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Developmental divergence of sleep-wake patterns in orexin knockout and wild-type mice

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Abstract

Narcolepsy, a disorder characterized by fragmented bouts of sleep and wakefulness during the day and night as well as cataplexy, has been linked in humans and nonhuman animals to the functional integrity of the orexinergic system. Adult orexin knockout mice and dogs with a mutation of the orexin receptor exhibit symptoms that mirror those seen in narcoleptic humans. As with narcolepsy, infant sleep-wake cycles in humans and rats are highly fragmented, with consolidated bouts of sleep and wakefulness developing gradually. Based on these common features of narcoleptics and infants, we hypothesized that the development of sleep-wake fragmentation in orexin knockout mice would be expressed as a developmental divergence between knockouts and wild-types, with the knockouts lagging behind the wild-types. We tested this hypothesis by recording the sleep-wake patterns of infant orexin knockout and wild-type mice across the first three postnatal weeks. Both knockouts and wild-types exhibited age-dependent, and therefore orexin-independent, quantitative and qualitative changes in sleep-wake patterning. At 3 weeks of age, however, by which time the sleep and wake bouts of the wild-types had consolidated further, the knockouts lagged behind the wild-types and exhibited significantly more bout fragmentation. These findings suggest the possibility that the fragmentation of behavioural states that characterizes narcolepsy in adults reflects reversion back toward the more fragmented sleep-wake patterns that characterize infancy.

Introduction

Narcolepsy has emerged in recent years as a newly recognized neurodegenerative disorder (van den Pol, 2000; Siegel *et al*., 2001; Taheri *et al*., 2002). Central to this reclassification has been the recent discovery of a neurotransmitter, orexin (or hypocretin) (de Lecea, *et al*., 1998; Sakurai *et al*., 1998), that is produced by neurons situated within the caudal hypothalamus that project to the locus coeruleus and other nuclei implicated in the regulation of sleep and wakefulness (Peyron *et al*., 1998). A narcoleptic phenotype is exhibited by dogs with a mutation of the orexin receptor (Lin *et al*., 1999) and in orexin knockout mice (Chemelli *et al*., 1999). Also, in humans, narcolepsy has been linked to deficient functioning of the orexinergic system (Peyron *et al*., 2000; Thannickal *et al*., 2000). Importantly, adult orexin knockout mice exhibit patterns of sleep and wakefulness that mirror those seen in narcoleptic humans (Chemelli *et al*., 1999; Willie *et al*., 2003; Mochizuki *et al*., 2004).

The development of narcolepsy has received relatively little attention. In humans, onset of the disorder occurs in early adulthood, with the presenting symptom in 90% of cases being excessive daytime sleepiness (Taheri *et al*., 2002). Cataplexy, a sudden loss of muscle tone in an otherwise awake individual, is a symptom specific to narcolepsy that usually appears later. In Doberman pinschers with an orexin receptor mutation that predisposes them to narcolepsy, cataplexy is first detected at 4 weeks of age and its frequency increases dramatically over the next several months (Riehl *et al*., 1998; John *et al*., 2004). In orexin knockout mice, video analysis of five subjects suggested the presence of cataplexy as early as 3 weeks of age, but the incidence was very low and sporadic (Chemelli *et al*., 1999). In none of these studies, however, were patterns of sleep and wakefulness measured across early development. Given that cataplexy appears to be a later-developing symptom of narcolepsy than is excessive daytime sleepiness, it is possible that measures of sleep and wake behaviour can provide a more reliable and sensitive measure of the onset of narcolepsy in orexin knockout mice.

As with narcolepsy, the sleep and wake bouts of infant humans (Kleitman & Engelmann, 1953) and rats (Gramsbergen *et al*., 1970; Blumberg *et al*., 2005b) are highly fragmented, characterized by rapid transitions between short-duration states. The question addressed here is whether the fragmented sleep and wake bouts observed in both narcoleptics and infants reflects, at least in part, deficient functioning of the orexinergic system. Specifically, we hypothesized that infant orexin knockout mice would lag behind wild-types with regard to the developmental consolidation of sleep and wake bouts.

