Chapter 4

Reduction in Munc18-1 expression leads to loss of atonia during REM sleep

Robert Hensbroek[a], Natasja de Bruin[b], Matthijs Verhage[a] and Berry Spruijt[a,c].

[a]   *Rudolf Magnus Institute for Neurosciences*
        Utrecht University Medical Center
        Universiteitsweg 100
        3584CG Utrecht
        The Netherlands

[b]   *NICI / Department of Psychoneuropharmacology and*
        Department of Comparative and Physiological Psychology
        University of Nijmegen
        P.O. Box 9104
        6500 HE Nijmegen
        The Netherlands

[c]   *Present address:*
        Animal Welfare Center
        Faculty of Veterinary Medicine
        Utrecht University
        Yalelaan 17
        3584CL Utrecht
        The Netherlands

Abstract

REM sleep is associated with vivid dream mentation, desynchronous cortical EEG and paralysis of the muscles (atonia). A number of disorders of REM sleep exists, one of which is REM sleep behavior disorder (RBD). RBD is characterized by the intermittent loss of atonia during REM sleep. Here, we report that munc18-1 gene-dose mutant mice show a phenotype that resembles RBD. This mutant shows intrusions of abnormal motor activity during sleep. This behavior consists of tail twitches, limb movements, body stretching, body rocking, body shaking and jumps, after which the mice often wake up. EEG recordings showed that this abnormal motor activity coincided with cortical desynchrony that had a high power at 5-8Hz, which is a feature of REM sleep. No indications of epileptic discharges were observed. Thus, our results show that a reduction of munc18-1 protein levels leads to abnormal motor behavior during REM sleep, and suggests that this mutant may prove to be a genetic model for RBD.

Introduction

Rapid eye movement (REM) sleep, also known as paradoxal sleep, is associated with ocular saccades, vivid dreaming, desynchronous cortical EEG with a predominance of theta rhythm, and atonia (paralysis) of the postural muscles. A number of disorders of REM sleep exist, such as narcolepsy and REM sleep behavior disorder. A prominent aspect of narcolepsy is cataplexy, which is the intrusion of atonia during wakefulness. The study of narcolepsy, and of REM sleep in general, was greatly aided by genetic canine models (Foutz et al., 1979;Baker et al., 1982;Siegel et al., 1991), that turned out to have mutations in the hypocretin/orexin receptor gene (Lin et al., 1999). Soon afterwards, a deficiency of hypocretin/orexin was demonstrated in human narcoleptics (Nishino et al., 2000;Peyron et al., 2000). Apparently the opposite of cataplexy is REM sleep behavior disorder (RBD). In this disorder, all the components of REM sleep are normal except for atonia, which is frequently lost and results in complex motor behaviors that are often injurious and associated with dreaming (Schenck et al., 1986). The exact prevalence is unclear, but recent studies suggest it might not be an uncommon condition (Schenck et al., 1993;Olson et al., 2000).

Atonia appears to be regulated by the pontine-medullar system. Lesions in pontine areas or the medial medulla disrupts atonia during REM sleep (Jouvet, 1980;Schenkel and Siegel, 1989;Shouse and Siegel, 1992). Furthermore, a portion of pontine and medullar cells fire at a high rates during REM sleep (el Mansari et al., 1989;Kanamori et al., 1980;Steriade et al., 1990;Kayama et al., 1992;Schenkel and Siegel, 1989). Interestingly, in narcoleptic dogs, a number of medullar neurons increase their firing rate only during REM sleep and during cataplectic attacks, which specifically implicates these neurons in atonia but not in other aspects of REM sleep (Siegel et al., 1991). Finally, electrical stimulation of the medial medulla causes paralysis in awake cats (Hajnik et al., 2000), which appears mediated by direct inhibitory connections to motor neurons in the spinal cord (Morales et al., 1987;Fort et al., 1993). Thus, there appears to be a neuronal system in the brainstem that is activated during REM sleep and specifically involved in the maintenance of atonia.

