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GAD67-GFP knock-in mice have normal sleep-wake patterns and sleep homeostasis

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Abstract

GABAergic neurons are important for controlling sleep and wakefulness but are difficult to identify, limiting their study. Knock-in mice with GABAergic neurons labeled by expression of green fluorescent protein (GFP) under control of the glutamate decarboxylase (GAD67/*Gad1*) promoter are now extensively used in neuroscience. However, it is unknown whether these mice have a normal sleep phenotype. Compared to adult wild type control mice (n=7), adult GAD67-GFP knock-in mice (n=7) had the same amount of NREM and REM sleep, a similar diurnal distribution of sleep, no NREM or REM sleep differences in EEG power, and normal sleep rebound following 6h sleep deprivation. Our results suggest GAD67-GFP knock-in mice are an excellent tool for study of GABAergic neurons involved in sleep-wake regulation.

Keywords

EEG; EMG; sleep deprivation; glutamate decarboxylase; green fluorescent protein; REM sleep; NREM sleep

Introduction

The inhibitory neurotransmitter γ -aminobutyric acid (GABA) is implicated in both rapid eye movement (REM) and non-rapid eye movement (NREM) sleep regulation [1,2]. GABA is synthesized by the enzyme glutamate decarboxylase (GAD), which exists in two forms referred to as GAD65 and GAD67, according to their molecular weight in kDa [3,4]. Both isoforms of GAD are co-localized in GABAergic neurons but GAD67 is more prominent in cell bodies while GAD65 tends to be found in axon terminals [5]. Selective gene knockout of each of the isoforms revealed that the loss of GAD67 markedly decreased brain GABA content in homozygotes and led to death during development, while the loss of GAD65 had relatively minor effects in neonates but led to spontaneous seizures in adults [6,7].

Different groups of GABAergic neurons in multiple brain areas spanning the neuraxis are involved in the generation and maintenance of sleep-wake states, as well as the generation of different frequencies of electroencephalographic (EEG) activity (delta, theta, gamma) which

are used to define them [1,2]. In order to study the properties of these neurons it is necessary to have a method to easily identify them. Recently, we generated GAD67-GFP knock-in mice using a gene targeting method via homologous recombination in ES cells [8]; in these mice green fluorescent protein (GFP) cDNA is inserted into exon 1 in one of the two GAD67 alleles (Heterozygous GAD67-GFP knock-in mice; now used extensively in neuroscience – full list of peer-reviewed papers using these mice is provided in Supplementary material 1). Since both the GFP and the GAD67 genes in the knock-in mice are identically controlled, the parallel GFP protein expression accurately reflects the expression of GAD67 in GABA neurons [8–11]. Using this line of GAD67-GFP knock-in mice, we provided a comprehensive anatomical and pharmacological description of GABA neurons around the mesopontine junction, an important region for REM sleep control [10]. Furthermore, we confirmed that, as expected, GFP is a selective and specific marker for GABAergic neurons [10]. It is, however, unclear if exogenous expression of GFP in GABA neurons *per se* will affect sleep-wake control in the mouse. Therefore, here we studied the characteristics of spontaneous sleep in GFP-GAD67 knock-in mice and their homeostatic sleep response after six hours of sleep deprivation.

Materials and methods

Animals

2–4 month old heterozygous GAD67-GFP (Δ neo) knock-in mice [8] (N=7, 2 males and 5 females) and their littermate controls (Swiss-Webster, N=7, 4 males and 3 females) were used. The transgenic animals termed GAD67-GFP knock-in mice originally carry a C57/BL6 background [8]. These mice were backcrossed to Swiss-Webster mice in the current study. The mice were bred in-house at the animal research facility of the Brockton campus of the VA Boston Healthcare System. The facility is approved by the American Association for Accreditation of Laboratory Animal Care and is under full-time veterinary supervision. Mice were housed under constant temperature (23°C) and a 12 h:12 h light–dark cycle (lights-on period from 7:00 h to 19:00 h) with food and water available *ad libitum*. All animals were treated in accordance with the American Association for Accreditation of Laboratory Animal Care's policy on care and use of laboratory animals. The experiments described here were approved by the Institutional Animal Care and Use committee of the VA Boston Healthcare system. Phenotyping of mice was achieved by examining the heads of the mice 1–2 days after birth under a fluorescence microscope at low power magnification. GAD67-GFP knock-in mice exhibited a striking green fluorescence in the brain and the olfactory bulb that can be visualized through the skull at this age, as described previously [10].