The examination of sleep and wakefulness in early infancy, especially in altricial infants such as rats and mice, has been limited by technical problems as well as concerns over the absence of state-dependent neocortical activity (Blumberg *et al*., 2005a). Recently, we have shown that the nuchal electromyogram (EMG) alone provides a reliable measure of sleep and wakefulness during early infancy in rats and, moreover, provides an adequate foundation for revealing the neural substrates of infant sleep (Karlsson & Blumberg, 2005; Karlsson *et al*., 2005; Seelke *et al*., 2005; Mohns *et al*., 2006). Building on work in adult mammals examining the statistical distributions of sleep and wake bouts (Lo *et al*., 2002; Lo *et al*., 2004), we have also described orderly developmental transitions in the statistical distributions of sleep and wake bouts in infant rats between

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postnatal day (P)2 and P21 (Blumberg *et al*., 2005b). Here, we adapt these techniques for use in infant mice over the first three postnatal weeks to describe a developmental divergence in sleep-wake patterning between orexin knockouts and wild-types.

Materials and methods

All experiments were performed under National Institutes of Health guidelines for the care of animals in research and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Subjects

All subjects were the male and female infant offspring of a founder population of orexin knockout mice (Mochizuki *et al*., 2004). Heterozygote breeding pairs were used to produce litters comprising knockout and wild-type infants. For this study, pups were tested on P2–4 (hereafter P4), P10–13 (hereafter P12), or P20–22 (hereafter P21). In total, 29 litters were produced from 12 fathers and 16 mothers, with no more than three litters being used from a given mother. A total of 19 P4, 21 P12, and 27 P21 subjects were included in the analyses presented here. At the time of testing, litter sizes were 2–9 pups (average 5–6 pups per litter) and body weights were 1.6–2.8 g at P4, 3.7–9.1 g at P12, and 6.0–13.4 g at P21. Mothers and their litters were housed and raised in standard laboratory cages ($28 \times 18 \times 14$ cm) in the animal colony at the University of Iowa. Food and water were available to the animals *ad libitum*. All animals were maintained on a 12-h light : 12-h dark schedule with lights on at 06:00 h. All experiments were conducted during the light period, with the exception of a group of P21 subjects that was tested during the dark period.

Surgery

On the day of testing, a pup was removed from the litter and weighed. Care was taken to ensure that the P4 and P12 subjects exhibited a milk band upon removal from the nest (milk bands are not detectable in the older subjects). Under isoflurane anaesthesia, bipolar stainless steel hook electrodes (50-μm diameter; California Fine Wire, Grover Beach, CA) were inserted bilaterally into the nuchal muscle and secured with collodion. After surgery, the pup recovered for at least 1 h before testing, either in a humidified incubator (maintained at 35 °C) or in the testing chamber.

For the P21 wild-type and knockout ($n = 6$ per group) subjects tested during the dark period, surgery was performed identically as during the light period, except under red-light illumination. Care was taken to ensure that pups were never exposed to white light after lights-off. Surgery typically began around 08:00 h and recovery was identical to that of P21 subjects tested during the light period.

Procedure and data acquisition

All pups were tested unrestrained inside an electrically shielded double-walled glass chamber (height 17.0 cm; i.d. 12.5 cm) with a Plexiglas lid. Air temperature inside the chamber was regulated at thermoneutrality for each age using a temperature-controlled water circulator (P4 37 °C; P12 36 °C; P21 34 °C). Access holes in the side of the chamber allowed for the passage of electrodes. A round platform constructed of polyethylene mesh was fitted inside the chamber and a perforated felt pad was placed on top of the mesh. For P21 (and one P12) subjects, a Plexiglas insert was constructed that divided the chamber into two halves and thereby allowed for the simultaneous testing of two subjects.

Nuchal EMG electrodes were connected to differential amplifiers (A–M Systems, Carlsborg, WA) and their signals were amplified (×10 000) and filtered (300–5000 Hz). EMG data were visualized by the experimenter during the test using a data acquisition system (BioPac Systems, Santa Barbara, CA). For subjects tested during the day, a microcamera was placed above the chamber lid for monitoring and recording of behaviour. EMG data were recorded directly to hard disk and at least 1 h of EMG and video data were recorded to digital videotape using a data recorder (DV8; WinTron Technologies, Rebersberg, PA).

All subjects acclimated to the testing chamber for at least 30 min before data recording began. For P4 and P12 subjects, data were recorded for 1 h. For P21 subjects, data were recorded for 6 h (For subjects tested during the dark period, data recording typically began at 09:30 h). In general, longer tests are needed for older subjects in order to acquire a sufficient number of sleep-wake cycles (Blumberg *et al*., 2005b). Also, for the P21 subjects, we were able to genotype more than 1 week before testing, thus allowing us to test only subjects of known genotype. For the P4 and P12 subjects, however, genotyping occurred after testing, thus requiring multiple littermates to be tested for 1 h and the discarding of data from heterozygotes. One P12 subject was genotyped before testing and was therefore tested for 3 h.