In the present paper, we have studied sleep in munc18-1 gene-dose (heterozygous) mutant mice. Munc18-1 is present in all neurons, and has been implicated in release of neurotransmitters and hormones (Garcia et al., 1995;Verhage et al., 2000;Voets et al., 2001). Previously, it was demonstrated that gene-dose mutants show a decrease of munc18-1 protein levels throughout the brain. This decrease did not affect basal transmission and paired pulse facilitation but caused an enhancement of tetanic depression, a blockade of post-tetanic potentiation and a severe impairment of mossy fiber LTP. Thus, it appears that a reduction of munc18-1 leads to impairments in secretion specifically during and after high neuronal activity (Chapter 2). Furthermore, this mutant showed hyperactivity in an open field task and deficits in a working memory task (Chapter 3).The present study was initiated to analyze the behavior of this mutant under conditions of rest in the home-cage. We report that munc18-1 gene-dose mutant mice did show higher behavioral activity in the home cage. Furthermore, when they appeared to be asleep, mutants displayed short episodes of abnormal motor activity, which appeared to coincide with REM sleep.

Material & Methods

Mice

Mutants were created by deleting the second exon of the munc18-1 gene by homologous recombination (Verhage et al., 2000). Subsequently, mutants were repeatedly backcrossed to 129S3 (also known as 129/SvImJ; Jax code: JR2448) for at least 6 generations, resulting in a line with a standardized genetic background. Mice were bred in our laboratory under standard conditions. At 25 days, they were weaned and housed with 2-4 mice of the same sex. Mice were kept at a 12h-light/dark cycle with lights on at 7:00 PM. Food (Hope Farm) and water was freely available. Heterozygotes were used for the experiments. Wildtype littermates served as controls. The experimenter was always blind with respect to genotype until the end of the experiments. Mice were genotyped by PCR, which was occasionally confirmed by western blot. All experiments were approved by the Ethical Committee of the Utrecht Medical Center.

Behavioral recordings

At the age of 3 months, mice were individually housed and allowed to habituate to this situation for 24 hours. The next day, during the fifth hour of the light cycle (from 0:00 to 1:00 A.M.) video recordings were made, which were analyzed afterwards. Two behavioral categories were used. The category inactive behavior was used to represent quiet rest and sleeping, which were defined as the lack of body movements. The category active behavior was used to measure active wakefulness and consisted of walking, digging, rearing, climbing, scanning, eating, drinking and grooming. Also, the number of motor bursts was counted. These motor bursts consisted of uncoordinated jumps and vigorous body shakes.

Detailed video-recordings were made from the mice that were used for the EEG recordings. These recordings were used to analyze episodes of abnormal motor activity during sleep in more detail. The categories were inactivity, tail movements, chewing, stretching, walking, body rocking (slow rocking motion of the whole body), body shaking (vigorous body movements) and jumps.

Electroencephalogram (EEG) analysis

2 wildtypes and 2 heterozygous mutants were implanted with cortical electrodes. Mice were anaesthetized with a Hypnorm (Janssen Pharmaceutica Inc., Beers, Belgium), Dormicum® (Roche Netherlands Inc., Mijdrecht, The Netherlands) and demiwater mixture (1:1:2), at a dose of 2-3µl per gram bodyweight, injected subcutaneously. After surgery, post-operative care was given with a subcutaneous injection of Temgesic® (Reckitt Benckiser plc, Berkshire, United Kingdom) at a dose of 1µl / gram bodyweight and an injection of saline (20µl/gr bodyweight). The electrodes were placed on the on the surface of the cortex: one in the frontal region (coordinates with the skull surface and bregma zero-zero (A 1.5, L –2.0), and the other in the parietal region (A –3.5, L –3.0) (Franklin and Paxinos, 1997). The ground electrode was placed over the cerebellum. After surgery, the animals were housed separately and were allowed to recover for at least 2 weeks.

