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Pharmacology, Biochemistry and Behavior 78 (2004) 765–773

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Altered sleep latency and arousal regulation in mice lacking norepinephrine

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Received 19 September 2003; received in revised form 12 May 2004; accepted 17 May 2004
Available online 2 July 2004

Abstract

Latency to sleep and the amount of sensory stimulation required to awaken an animal are measures of arousal threshold, which are ultimately modulated by an arousal regulation system involving many brain areas. Among these brain areas and network connections are wake-promoting nuclei of the brainstem and their corresponding neurotransmitters, including norepinephrine (NE). In this study, we used mice that are unable to produce NE to study its role in regulating sleep latency after a variety of interventions, and to study arousal from sleep after sleep deprivation (SD). Sleep latency was measured after gentle awakening or after injections of saline, caffeine or modafinil. Sleep latency was also measured before and after partial restoration of NE pharmacologically. Arousal threshold was measured by recording the number of decibels of white noise required to wake each mouse from NREM sleep after 0, 3 and 3 + 3 h SD (3 h SD followed by < 2 min sleep, followed by an additional 3 h SD). Results showed that when mice were awakened without being touched, there were no differences in sleep latency between the genotypes. However, after an injection of saline, the control mice increased their sleep latency, whereas the NE-deficient mice did not. There were no group differences in sleep latency after treatment with either stimulant. The sleep latency difference between the genotypes was ameliorated by partial restoration of NE. The arousal threshold experiments revealed that significantly more noise was required to wake the NE-deficient mice after 3 and 3 + 3 h of SD. These findings show that mice lacking NE fall asleep more rapidly only after a mild stressor, such as an intraperitoneal injection. NE-deficient mice are also more difficult to wake up using audio stimulation after SD. The results presented here suggest that NE promotes wakefulness during transitions between sleep and wake under conditions involving mild stress and SD, but not under baseline circumstances.

Keywords: Sleep; Sleep latency; Knockout; Mouse; Arousal; Sleep deprivation; Caffeine; Modafinil

1. Introduction

Throughout their daily lives, mammals experience three states of consciousness: wake, NREM sleep and REM sleep. The transitions between these states are physiologically regulated, and have evolutionary significance. For instance, studies have shown that autonomic responses to spontaneous and elicited awakenings from sleep are greatly and transiently elevated, which probably serve to heighten arousal in preparation for danger (Horner et al., 1997; Trinder et al., 2003). In addition, the noradrenergic neurons of the locus coeruleus (LC) spontaneously fire not only during heightened periods of arousal, but they increase their firing just prior to a change in state between NREM sleep and wake, suggesting that the neurotransmitter norepinephrine (NE) participates in this alerting behavior upon waking (Aston-Jones and Bloom, 1981). In the present study, we investigate if NE plays a critical role in the arousal regulation of mice genetically deficient in NE. Because NE is traditionally thought of as a "wake-promoting" neurotransmitter, we hypothesized that the NE-deficient mice would have a shorter sleep latency after being awakened gently or with an injection of saline, and that they would require a higher level of noise to awaken them from sleep. We also tested their sleep latency after injections of common stimulants, to determine if the lack of NE altered the "normal" behavioral reaction to these two drugs.

The noradrenergic and serotonergic neurons of the reticular activating system of the brainstem, along with histaminergic neurons from the hypothalamic tuberomam-

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doi:10.1016/j.pbb.2004.05.008

milary nucleus, have been shown by electrophysiology to have high activity during the waking state, low activity during NREM sleep and to be nearly quiescent during REM sleep (Aston-Jones and Bloom, 1981; McGinty and Harper, 1976; Steininger et al., 1999). Among these "wake active" nuclei is the noradrenergic LC. Increased NE levels and/or LC firing is highly associated with periods of increased arousal or attention, and cortical activation (Aston-Jones and Bloom, 1981; Aston-Jones et al., 1994; Berridge and Foote, 1991; Berridge et al., 1993; Foote et al., 1991; Kodama et al., 2002; Rajkowski et al., 1994; Smith and Nutt, 1996). In addition, these "wake active" nuclei have anatomical and functional connections with the NREM sleep-generating ventral lateral preoptic area (VLPO), which uses the inhibitory neurotransmitter GABA to inhibit these nuclei during sleep (Gallopin et al., 2000; Nitz and Siegel, 1997; Sherin et al., 1996, 1998; Szymusiak et al., 1998). There is also strong evidence showing a causal reciprocal relationship between the activity of the reticular activating system (including NE in particular) and the cholinergic pedunculopontine tegmentum in the generation of REM sleep (Crochet and Sakai, 1999; Hobson et al., 1975; Jones, 1991; Monti et al., 1988; Sakai, 1988; Singh and Mallick, 1996).