Genotyping

Tail snips for genotyping were acquired either after testing (for all P4 subjects and all but one P12 subjects) or at least 6 days before testing. DNA was extracted and mice were genotyped using PCR with a neo primer (knockout forward), 5′-TGATATTGCTGAAGAGCTTGGCGG, or a genomic primer (wild-type forward), 5′-GACGACGGCCTCAGACTTCTTGGG, and a genomic primer common to knockouts and wild-types (reverse primer), 3′-ACTAGCCCTTCCCTCCACAGA.

Data analysis

As described previously (Karlsson *et al*., 2004; Blumberg *et al*., 2005b), EMG signals were digitized at 1 or 2 kHz using a data acquisition system (BioPac Systems Inc., Santa Barbara, CA). Digitized signals were integrated and full-wave rectified. Then, the EMG signal was dichotomized into bouts of sleep (i.e. atonia/hypotonia) and wake (high muscle tone) as follows. The amplitude of five 1-s segments of noise-free, uninterrupted atonia and high-tone periods was measured for each pup, averaged, and the midpoint between the two was calculated. A bout of sleep and wake was defined as a period in which muscle tone was below or above, respectively, the midpoint value for at least 1 s. For P21 subjects, we found that increasing the sleep-bout criterion from 1 to 3 s more accurately reflected behaviour at this age (as judged by comparing video and EMG records) and improved interrater reliability.

Three experienced scorers, typically blind to the subject's age and genotype, performed the analyses. When two scorers analysed the same record, interrater reliabilities were high, usually exceeding 80% and often exceeding 90%. When a record was deemed too unclear to score or when two scorers were in substantial disagreement (i.e. interrater reliability < 80%), that record was excluded.

For the analyses described above, data were imported into Statview 5.0 (SAS, Cary, NC), JMP 5.0 (SAS, Cary, NC), and

DeltaGraph 5.5 (SPSS, Chicago, IL). Because genotyping was performed after testing for the P4 and P12 subjects, data from littermates of the same genotype were often collected. Because it is inappropriate to treat these littermates as independent subjects within the same experimental group (Abbey & Howard, 1973; Holson & Pearce, 1992), their data were always averaged before statistical analyses were performed. At P4 and P12, as many as four same-genotype littermates were tested. Because the P21 subjects were genotyped before testing, no more than one wild-type and one knockout pup was selected from each litter.

Two-factor analysis of variance (ANOVA), with age and genotype as factors, was used to test for differences in sleep and wake variables. When appropriate, unpaired *t*-tests were used for *posthoc* tests. Additional ANOVAs were performed to test for light–dark differences in sleep and wake behaviour at P21. *Posthoc* tests included planned comparisons (i.e. unpaired *t*-tests) to examine within-genotype circadian differences.

In order to assess the statistical distributions of all sleep and wake bouts, log-survivor analyses were performed (Blumberg *et al*., 2005b). Survivor distributions were produced from each pup and from pooled data at each age. Data were plotted using log–log and semilog coordinates and regression analyses were performed on the data for each subject to assess the r^2 values and therefore the degree of fit of the data to power-law and exponential distributions, respectively (Blumberg *et al*., 2005b). Three-factor ANOVAs, with age, genotype, and distribution (i.e. exponential or power-law) as factors, were used to test for differences in r^2 values. When appropriate, paired *t*-tests were used for *posthoc* tests to assess within-age, between-distribution differences in r^2 values.

For all tests, alpha was set at 0.05. Means are presented with their standard errors (SEM).

Results

As shown in Fig. 1A, mean sleep bout durations during the light period increased dramatically between P4 and P12 and then levelled off between P12 and P21. ANOVA revealed a significant effect of age ($F_{2,30} = 43.6$, $P < 0.0001$) and genotype ($F_{1,30} = 7.0$, $P < 0.05$), but not a significant age–genotype interaction ($F_{2,30} = 1.0$). *Posthoc* tests revealed that at P21, but not P12, wild-type mice were exhibiting significantly longer mean sleep durations than were the orexin knockouts.