Surgery

Under 2% isoflurane general anesthesia, mice were implanted with two stainless steel electromyogram (EMG) electrodes and two stainless-steel EEG electrodes (Plastics One, Roanoke, VA). EMG electrodes were placed into the dorsal neck muscles. EEG electrodes were placed over the frontal and parietal cortices. All electrodes were fixed on the skull with 3M P-10 resin bonded ceramic (3M Dental Products, St. Paul, MN). The leads from all electrodes were routed to a Teflon pedestal (Plastics One).

Sleep Recording and Sleep Deprivation

After surgeries animals were housed in individual Plexiglas cages. Recording cables were connected to the mice one week post-surgery. After at least an additional week of recovery and habituation in the recording room, a 24-hour baseline sleep recording beginning at dark onset was obtained. After baseline recording, mice were sleep deprived for 6 hours from 10AM to 4PM the following day via gentle handling, which involved auditory stimulation,

such as tapping on the side of the cage, or tactile stimulation, such as gentle brushing of the mouse's back with a piece of gauze or cotton ball. Sleep recordings were restarted at the end of sleep deprivation and continued until the next light onset.

Recording and Analysis

The sleep-wakefulness states were scored visually offline in 10 sec epochs of Wakefulness, NREM and REM using standard criteria [12]. The amount of time spent in each state was calculated as a percentage in 3 hour blocks. For EEG power spectral analysis, the EEG power density was calculated in 1-Hz intervals in the 0 to 20-Hz range for each behavioral state. The sum of the values obtained for the 1-Hz frequency bin in each mouse was normalized to 1. Each corresponding 1-Hz bin value was expressed as a relative percentage of the total. EEG slow wave (delta) activity (SWA, 0.5–4.0 Hz) changes during NREM sleep during a 6 hour time period immediately following sleep deprivation was calculated as percentage change compared to the corresponding baseline levels (same time period on the previous day).

Statistical Analysis

Data concerning the time spent in each vigilance state was analyzed by two-way repeated ANOVA followed by paired comparisons. Sleep and EEG SWA changes after sleep deprivation were analyzed by paired t-tests. A value of $p < 0.05$ was accepted as statistically significant.

Results

GAD67-GFP knock-in mice exhibited typical polysomnographic features that are seen in wild type mice and other mouse and rat strains (Fig. 1), i.e. they showed low-voltage fast EEG activity and high muscle tone during waking; high amplitude, slow wave activity (SWA) and reduced EMG tone characteristic of NREM sleep; and low-voltage theta frequency EEG activity coupled with muscle atonia typical of REM sleep. Both GAD67-GFP knock-in mice (N=7) and wild type mice (N=7) displayed diurnal variations of sleep typical of nocturnal rodents that sleep more during the daytime (0700–1900 h: % NREM 57.1 ± 4.0 in wild-types, 51.5 ± 2.1 in GAD67-GFP; % REM 6.8 ± 0.4 in wild type and 6.8 ± 0.7 in GAD67-GFP) and less at night (1900–0700 h: % NREM 41.3 ± 3.5 in wild-type and 35.5 ± 3.1 in GAD67-GFP; % REM 5.0 ± 0.5 in wild type mice 4.6 ± 0.4 in GAD67-GFP mice). Both strains had similar patterns and amounts of NREM sleep ($F(1,12)$, $P=0.08$) and REM sleep ($F(1,12)$, $P=0.47$) (Fig. 2). In addition, GAD67-GFP knock-in mice had the same average EEG power spectral patterns in NREM sleep and REM sleep over a 24 hour time period as those seen in wild type mice, which showed the greatest power in the low frequency ranges (1–4 Hz) for NREM sleep and a characteristic theta frequency peak (4–8 Hz) for REM sleep (Fig. 3).