EEG recordings were made for 12 hours, beginning at the start of the light-phase. Mice were put in a transparent recording cage and connected to EEG leads. The EEG (in a bandwidth between 1 and 100Hz, sample frequency of 256Hz) was amplified, digitized, monitored and stored for off-line analysis

using the WINDAQ system (DATAQ Instruments, Akron, OH). Simultaneously, continuous video recordings were made. EEG traces were analyzed using Fast Fourier Transformatio n (FFT).

Statistics

The behavioral data was analyzed using univariate ANOVA for parametric data, and Kruskall Wallis and Mann Whitney U for non-parametric data.

Results

Mutants show higher levels of behavioral activity and abnormal motor bursts.

Previously, it was observed that munc18-1 gene-dose mutants showed hyperactive behavior in the open-field task (Chapter 3). The present study was started to determine whether this hyperactive behavior persisted in the home-cage. Munc18-1 gene-dose mutant mice were monitored in the home-cage for a period of 60min during the fifth hour of the light phase. Munc18-1 gene-dose mutants showed a five-fold increase in active behavior (p<0.001)(Table1). Surprisingly, heterozygotes showed occasional bursts of motor activity that consisted of jumps and vigorous body shakes. These bouts of abnormal activity occurred in all munc18-1 gene-dose mutants (ranging from 1 to 9 bursts), but were

Table 1. Behavior during the light phase in wildtypes (n = 5 males and 6 females) and munc18-1 gene-dose mutants (n = 3 males and 5 females). Shown are means and SEM. Mutants showed more active behavior then wildtypes ($F_{1, 15}$ = 25.2, p<0.001). No other effects on active behavior were detected (sex: $F_{1, 15}$ = 0.05, n.s., sex*genotype: $F_{1, 15}$ = 0.61, n.s.). Heterozygotes showed occasional bursts of motor, which was never observed in wildtypes (Z=-4.05, p<0.001).

	wildtype	munc18-1	statistics
active behavior (min per hour)	3.4 ± 1.2	17.8 ± 2.6	p < 0.001
motor bursts (number per hour)	0	4.4 ± 1.0	p < 0.001

never observed in wildtype mice (p<0.001). No differences were observed between males and females. Motor bursts are preceded by periods of u ncoordinated motor activity.

The motor bursts were further analyzed using a new set of video recordings of individual mice that allowed a more detailed analysis. During the whole recording period, wildtype mice never showed conspicuous motor activity. Again, gene-dose mutants showed motor bursts, and these motor bursts were usually preceded by short episodes of motor activity that was of a less vigorous nature. Behavior during these episodes had an uncoordinated and undirected appearance, and consisted of raising and twitching of the tail, chewing, body stretching, short walks, body rocking, and were often concluded by body shakes (28%) or jumps (41%) through the cage (Fig.1). On occasion, abnormal grooming behavior was observed. During this abnormal groomi ng the paws did not touch the face. Prior to these episodes of abnormal behavior, mice always had their eyes closed, were completely inactive and seemingly asleep. Also, during the episodes of abnormal motor behavior they kept their eyes closed
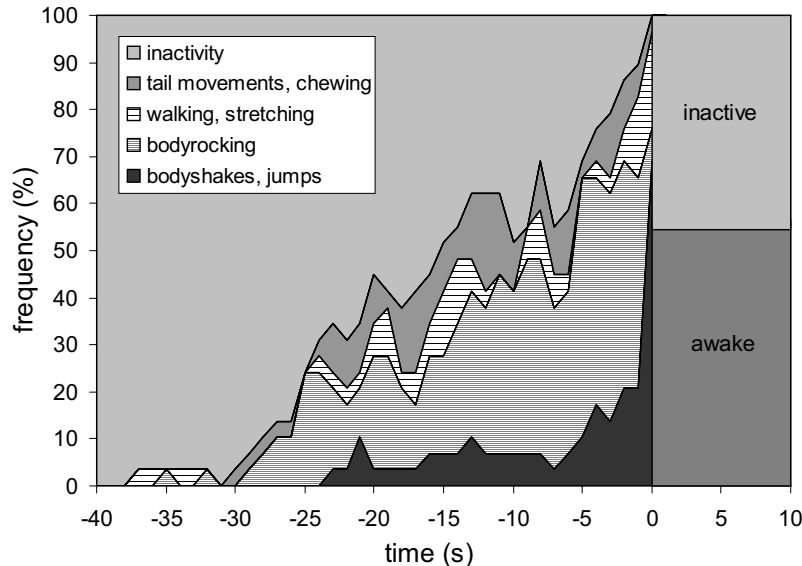
Figure 1. Description of abnormal motor behavior. 29 of these episodes from videorecordings of 2 male gene-dose mutants were collected and analyzed. These episodes were aligned at the moment these episodes of abnormal behavior ended (at t=0). Behavior was classified in a mutually exclusive fashion in the categories inactivity, tail movements and chewing, walking and stretching, body rocking, and finally vigorous bodyshakes and jumps. The graph shows that the latter categories were present to a greater extent towards the end. When these episodes ended, mice became inactive and seemingly asleep in 45% of the episodes. In 55% of the episodes they appeared to have been awakened.