As shown in Fig. 1B, mean wake bout durations did not increase substantially until P21, and then only in the wild-type mice. Although ANOVA revealed a significant effect of age ($F_{2,30} = 7.5$, $P < 0.005$), there was no significant effect of genotype ($F_{1,30} = 2.1$) and no significant age–genotype interaction ($F_{2,30} = 1.9$). The effects of shortened sleep and wake bouts in the knockout mice at P21 combined to produce significantly more sleep-wake cycles per min, as shown in Fig. 1C. For this measure, ANOVA revealed a significant effect of age ($F_{2,30} = 107.8$, $P < 0.0001$) and genotype ($F_{1,30} = 7.3$, $P < 0.05$), but not a significant age–genotype interaction ($F_{2,30} = 0.7$). As shown in Fig. 1D, the mean percentage of time spent awake decreased significantly with age ($F_{2,30} = 46.7$, $P < 0.0001$) but, importantly, did not differ between genotypes (genotype $F_{1,30} = 0.7$; age × genotype $F_{2,30} = 0.6$).

Survivor distributions for pooled sleep bout data at P4, P12, and P21 are presented in Fig. 2A (left panel) for wild-type (solid lines) and knockout (dotted lines) mice. It can be seen that the data at all three ages are best described by an exponential function [such that the frequency distribution $f(t)$ of bout durations of duration t was proportional to $e^{(-t/\tau)}$, where τ is the characteristic time scale], as they fall along a straight line on the semilog plot. By P21, the data for the wild-types and knockouts have diverged significantly from each

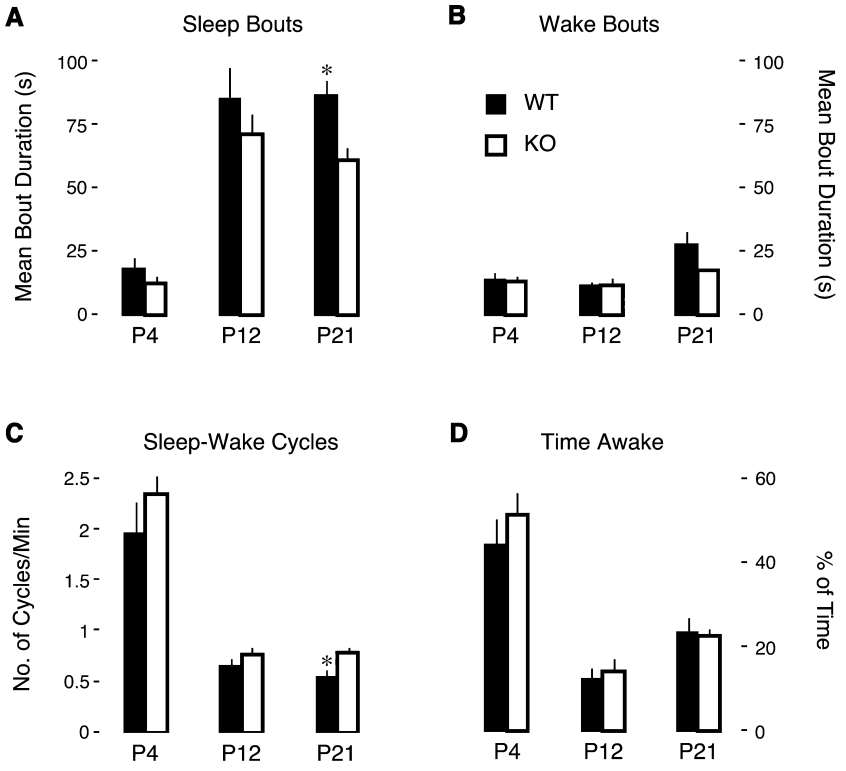

FIG. 1. Mean sleep (A) and wake (B) bout durations for wild-type (WT) and knockout (KO) mice at P4, P12, and P21. Mean number of sleep-wake cycles per min (C) and percentage of time awake (D) for the same subjects. *Significant between-genotype difference. Mean + SEM.