After 6 h of sleep deprivation (from 1000 to 1600 h), there was a significant increase of NREM sleep (Figure 4) in the subsequent 6 hour period (1600–2200 h) in both GAD67-GFP knock-in mice ($p=0.004$) and wild type mice ($P=0.02$). Both strains had a similar amount of NREM sleep in the first 6 hours after sleep deprivation (wild type, 47.6 ± 1.7 %; GAD67-GFP, 49.2 ± 2.1 %), although the GFP mice had a larger percentage increase (wild-type, 14.9% increase; GAD67-GFP mice 39.9 % increase) due to a relatively lower baseline level (wild-type, 41.5 ± 2.9 %; GAD67-GFP mice 35.2 ± 3.4 %). There was also a non-significant increase of REM sleep in both strains of mice (wild-type, 5.4 ± 0.6 % in baseline to 5.6 ± 0.3 % after SD; GAD67-GFP, 4.5 ± 0.8 in baseline to 5.2 ± 0.8 % following SD; $p > 0.05$). EEG SWA during NREM sleep significantly increased in both strains of mice (wild-type,

137.8 ± 13.1 % of baseline; GAD67-GFP mice 133.1 ± 15.0 %; P=0.02 for both strains, Wilcoxon Signed Rank Test, Fig. 4).

Discussion

Our major finding here is that GAD67-GFP knock-in mice have normal spontaneous sleep-wake patterns and sleep homeostasis. The GAD67-GFP knock-in mice had EEG characteristics indistinguishable from wild type controls, as verified by visual observation and spectral analysis.

It is generally established that controlled expression of GFP in neurons of transgenic mice is biologically inert [13]. Prolonged expression of GFP had no detectable effect on synaptic structure over a 9-month period [14]. Transgenic mice neurons that expressed GFP also had no apparent effect on cellular physiology [15]. Another strain of mice (GIN mice) that express GFP in a subpopulation of GABAergic neurons had no obvious physical or behavioral abnormalities [16]. Similarly, no abnormality in the brain was found at the macroscopic level in the GAD67-GFP knock-in mice used here [8]. Since GAD67-GFP mice lose one copy of the GAD67 gene, it is not surprising that GABA content in those mice is lower at birth [8]. However, this reduction is transient and is reversed in 7 weeks [8]. Since the mice used in our recordings were at least two months old, their brain GABA content is expected to be at a normal level.

Our study confirms this line of mice has sleep characteristics comparable to their wild type littermates. Both male and female mice displayed similar sleep patterns and thus, the data collected from them were pooled together. Subtle sex differences in sleep and sleep EEG in mice have been reported in some strains [17–19]. It is possible that we could identify such differences in GAD67-GFP knock-in mice following more polysomnographic recording and analysis. On the other hand, previous research has established that sleep in both male and female mice is strongly influenced by the genetic background [20,21]. Furthermore, homeostatic sleep characteristics do not seem to differ between different sexes, and the estrous cycle alone had an insignificant influence on sleep [19,21].