Given the participation of NE in the wake- and alertness-promoting systems in the brain, it follows that animals deficient in NE might have altered wake states or altered transitions to and from the waking state. Thomas et al. (1995) created mice that are chronically deficient in NE and epinephrine, by genetically targeting the dopamine β-hydroxylase gene; these mice are referred to here as either NE-deficient or "knockout" mice. The mice require pharmacological restoration of NE prenatally for survival, but after birth they develop normally without intervention. The mice are deficient in epinephrine also, but because there are very few adrenergic nuclei in the central nervous system, and sleep is a centrally generated state, we are primarily interested in how NE acts in the brain to modulate the sleep and wake states.

Stress is a variable that can have large and wide-ranging effects on animal behavior. Acute stressors, such as restraint stress or footshock, can increase sleep latency after the stressor is discontinued (Gonzolez et al., 1995; Koehl et al., 2002; Vazquez-Palacios and Velazquez-Moctezuma, 2000), although many studies do not report sleep latency (Marinesco et al., 1999; Meerlo et al., 2001). Rodents experience increases in measures of physiological stress in response to intense stressors, such as restraint or cold stress (Lenox et al., 1980; Meerlo et al., 2001); however, laboratory mice routinely experience less intense stressors, such as handling, cage changing and injections.

In the physiological response to stress, NE contributes to the stimulation of the hypothalamic–pituitary–adrenal axis (Pacak et al., 1995; Tsigos and Chrousos, 2002). We have previously shown that NE-deficient mice have shorter sleep latencies after an injection of saline or a low dose of amphetamine; however, it is unclear if this phenotype is associated with the stress of handling (Hunsley and Palmiter, 2003). In the present study, sleep latency after a saline injection is compared with gentle awakening that does not include handling. In addition, the present study measures sleep latency both at the beginning of the light portion of the light/dark cycle and at the end, to determine if the sleep latency difference in the knockouts is influenced by a previous period of dark or light.

Another measure of arousal threshold is the amount of stimulation required to awaken an animal from sleep. In the current study, we recorded the amount of noise, in decibels, which was required to awaken mice from NREM sleep. In humans, investigators have studied arousal from all stages of sleep due to external noise generation (Bonnet and Moore, 1982; Mullin and Kleitman, 1938). In general, arousal threshold from sleep varies according to time spent asleep and sleep stage (Bonnet and Moore, 1982), and is modifiable by sleep-promoting drugs (Cluydts et al., 1995; Saletu and Grunberger, 1981; Saletu et al., 1980). Because NE-deficient mice are lacking a neurotransmitter that has traditionally been associated with wake-promotion, we predicted that the knockouts would be more difficult to wake up from NREM sleep than the controls. Several hours of sleep deprivation (SD) were included to potentially exacerbate this phenomenon.

Caffeine and modafinil, which are CNS stimulants, increase wake and locomotion in mice (Duteil et al., 1990; Edgar and Seidel, 1997; Wisor et al., 2001). These stimulants utilize a variety of wake-promoting systems and associated neurotransmitters, including adenosine, dopamine, serotonin, hypocretin/orexin, acetylcholine and NE (Chemelli et al., 1999; Duteil et al., 1990; Hadfield and Milio, 1989; Kirch et al., 1990; Porkka-Heiskanen et al., 1997; Strecker et al., 2000; Wisor et al., 2001). Because NE-deficient mice only lack NE, these stimulants should still increase waking behavior through the remaining, intact systems. Therefore, we sought to test the hypothesis that stimulation of nonnoradrenergic wake-promoting pathways would cause the knockout mice to exhibit a sleep latency duration that was similar to the control mice.