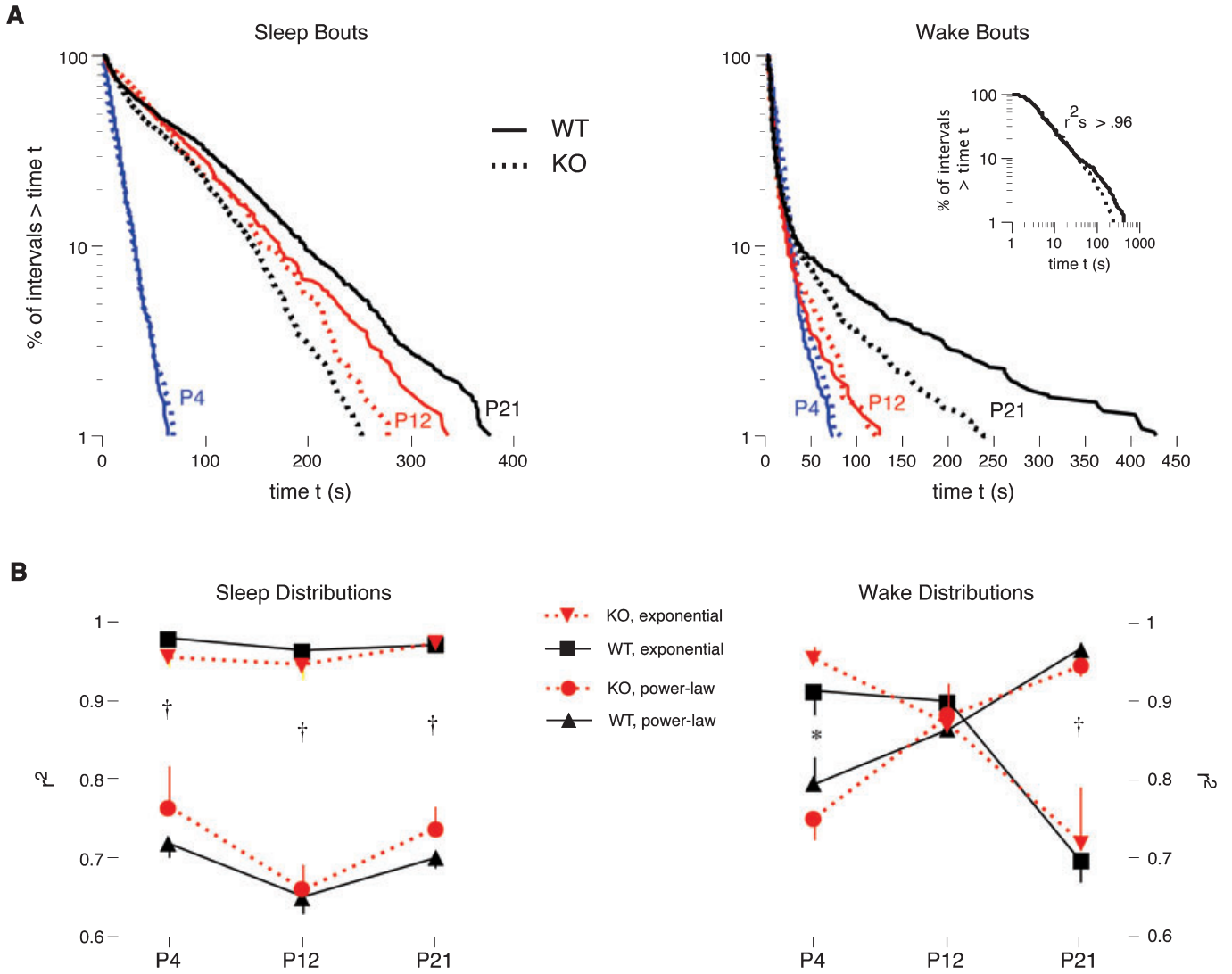

FIG. 2. (A) Survivor plots of sleep and wake bout durations for wild-type (WT; solid lines) and knockout (KO; dashed lines) mice at P4 (blue), P12 (red), and P21 (black). Straight lines on these plots indicate that the data follow an exponential distribution. The inset is a replotting of the P21 wake data using log–log coordinates; straight lines on these plots indicate that the data follow a power-law distribution. Individual data points were pooled across all subjects; P4, $n = 1086$–1398; P12, $n = 424$–545; P21, $n = 1674$–2002. (B) Values of r^2 produced using regression analysis of survivor data from the wild-type (WT; black) and knockout (KO; red) mice at P4, P12, and P21. For each individual pup, the degree of fit of the data to power-law and exponential distributions was determined, yielding a value of r^2 that was then averaged across subjects at each age. †Significant within-age, between-distribution difference in r^2 values for both wild-types and knockouts. *Significant within-age, between-distribution difference in r^2 values for knockouts only. Mean ± SEM.

