Kruskal-Wallis from SciPy (*98*) followed by Dunn's multiple comparison test with FDR-BKY correction was performed using scikit-posthocs (*100*) instead of a one-way ANOVA. For the two-way ANOVA, a generalized least squares model was fitted using statsmodels (*99*), followed by Dunn's multiple comparison and FDR-BKY correction using scikit-posthocs (*100*). We evaluated whether a sex-specific effect was present in all our analyses by performing a two-way ANOVA with a one-step Bonferroni correction for both sexes. Sex was self-reported in both ALS cohorts.

For survival, a Kaplan-Meier model was fitted using lifelines (*101*), and a log-rank test with Wilcoxon weighting was performed using SciPy (*98*). Simpson's rule and Spearman's correlation coefficient, from SciPy (*98*), were used to determine the AUC and correlations on nonparametric data. PCAs were performed using scikit-learn package (*102*).

Data are presented as violin plots with all points and expressed as average ± interquartile. Plots were generated using Seaborn and Matplotlib packages (*103*). Results were deemed significant when their adjusted $P < 0.05$. Here, only corrected *P* values are shown.

Supplementary Materials

The PDF file includes:
Materials and Methods
Figs. S1 to S16
Tables S1 and S2

Other Supplementary Material for this manuscript includes the following:
Data file S1
MDAR Reproducibility Checklist

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