2. Methods

2.1. Animals

Mice lacking NE and Epi (knockouts) were created by inactivating the dopamine β-hydroxylase gene (*Dbh*; Thomas et al., 1995), and maintained on a mixed C57BL/6J and 129/SvCPJ hybrid background. Heterozygous mice were used as controls because they have normal NE levels (Thomas et al., 1998). Control and knockout mice were bred under specific pathogen-free conditions according to a protocol that allows production of the knockout animals (Thomas et al., 1995). Adult mice were

housed in a modified specific pathogen-free animal room appropriate for sleep studies. The mice were maintained on a 12:12 L/D cycle, with food, water and nesting material available at all times. All the mice were group housed unless otherwise noted, with two knockouts and two controls per cage.

All female mice were used for these experiments. We have observed that male *Dbh* knockouts do not survive surgery using ketamine/xylazine anesthetic at a high enough rate to justify their use. Estrous cycle stage was not taken into account, primarily because the mice were housed in an isolated room where no male mice were present. Ovulation is either suppressed in group-housed female mice, or they do not cycle at all (Champlin, 1971; Clee et al., 1975; Whitten, 1959). Regardless, the sleep changes described in several studies with proestrus females reveal changes only at night (Fang and Fishbein, 1996; Schwierin et al., 1998; Zhang et al., 1995), whereas in the present study, we were interested in sleep latency (not sleep amounts) during the daytime. Only one published study used female mice, and the effects of estrus on sleep were minimal and dependent on strain (Koehl et al., 2003).

The mice in Experiment 1 had not received any drug injections (except for surgery anesthetic) prior to the saline injection and the gentle waking procedures. The mice were approximately 5 months old for this experiment. The same group of mice then received modafinil and caffeine, in that order, and were approximately 6 and 7 months of age, respectively. Each mouse had 3–4 weeks between the two drugs. For Experiments 2 and 3, a different group of mice was used. They were first subjected to the noise and SD in Experiment 3 and were drug naïve at that time. Two to 3 months later, they received the injections of amphetamine and DOPS as described in Experiment 2. Although sleep stage percentages are known to change according to age, because we were only measuring sleep latency and the ages of the mice were the same between genotypes, we were less concerned about age-related sleep changes.

All procedures adhered to the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences Press, Washington, DC, 1996) and were approved by the University of Washington Institutional Animal Care and Use Committee.

2.2. Surgery

Female NE-deficient and control littermates were implanted with EEG and EMG electrodes between 2 and 4 months of age. Animals were anesthetized with a mixture of ketamine and xylazine (13 and 0.88 mg/ml, respectively, at a volume of 10 μl/g) and four miniature stainless steel screws (0.9 mm diameter, Small Parts, Miami Lakes, FL) attached to 36-gauge, Teflon-coated solid silver wires (Cooner Wire, Chatsworth, CA) were inserted into the skull. These electrodes were placed over the right frontal cortex (approximately 0.5 mm anterior to bregma, approximately 0.5 mm lateral to the central suture), right and left parietal cortex (approximately 1 mm posterior to bregma, approximately 2–3 mm lateral to the central suture) and cerebellum (approximately 0.5 mm posterior to lambda, in line with the central suture). The left parietal electrode served as the ground. The wires were crimped to a small 6-channel connector (Microtech, Boothwyn, PA) that was attached to the skull with dental acrylic. Two of the same silver wires were sewn through the nuchal muscles and also crimped to the headpiece, serving as the EMG electrodes. After the surgery, mice were singly housed and allowed to recover with their cage placed halfway on a heating pad (35–40 °C). After 24 h of recovery, mice were rehoused with two to four littermates. At least 3 weeks of postsurgical recovery preceded the sleep recordings.

2.3. Recording procedure

One week prior to the sleep recordings, mice were singly housed. At least 24 h before the recording, two knockout and two control mice were placed in separate high-walled, open-topped polycarbonate mouse cages containing bedding and nesting material. Food and water were available ad libitum. A cable (20 cm long) was attached to the headpiece on the mouse and then to a commutator/swivel (Plastics One, SL6C/SB lowest torque, Roanoke, VA), which was connected to a model 15 Grass polygraph (Astro-Med, West Warwick, RI) that digitally recorded EEG and EMG activity at a rate of 200 samples/s. The cable was constructed from six, 38-gauge, stainless steel Teflon-coated wires plus one wire (1 mm diameter) that was stiffer and provided support (Cooner Wire). Cyanoacrylate glue was used to cement the wires at both ends of the cable (approximately 1 cm at each end). The cable was lightweight (approximately 1 g experienced by the mouse), and the mice were able to move, groom, eat and drink freely. Because of this, they habituated to the recording chamber and cable within a few hours. EEG/EMG data were recorded and processed using the Grass-Telefactor PolyviewPro (Astro-Med) data acquisition software for Windows 98.