Sleep is regulated by the interaction of a circadian drive (termed process C) and a homeostatic drive (termed process S) [22]. Process C affects the timing of sleep. Thus, for nocturnal rodents such as mice, sleep is more frequent and of longer duration during the daytime. The fact that the GAD67-GFP knock-in mice slept more during daylight hours and had identical patterns of NREM and REM sleep as the wild type mice implies a normal circadian control mechanism. EEG SWA is the hallmark of NREM sleep and is an index of sleep and of Process S. It has been shown that sleep deprivation in mice induces a sleep rebound and enhanced EEG SWA during NREM sleep, especially during the first 6 hours after sleep deprivation [23,24]. Our results are consistent with previous observations and thus, it appears that GAD67-GFP knock-in mice have normal sleep homeostasis.

The theta rhythm is a prominent component of the EEG during REM sleep and during active waking (accompanied by movement) in rodents. GABAergic neurons in the septum, hippocampus and cortex play an important role in theta rhythm generation [25]. Our finding of prominent theta in the EEG during REM sleep (figures 1 and 3) suggests that these neurons are functioning normally in the GAD67-GFP knock-in mice. Thus, GFP can be used to identify and study these and other GABAergic neurons involved in the theta rhythm.

Sleep is thought to be regulated by multiple, interacting brain areas using different neurotransmitters. Amongst these neurotransmitters, GABA appears to be one of the most important, as reflected by the many drugs acting on GABA receptors which act as tranquilizers, sedatives or anesthetic agents. Our previous in vitro study [10] and our

findings here confirms the GAD67-GFP knock-in mouse as an ideal tool to investigate the role of GABAergic neurons in sleep-wake regulation.

Conclusion

We demonstrate here that GAD67-GFP knock-in mice have normal spontaneous sleep-wake patterns and a normal homeostatic sleep response after sleep deprivation. Thus, they are a useful tool for investigating the role of GABAergic neurons in the control of sleep related behavior both *in vivo* and *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Normal polysomnographic features of rapid-eye-movement (REM) sleep, non-REM (NREM) sleep and wakefulness are present in GAD67-GFP knock-in mice

Low-voltage theta frequency electroencephalogram (EEG) activity coupled with muscle atonia typical of REM sleep, high amplitude, slow EEG waves and reduced electromyogram (EMG) activity characteristic of NREM sleep and low-voltage fast EEG activity and high muscle tone typical of the waking state were observed (representative examples from one mouse are illustrated).

Figure 2. Similar diurnal distribution of sleep of wild type (WT, n=7; open circles) and GAD67-GFP knock-in mice (GFP, n=7; closed circles) across a 24 hour light-dark cycle
 The top panel shows the percentage of time spent in NREM sleep, and the bottom panel shows the percentage of time spent in REM sleep. The dark period is marked with a horizontal black bar. Note the increased sleep during the light period typical of nocturnal rodents in both strains of mice. No significant differences between strains were observed. Data are presented as mean $\pm$ SEM.

Figure 3. GAD67-GFP knock-in mice have the same EEG power spectrum characteristics in NREM sleep (top) and REM sleep (bottom) as seen in wild type mice
 Relative average EEG power over 24 hours in the 0–20 Hz band (1 Hz bins) of wild type (n=7; open circle) and GFP (n=7; closed circle) mice are expressed as a percentage of total power across a 24 hour period. Note the peak power in the delta range (0–4 Hz) during NREM sleep and the peak power in the theta band (4–8 Hz) during REM sleep in both strains. No significant differences between strains were observed. Data are expressed as mean±SEM.

Figure 4. Both GAD67-GFP knock-in mice and wild type mice have a homeostatic sleep rebound after a 6 hour sleep deprivation

Top panel: Compared to baseline levels at the same time point, the percentage of NREM sleep significantly increased ($p < 0.05$; paired t-test, asterisks) in both wild type and GAD67-GFP knock-in mice following sleep deprivation (1000 to 1600 hr) in the following 6 hour period (1600 to 2200 hr). Bottom panel: EEG slow-wave activity (SWA, 0–4 Hz) was enhanced by over 30% in both strains of mice after sleep deprivation (baseline level was normalized as 100%). Data are presented as mean $\pm$ SEM ($N = 7$ for each strain).