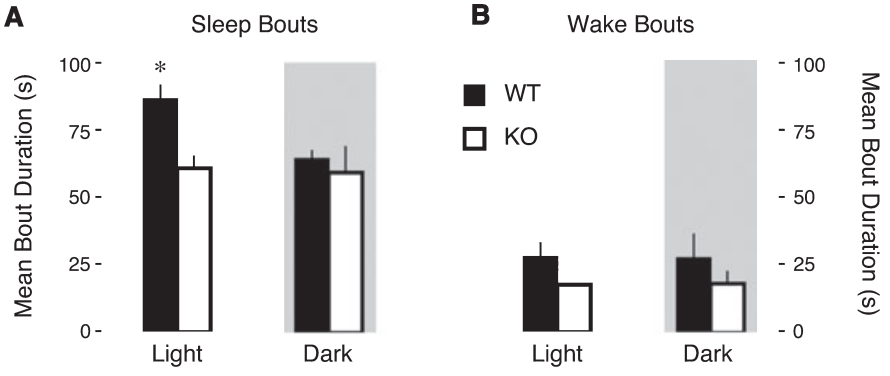

FIG. 3. Mean sleep (A) and wake (B) bout durations for wild-type (WT) and knockout (KO) mice at P21 for subjects tested under light and dark conditions. *Significant between- and within-genotype difference. Mean + SEM.

other such that the 95% confidence intervals for the two distributions do not overlap at intervals greater than 25 s (data not shown).

Figure 2B (left panel) presents r^2 values based on regression analyses performed for each pup when data were plotted using semilog or log–log coordinates. Most importantly, the three-factor ANOVA revealed a significant effect of distribution ($F_{1,60} = 410.9, P < 0.0001$) on r^2 values. Moreover, paired *t*-tests revealed that an exponential distribution provided a significantly better fit to the data than did a power-law distribution at each age and for each genotype.

Whereas survival data that follow an exponential distribution fall on a straight line in a semilog plot, those that follow a power-law distribution [such that $f(t) \approx t^{-\alpha}$, where α is a characteristic power-law exponent] fall on a straight line in a log–log plot. As shown in Fig. 2A (right panel), survivor distributions for pooled wake data exhibit different distributions depending on age. First, at P4, and to a lesser extent at P12, the data for both wild-types and knockouts fall on overlapping straight lines, indicative of an exponential distribution. By P21, the distributions are now skewed at intervals greater than 30 s and, in addition, the distributions for the wild-types and knockouts no longer completely overlap. Specifically, the 95% confidence intervals do not overlap at intervals greater than 75 s, thus indicating that the two distributions arise from separate populations (data not shown).

Figure 2B (right panel) presents r^2 values based on regression analyses performed for each pup when wake bout data were plotted using semilog or log–log coordinates. The three-factor ANOVA revealed only a significant age–distribution interaction ($F_{2,60} = 34.2, P < 0.0001$). In particular, although the data distribute exponentially at P4, paired *t*-tests revealed that, by P21, the data now follow a power-law distribution. This transition is illustrated in the inset in Fig. 2A (right panel), which presents the P21 data replotted on log–log coordinates to reveal that they now fall on a straight line.

Figure 3A presents again the data for P21 subjects during the light period but now contrasted with the data for P21 subjects tested during the dark period. ANOVA revealed a significant effect of genotype ($F_{1,23} = 5.8, P < 0.05$) but neither a significant effect of light cycle ($F_{1,23} = 3.3, P = 0.08$) nor a significant genotype × light–cycle interaction ($F_{1,23} = 2.6$). Planned comparisons did indicate, however, that mean sleep duration for the wild-types, but not the knockouts, was significantly higher during the day than during the night.

Figure 3B contrasts the light–dark P21 data for mean wake duration. None of the ANOVAs reached statistical significance (genotype $F_{1,23} = 3.7, P < 0.07$; light cycle $F_{1,23} = 0.0$; genotype × light–cycle interaction $F_{1,23} = 0.0$). ANOVA did reveal that knockouts cycled more rapidly between sleep and wakefulness than did wild-types; though the main effect of genotype was significant ($F_{1,23} = 6.7, P < 0.05$), there was no effect of light cycle ($F_{1,23} = 1.8$) and no genotype × light–cycle interaction ($F_{1,23} = 0.1$). Finally, the percentage of time awake was indifferent to genotype and light cycle (genotype $F_{1,23} = 0.1$; light cycle $F_{1,23} = 0.3$; genotype × light–cycle interaction: $F_{1,23} = 0.1$).