2.4. Analysis of sleep recordings

Sleep/wake data were visually scored for the states of wake, NREM sleep and REM sleep. Scoring was done with the Grass Rodent Sleep Stager program, which facilitates manually scored 10-s epochs. During the scoring procedure, the high-pass digital filters were set at 1 and 30 Hz for EEG and EMG, respectively, and the low-pass filters were set at 15 and 100 Hz for EEG and EMG. Wake was considered to be higher frequency (>10 Hz), lower amplitude, with medium to high muscle activity. NREM was considered to be lower frequency (1–4 Hz), higher amplitude, and low to medium muscle activity. REM consisted mostly of theta waves (5–9 Hz) at a low amplitude, with low muscle activity. Each epoch had to

contain at least 50% of wake, NREM or REM for it to be labeled that state.

2.5. Drugs

Caffeine (Sigma, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS). Modafinil (Cephalon, West Chester, PA) was suspended in 0.25% methylcellulose and PBS, and mixed thoroughly before the intraperitoneal injection.

L-threo-3,4-dihydroxyphenylserine (DOPS; Sumitomo Pharmaceuticals, Osaka, Japan) is a synthetic amino acid that can be converted to NE by aromatic L-amino acid decarboxylase, bypassing the need for dopamine β-hydroxylase, the enzyme that normally converts dopamine into NE (Thomas et al., 1998, 1995). DOPS was prepared at 10 mg/ml as described (Thomas et al., 1995). Carbidopa (Sigma) was added to the DOPS solution at 5 mg/ml, along with 2 mg/ml ascorbic acid (Sigma); this cocktail was injected subcutaneously at a dose of 25 μl/g body weight.

2.6. Experiment 1

2.6.1. Measuring sleep latency

The time between the intervention (injection or verbal awakening) and the first two epochs of NREM sleep was measured. The epochs of NREM sleep had to be followed by a large bout of sleep (at least 2 min of continuous sleep, followed by a 10-min period consisting of 75% sleep or more) and not an isolated sleep period surrounded by wake.

2.6.2. Wake and saline injections

At the beginning of the light part of the light/dark cycle, mice were awakened by the experimenter entering the recording room and gently arousing them if they were asleep. This was done by speaking in a normal voice, and if a mouse did not awaken, the experimenter gently tapped the cage with a pen. Mice were not touched and the experimenter left the room within 1 min. The same procedure was repeated approximately 8 h later. The next day, at the beginning of the light period, the experimenter entered the room and picked up each mouse in succession (cable still attached) and gave it an intraperitoneal injection of 0.25 ml PBS. Eight hours later, the procedure was repeated. There were eight knockouts and eight control mice used for this experiment, except for the "morning saline" injection group, where only six of each genotype were used.

2.6.3. Drug injections

At the beginning of the light part of the light/dark cycle, mice were injected with caffeine (25 mg/kg, knockouts = 11, controls = 10), or modafinil (90 mg/kg, $n = 8$). The same mice were used for each drug, with 3–4 weeks between different drugs.

2.7. Experiment 2

Shortly after lights on, the EEG recording was started and the mice were given an intraperitoneal injection of 0.25 mg/kg amphetamine (Sigma). Approximately 3 h later, the mice were given a subcutaneous injection of 250 mg/kg DOPS; 5 h later, the mice were injected with the same concentration of amphetamine. Sleep latency was measured from the EEG recording after the injections of amphetamine. Eight knockout mice and seven control mice were used for this experiment.

2.8. Experiment 3

2.8.1. Apparatus

The mouse was housed in a high-walled recording cage and attached to the cable and commutator as in the previous experiments. Adjacent to the recording cage was another cage containing bedding and a sound-level meter (Radio Shack, Fort Worth, TX). A speaker (Vita tweeter, model #D19TD-05-08, 9.25 cm diameter, Ruark Acoustics, Essex UK) was suspended 35 cm above both of the cages, so that the same level of noise was detected in both the cage containing the mouse and the cage with the sound-level meter (± 1 dB). A white noise generator, switch and amplifier were used to provide the white noise. The noise was generated at a 50% duty cycle, 2.5 ms rise/fall time, at a rate of 4 bursts/s.