and these episodes were often interrupted by short periods of complete inactivity. Thus, this abnormal motor activity appeared to occur during sleep. Jumps almost always caused awakening (92%), the other classes of behavior were usually followed by inactivity (41% resulted in awakening). In general these episodes of abnormal behavior caused awakening in approximately half (55%) of the cases, but in a large percentage of cases (45%) mice resumed inactive behavior with closed eyes. Thus, these behavioral observations suggest that this abnormal motor behavior occurred during sleep. This hypothesis was further explored using EEG recordings.

Motor activity occurred during theta rhythm

In order to determine if the motor acts occurred during sleep, and if so, to determine the phase of sleep, mice were prepared for EEG analysis. The mice were videotaped for 12 hours and continuous EEG measurements were made. During inactivity, wildtype mice showed periods of large, irregular waves and periods of small, regular waves, which are characteristics of slow-wave sleep and REM sleep, respectively (Hobson et al., 1998). Gene-dose mutants also showed episodes of inactivity with synchronous or desynchronous EEG but, as observed before, these periods of inactivity were frequently interrupted by episodes of abnormal motor activity. Corresponding EEG traces were collected and revealed that these episodes of abnormal motor activity coincided with cortical desynchrony. Furthermore, after Fast Fourier Transformation (FFT) the traces showed a predominant theta rhythm of 5-8Hz (Fig.2). As mentioned, theta rhythm is a feature of REM sleep and these results indicate that the gene-dose mutant mice show REM sleep without atonia. Representative examples of individual EEG traces and corresponding FFT of heterozygous mutants are shown in Fig.3. The first and second traces were collected when mice were inactive and had their eyes closed. Fig.3A shows an example of synchronous waves. Note the large, irregular waves and the predominance of a 1-4 Hz
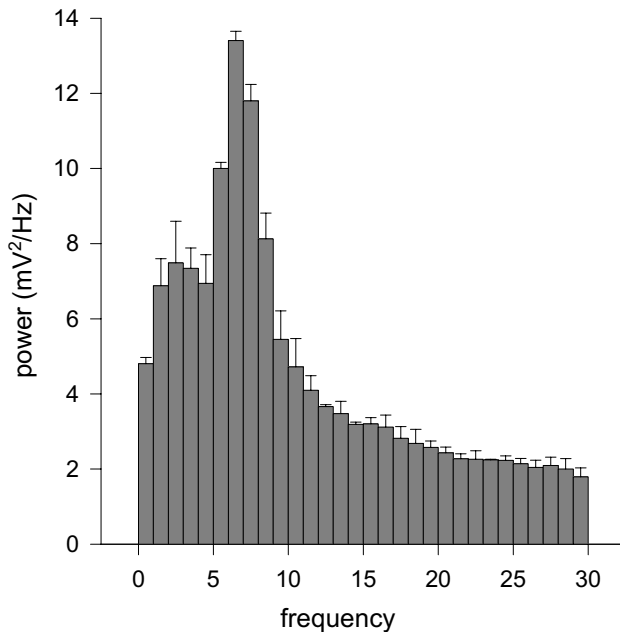
Figure 2. Fast Fourier Transformation of EEG traces that coincided with the episodes of abnormal motor behavior. 10 episodes of abnormal behavior that lasted at least 10s were collected from each mouse. FFT was applied and the 10 episodes were averaged for each mouse. Shown are means ± SEM (n = 2 males).