Discussion

As every attentive parent is aware, newborns cycle rapidly between sleep and wakefulness and only gradually, over the first several months, do ultradian rhythms consolidate and circadian patterns strengthen (Kleitman & Engelmann, 1953; Jenni *et al.*, 2006). Similar processes occur in rats over the first three postnatal weeks (Gramsbergen *et al.*, 1970; Frank & Heller, 1997; Blumberg *et al.*, 2005b). Because narcolepsy, a disorder typically found in adults, is also characterized by increased sleep and wake fragmentation (Taheri *et al.*, 2002), we hypothesized that sleep-wake fragmentation in narcoleptics and infants reflects in part a shared deficit in neural function. With the discovery of orexin's causative role in narcolepsy (Siegel *et al.*, 2001), the advent of orexin knockout mice (Chemelli *et al.*, 1999), and recent advances in our ability to monitor and analyse behavioural states in altricial infants (Blumberg *et al.*, 2005b), we were in a position to test this hypothesis directly. We predicted that the knockouts would lag behind the wild-types with regard to their consolidation of sleep and wake bouts across early development.

We report here that both knockout and wild-type mice exhibited decreasing fragmentation of sleep and wake bouts between P4 and P21. Moreover, similar to what was shown recently in infant rats (Blumberg *et al.*, 2005b), mice of both strains exhibited orderly

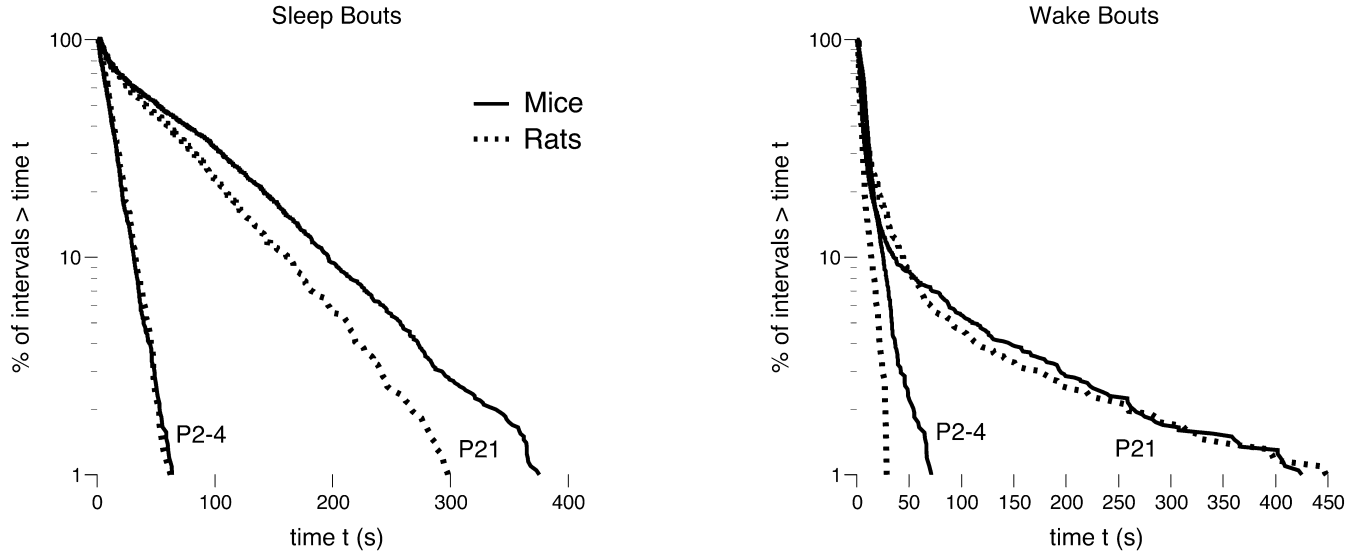

FIG. 4. Survivor plots of sleep and wake bout durations for the P2–4 and P20–22 wild-type mice (solid lines) in the present study and P2 and P21 Norway rats (dotted lines) from a previous study (Blumberg *et al.*, 2005b). The data for the mice are replotted from Fig. 2. All plots were constructed using data pooled from multiple subjects. Recall that the gestation length of mice is 3 days shorter than that of rats. Regardless, the distributions are remarkably similar, including the development of wake-related power-law behaviour by P21.

changes in the statistical distributions of sleep and wake bouts, including the wake-bout transformation from exponential to power-law behaviour (see Fig. 2). Against this backdrop of orderly, orexin-independent developmental change, by P21 the knockouts were lagging behind the wild-types with regard to the developmental consolidation of sleep and wake bouts. These knockouts also appeared to lag behind the wild-types in the onset of circadian rhythmicity, exhibiting similar mean sleep bout durations during the light and dark periods as the wild-types exhibited increased sleep bout durations during the light period (see Fig. 3A). Given the interrelations of circadian rhythmicity and sleep-wake consolidation (Dijk & Czeisler, 1995), it is possible that some aspects of decreased sleep-wake fragmentation in infancy are related to the development of circadian regulation of behavioural states (Frank & Heller, 1997).