2.8.2. Procedure

Mice were singly housed 1 week before testing. Thirty-six to 48 h before the morning of testing, one mouse was attached to the cable and hooked up to the commutator/swivel. Food and water were supplied ad libitum. After lights on, the morning of testing, the mouse was observed and allowed to fall asleep for 1–2 min, as evidenced by the appearance of slow waves on the EEG as well as behaviorally. Then, the noise generator was switched on, and the gain was gradually increased at a rate of 1 dB/s until the mouse awakened. For the mouse to be considered "awake," it had to stay awake (low-amplitude, high-frequency EEG) for more than 3 s. The mouse was then allowed to fall asleep again, and the same procedure was repeated. If the mouse did not fall asleep within 1 h, this baseline time point was skipped. The mouse was then deprived of sleep for 3 h. Mice were kept awake by cage tapping, gentle jostling of the cage, and introducing new bedding and food into the cage one piece at time; care was taken not to touch them. After 3 h of SD, the same procedure for awakening the mouse with white noise was followed. After the arousal threshold was determined at the 3-h SD point, the mouse was deprived of sleep for three additional hours (the 3 + 3-h time point) and the white noise procedure repeated.

At both the 3- and 3 + 3-h SD time points, some mice did not wake up when the noise was raised to 85–87 dB.

This was the upper limit to the noise generating equipment; thus, when a mouse reached this level of noise without waking up, it was timed to see how long it could sleep with the noise production at that level. If the mouse did not awaken after 2 min, the noise was discontinued and the mouse was awakened manually.

If during any testing period the mouse entered REM sleep, it was allowed to finish its REM cycle and then return to NREM sleep before being tested. It was too difficult to wait for each mouse to enter REM sleep to determine how much noise was required to wake it from this less abundant sleep state.

2.9. Statistics

For Experiment 1, a one-way ANOVA test (Statistica software, Statsoft) was used to analyze potential differences between the genotypes for each method of waking, in both the morning and the evening. In addition, we wanted to determine if there was an interaction between method of waking and genotype, (e.g., if the knockouts reacted differently to the method of waking than the control mice) so a 2×2 ANOVA was performed to obtain this interaction statistic.

For Experiment 2, a repeated-measures ANOVA was used to test for genotype differences, because the same group of mice was tested for sleep latency before and after DOPS administration. Post hoc tests (Tukey unequal N HSD) were done to determine differences between genotypes at each time point.

For Experiment 3, a repeated-measures ANOVA for all three time points and a test for linear trend were done, to determine if the genotypes changed their tolerance for the noise at the same rate over time.

3. Results

3.1. Experiment 1

3.1.1. Sleep latency after gentle waking or saline injection

In this experiment, we investigated any potential differences in sleep latency between the genotypes according to method of waking, and between the morning/evening time points. When the mice were gently awakened (as described in Methods), there was no significant difference in latency to sleep between the genotypes either in the morning, or in the evening. However, when mice were handled and administered an intraperitoneal injection of saline (thereby introducing an element of stress), knockout mice fell asleep significantly faster both in the morning and in the evening (Fig. 1). When using method of waking and genotype as the two independent variables in a 2×2 ANOVA, there was a significant Genotype × Method of Waking interaction [$F(1,24) = 8.3$, $P < .01$], indicating that the knockout mice responded differently to the two methods of waking than the control mice did, which can also be seen in Fig. 1.