rhythm in the FFT figure. These are characteristics of slow-wave sleep (Hobson et al., 1998). Behavioral abnormalities never coincided with such EEG traces indicating that mutants had normal slow-wave sleep. Fig.3B shows an example of desynchronous EEG. The FFT shows a predominance of theta (5-8 Hz) rhythm. The combination of behavioral inactivity, closed eyes, and predominant theta rhythm suggests that mutants do show episodes of normal REM sleep. Furthermore, these periods of normal REM sleep appeared to outnumber the episodes with abnormal motor activity. Fig.3C shows a trace of sleep with motor activity, which in this case consisted of chewing and vigorous body shakes. Note the resemblance with Fig.3B, and the predominance of theta rhythm. Furthermore, sharp wave discharges such as those typical for epileptic attacks (Montagna et al., 1997) were not present. Fig.3D shows a trace taken from an episode of body shakes that was concluded with a violent jump. Again, this trace shows cortical desynchrony with a predominance of theta rhythm. The blow-up shows that during, and immediately before the jump, erratic waves were not present. Sharp wave discharges were never observed throughout the recording period. Thus, the abnormal motor behavior in munc18-1 gene-dose mutants was unrelated to epileptic attacks.

Discussion

Munc18-1 gene-dose mutants displayed episodes of abnormal motor activity that intruded on periods of rest. All mutants showed it, but none of the wildtypes (approximately 40 hours of behavioral recordings of wildtypes were made), and males and females were affected to a similar degree. Furthermore, this phenotype was observed in young adults of 3 months of age. Thus, this phenotype displays an autosomal dominant inheritance pattern that has full penetrance at an early age. EEG
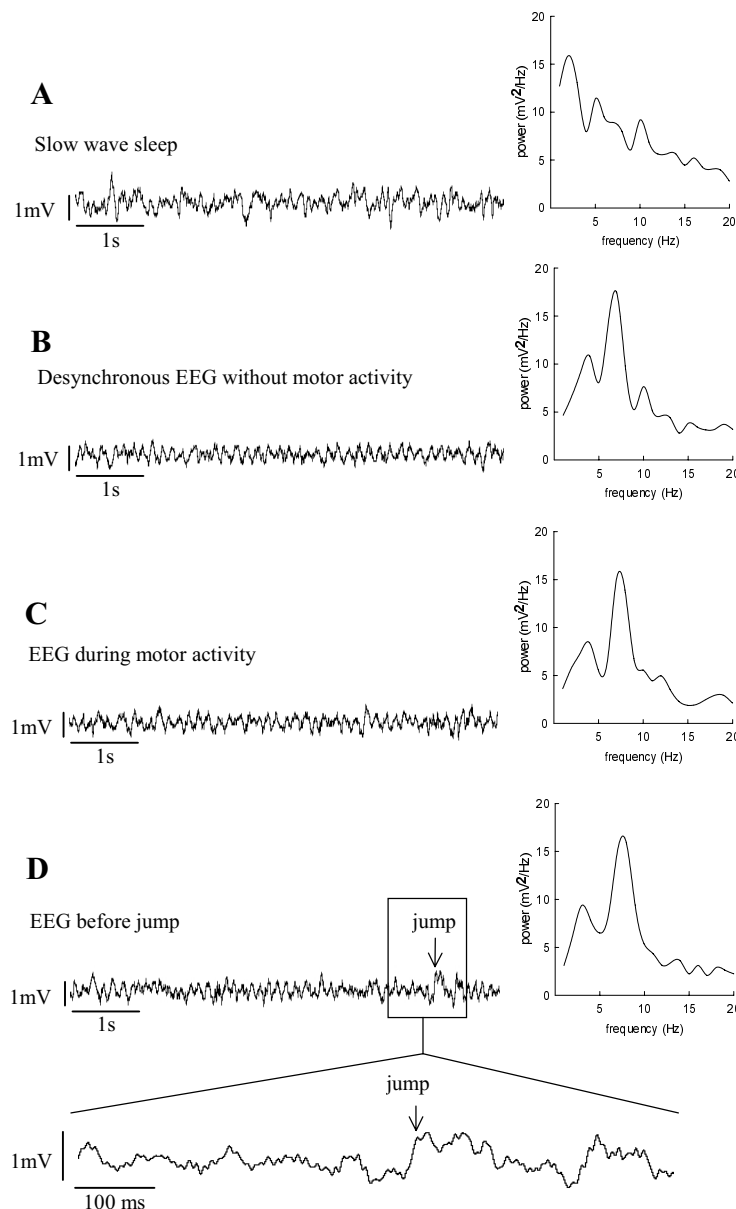
Figure 3. Individual EEG traces with FFT. **A.** example of synchronous EEG during a period of inactivity. The EEG trace on the left shows large, irregular waves and FFT on the right shows a predominance of delta waves (1-4 Hz). **B.** example of desynchronous EEG during behavioral inactivity. The FFT shows a predominance of theta waves (5-8 Hz). **C.** example of abnormal motor activity without a jump. The FFT shows cortical desynchrony and a predominance of theta rhythm. **D.** example of an episode of bodyshakes that was concluded with a violent jump. Again, this trace shows cortical desynchrony with a predominance of theta rhythm. The blow-up shows the trace immediately before and after the jump.