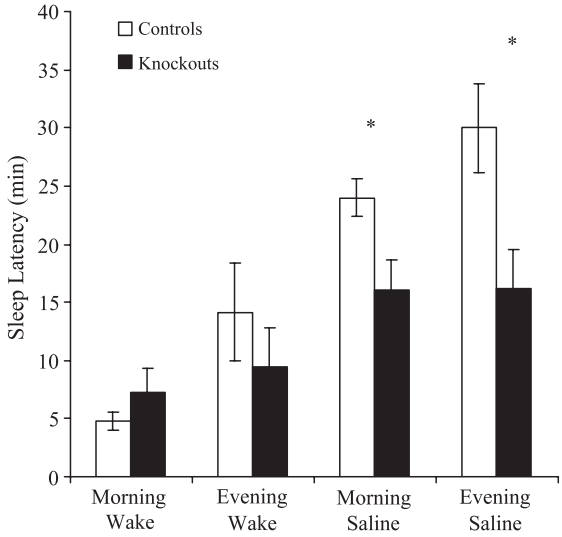

Fig. 1. Sleep latency in the morning and evening, after gentle awakening (wake), and after saline injection (saline). All groups $n = 8$, except morning saline, $n = 6$. * $P < .05$. Error bars express S.E.M.

3.1.2. Stimulant injections

Both stimulants had a major effect of increasing sleep latency in knockout and control mice; however, there was no difference in sleep latency between the genotypes for either stimulant tested (Fig. 2). Control mice and knockouts did not exhibit differences in sleep latency after the dose of caffeine (25 mg/kg) or after modafinil (90 mg/kg). However, four out of the eight knockout mice exhibited a short sleep period (1–3 min) 10–20 min after injection, then woke up and were awake for 2–3 h. This short sleep bout was not included in the analysis because it was not the beginning of the sustained sleep that normally occurs in the light part of the light/dark cycle. No control mice exhibited this behavior.

3.2. Experiment 2

The goal of this experiment was to restore NE in the knockouts by administering DOPS, a synthetic NE precursor, and carbidopa, to prevent peripheral production of NE. Before the DOPS administration, the knockout mice fell asleep faster after the injection of amphetamine than the control mice ($P < .05$), as shown in Fig. 3. After DOPS administration, there was no significant difference between the groups ($P = .4$).

This low dose of amphetamine did not increase sleep latency in the knockouts, and had only a marginal effect on the controls, as compared to each genotype's morning saline injection from Experiment 1 [knockout first amphetamine injection = 12.1 ± 1.4 min, knockout morning saline = 16.1 ± 2.5 min, $F(1,12) = 2.1$, n.s.; control first

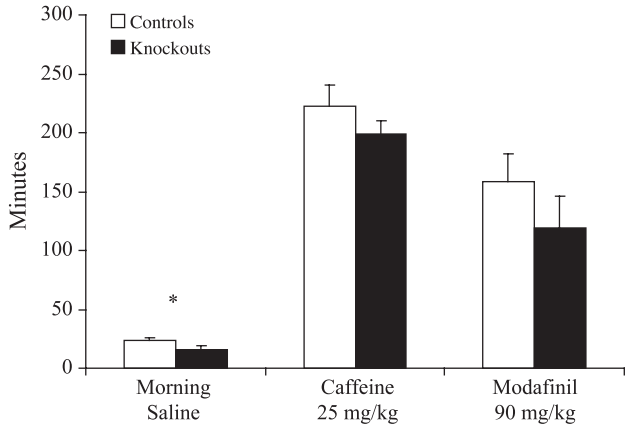

Fig. 2. Sleep latency after injections of saline ($n=6$), 25 mg/kg caffeine (knockouts = 11, controls = 10), and 90 mg/kg modafinil ($n=8$). All injections were done in the morning, shortly after lights on. For comparison, the morning saline data are repeated from Fig. 1. *$P<.05$. Error bars express S.E.M.

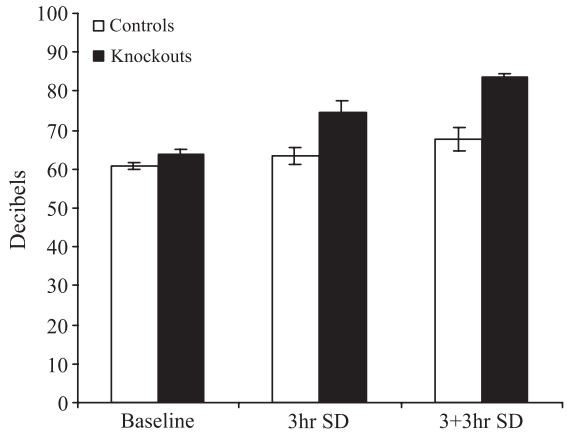

Fig. 4. Number of decibels of white noise required to wake mice from NREM sleep after no SD (baseline), 3 h of SD, and 6 h of SD. The Time × Genotype interaction was significant ($P<.01$). Knockouts = 10, controls = 9. Error bars express S.E.M.

amphetamine injection = 36.8 ± 6.1 min, control morning saline = 24 ± 1.6 min, $F(1,11)=3.6$, $P<.10$].

3.3. Experiment 3

This experiment tested whether the NE-deficient mice required more noise to wake up from sleep, after three different levels of SD. The repeated-measures ANOVA revealed a significant main effect of genotype [$F(1,14)=11.7$, $P<.01$], a significant main effect of time [$F(2,28)=24.2$, $P<.001$], and a significant Genotype × Time effect [$F(2,28)=5.9$, $P<.01$]. In addition, the test for a linear trend also revealed significance for the Linear Trend × Genotype interaction [$F(1,14)=14.0$, $P<.001$]. These results show that over all time points, the knockouts required more noise to wake them up, and there was also a difference in the amount of noise required to wake up all of the mice as the SD increased. Most importantly, however, the results demonstrate that the knockouts in particular required significantly more noise to wake up as the SD increased, as seen in Fig. 4.

At the 3 + 3-h time point, 6 of the 10 knockouts stayed asleep up to the maximum of 85–87 dB of white noise, and 5 of them slept through it for 2 min before the experiment was terminated. None of the control mice tolerated this level of white noise while asleep.

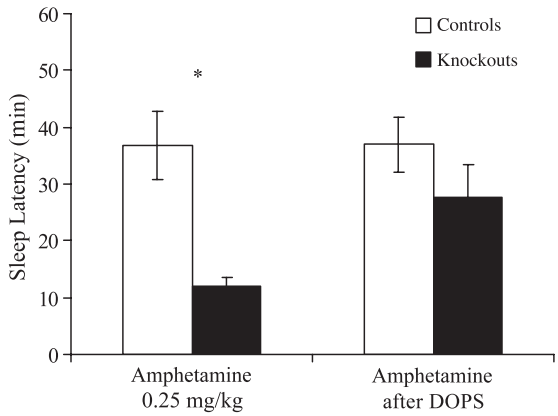

Fig. 3. Sleep latency after a low dose of amphetamine, before and after DOPS administration. *$P<.05$. Knockouts = 8, controls = 7. Error bars express S.E.M.

4. Discussion

We have shown previously that NE-deficient mice have a shorter sleep latency than controls after injection of low-dose amphetamine or saline (Hunsley and Palmiter, 2003). The results presented here suggest that this difference is mediated by some aspect of handling and/or injection, because when the mice were awakened using methods that were minimally stressful (no touching involved), there was no significant difference in latency to sleep between the genotypes. This was observed both at the beginning of the sleep cycle when sleep debt was high, as well as in the evening after the mice had at least eight uninterrupted hours to sleep. However, when mice were handled and injected with saline, there was a significant difference in sleep latency both in the morning and in the evening. Statistical analyses indicated that the two groups of mice reacted differently to the two methods of waking; the control group increased their sleep latency in response to the more stressful procedure, which is generally considered to be the normal response. Thus, it appears as if the knockout mice were less sensitive to the stress of injection compared to the controls. Perhaps this is due to the activation of the HPA axis and the sympathetic nervous system, which promotes vigilance and prepares the organism for action. Because of their genetic defect, the knockouts are unable to produce and mobilize NE, which is a main component of the sympathetic nervous system. But when NE is restored using

DOPS, the sleep latency difference between the genotypes after amphetamine injection disappears. Other aspects of the stress–response system in the knockouts are still functional, however. The knockouts have higher resting corticosterone levels (Ste. Marie and Palmiter, 2003), and they also have higher corticosterone levels after 2 min of restraint stress (unpublished data). Taken together, these data suggest that NE is likely the primary mediator of the difference in sleep latency between the genotypes, and that chronically elevated corticosterone levels are not sufficient to cause a normal behavioral response to this mildly stressful procedure in the knockouts.