measurements showed that the abnormal motor activity coincided with cortical desynchrony with a predominance of theta rhythm. No signs of epileptic attacks were observed. Thus, the munc18-1 gene-dose mutant appears to have abnormal motor activity during REM sleep.

During REM sleep the brain is highly activated and a suppression of motor output is necessary to prevent uncoordinated motor activity. This suppression seems to be mediated by a specialized system in the pontine-medullar region (Jouvet, 1980;Schenkel and Siegel, 1989;Shouse and Siegel, 1992). A proportion of medullar neurons fire at high discharge rates only during atonia (Siegel et al., 1991) and it appears that these neurons suppress motor activity by direct glycinergic inhibitory connections to motor neurons in the spinal cord (Morales et al., 1987;Fort et al., 1993). Munc18-1 gene-dose mutant mice show reductions in munc18-1 protein levels in the brainstem (Chapter 2). These mutants had normal slow-wave sleep, indicating that their phenotype was specific for REM sleep. Also, REM sleep

was often normal, indicating that the mechanisms that enable atonia were still present. However er, longer periods of REM sleep require longer periods of atonia, and consequently longer periods of high neuronal activity (Siegel et al., 1991). Thus, the appearance of abnormal motor activity during REM sleep may be caused by impairments in maintaining secretion during high neuronal activity resulting in the gradual loss of motor inhibition during REM sleep.

REM sleep behavior disorder (RBD) is characterized by a frequent loss of atonia during REM sleep, and results in complex motor behaviors that are often injurious and associated with dreaming (Schenck et al., 1986). The munc18-1 gene-dose mutant displays a phenotype that resembles RBD and may prove to be a valuable genetic model for this disorder. The phenotype that we have described is fully penetrable in both males and females, and already present at an early age. This, however, is not the case in human patients with RBD. Onset of this disorder is at approximately 60 years of age, there is a strong male preponderance and familial cases have not been observed (Schenck et al., 1993;Olson et al., 2000). Thus, there is no sign of a dominant inheritance pattern as that observed in the gene-dose mutant mice.

In summary, a reduction of munc18-1 in munc18-1 gene-dose mutants leads to abnormal motor activity during REM sleep. This phenotype may have been caused by the loss of atonia as a result of impairments in secretion during high neuronal activity. The munc18-1 gene-dose mutant may prove to be a mouse model for RBD on the physiological level. Further research is required to unravel more of the molecular mechanisms that regulate atonia during REM sleep.