Both caffeine and modafinil increased wakefulness substantially in both genotypes. Because neither stimulant relies on NE for its only mode of action, this was not surprising. However, the drugs ameliorated the sleep latency difference seen in the knockouts under the saline-injection conditions. After injection of modafinil, though, an interesting phenomenon occurred in half of the knockouts tested. Four of the NE-deficient mice fell asleep very briefly 10–20 min after the injection, which is consistent with the sleep latency after saline injection. But sleep was sustained for only 1–3 min, after which the mice awoke and stayed awake for several hours. Because this behavior only occurred in half of the knockouts, but in none of the control mice, it is difficult to draw any conclusions without further study. Perhaps the NE-deficient mice are less sensitive than the control mice to any initial wake-promoting effects of this stimulant as the drug levels rise in their bloodstream after injection. We have previously shown that NE-deficient mice are less sensitive to the wake-promoting effects of low doses (0.5 and 1.0 mg/kg) of another stimulant, amphetamine (Hunsley and Palmiter, 2003). Alternatively, the behavioral effects of modafinil may be delayed in the NE-deficient mice due to an unknown physiological mechanism, and they may simply have more time after the injection in which to fall asleep. Nevertheless, because latency to sustained sleep was similar in knockouts and controls, we conclude that the wakefulness promotion by caffeine or modafinil does not depend on NE.

NE-deficient mice required more noise than the control mice to wake them up as the lengths of SD increased. In fact, over half of the knockouts were able to sustain up to 2 min of NREM sleep through 85–87 dB of white noise blaring into their cage at the 3 + 3-h time point, whereas none of the control mice reached this level of noise without waking up. As a reference, the noise generated from a lawn mower is approximately 90 dB. Conversely, the control mice consistently awoke at similar levels of white noise even as the SD increased. The difference in tolerated noise levels between the genotypes is impressive, especially considering that it is measured on a logarithmic scale. Because the amount of noise required to awaken the two groups of mice at the baseline time point was so similar between the genotypes (Fig 4), the increase in arousal threshold appears to be dependent on prior wakefulness. This suggests that the arousal threshold of the knockouts is altered in response to SD. It is possible that there are NE-dependent neural pathways that interact with other molecule(s) or pathways during SD, to promote wakefulness. Consequently, NE may be necessary for an organism to maintain vigilance and resist sleep during periods of prolonged wakefulness and after stressful events, and wake up easily if it should fall asleep. This notion is congruent with results from studies showing that immediately prior to wake onset, organisms exhibit EEG activity characteristic of increasing arousal, and immediately after wake onset, they experience a higher level of alertness and autonomic activation than during other times of the day (Aston-Jones and Bloom, 1981; Horner et al., 1997; Trinder et al., 2003). These results suggest that the central wake-promoting systems prepare the organism for potential threat by increasing arousal upon waking. Therefore, when NE is absent, the arousal threshold shifts away from wake promotion, allowing the occurrence and propagation of NREM sleep during sensory stimulation that would normally cause wake.

Saper et al. (2001) propose a model to describe the change between wake and sleep, the "sleep–wake switch," which is useful in understanding the reciprocal relationship between behavioral state-generating brain areas. Briefly, when homeostatic and/or circadian factors drive the initiation of sleep, the VLPO becomes active and begins to inhibit the wake-promoting areas in the brainstem and hypothalamus, and NREM sleep ensues. Conversely, upon waking, the wake-promoting nuclei inhibit the sleep-generating VLPO, reinforcing their own activity. So each part of the "switch" inhibits the other, creating a "feedback loop that is bistable" (p. 728). The authors predict that if one portion of either of these groups was compromised, the stability between the states might be altered. Within this model, the NE-containing LC is one of the wake-promoting regions in the reticular activating system of the brainstem. If NE were not present, it could decrease the wake-promoting ability of the "wake active" portion of the loop, and one of many possible outcomes could be a change in sleep latency.

In summary, mice genetically deficient in NE exhibit altered arousal regulation when perturbed by mild stress or SD. These results provide additional evidence that NE plays a specific role in modulating the transitions between sleep and wakefulness (and vice versa), but it may not be essential for these transitions under normal circumstances.

Acknowledgements

We acknowledge W. Curtis and M. Day for assistance with experimental procedures, and W. Curtis for animal care and data processing. We also thank C. Landis and the department of Biobehavioral Nursing at the University of Washington for the generous use of their animal sleep recording facilities, V. Denenberg for statistical consultation, D. Mills and E. Rubel for the use of their noise generating

equipment, Sumitomo Pharmaceuticals for their donation of DOPS, and Cephalon for their donation of modafinil.

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