The mean percentage of time awake decreased significantly with age, from a value of 47% at P4–14% and 22% at P12 and P21, respectively (Fig. 1D). In contrast, a value of approximately 40% has been reported for adult knockout and wild-type mice during the light phase (Mochizuki *et al*., 2004); in addition, P2–21 rats were found to be awake 25–35% of the time (Blumberg *et al*., 2005b). It is not clear what is responsible for the more variable wake percentages seen here or the relatively high value at P4. Testing over a range of air temperatures may help to resolve this issue. It should be stressed, however, that mean wake percentage did not differ between the knockouts and wild-types.

In humans, excessive daytime sleepiness is the presenting symptom of narcolepsy in 90% of cases and cataplexy is the presenting symptom in only 10% of cases (Taheri *et al*., 2002). Nonetheless, cataplexy has received exclusive attention in previous developmental studies in dogs and mice (Riehl *et al*., 1998; Chemelli *et al*., 1999; John *et al*., 2004). Interestingly, from the perspective of cataplexy, narcolepsy entails the onset of a distinct, pathological symptom. In contrast, we have shown here that, from the perspective of sleep and wakefulness, narcolepsy in orexin knockout mice (and likely mutant Doberman pinschers as well) entails retention of the more fragmented patterns of sleep and wakefulness normally expressed by infants. Accordingly, we hypothesize that adult-onset human narcolepsy entails reversion back toward the infantile pattern of fragmented sleep and wakefulness. It must be stressed, however, that because significant sleep-wake bout consolidation occurs in the absence of a functional orexinergic system, individuals neither retain nor revert to their earliest infantile sleep-wake patterns. Finally, it should be noted that the cascade of mechanisms that might link sleep-wake fragmentation with cataplexy remains to be examined.

In both rats and mice *in vitro*, orexin is able to modulate synaptic activity as early as the day of birth, at which time orexin receptor mRNA is detectable (van den Pol *et al*., 2001; van den Pol *et al*., 2002). But the orexin system exhibits substantial developmental changes postnatally in rats. Specifically, prepro-orexin mRNA is only weakly expressed in the hypothalamus through P15, increases dramatically through P20, and largely stabilizes thereafter (de Lecea, *et al*., 1998; Yamamoto *et al*., 2000). Importantly, as shown in Fig. 4, the timing of sleep-wake developmental events seen here in mice is similar to that found in a previous study in rats (Blumberg *et al*., 2005b). Thus, we hypothesize that increased availability of orexin during the third postnatal week accounts for the differences in sleep-wake patterning observed here between orexin knockout and wild-type mice.

In a previous study using 11-week-old mice from the same population of animals used in the present study, it was concluded that the fragmented sleep and wakefulness of orexin knockouts reflects behavioural state instability produced by lowered transition thresholds between states (Mochizuki *et al*., 2004). Our results with infants are broadly consistent with that conclusion. It should be noted, however, that comparisons between that earlier study and the present one make clear that the sleep-wake patterns of knockouts and wild-types diverge further after P21. A substantial proportion of this further divergence will likely be due to the nighttime increase in mean wake bout durations in wild-types (Mochizuki *et al*., 2004), which was not yet detected at P21. In future work, as behavioural state development in these animals is explored beyond P21, it will be important to use uniform testing procedures and methods of analysis.

The most salient circadian difference detectable at P21 was the flattening of the light–dark difference in sleep bout duration in the orexin knockouts in relation to the wild-types. This flattening is consistent with (i) the effects seen in adult rats after orexin-specific (Gerashchenko *et al*., 2001) and nonspecific (Chou *et al*., 2003) lesions of the orexin-concentrating lateral and dorsomedial hypothalamus (DMH), and (ii) the notion that the DMH, through its interactions with the suprachiasmatic nucleus (SCN), modulates circadian rhythmicity (Aston-Jones *et al*., 2001; Saper *et al*., 2005).

The decreasing fragmentation of sleep-wake organization across early infancy reflects, at least in part, the increasing modulatory control of hypothalamic mechanisms over the basic brainstem circuit that governs sleep and wakefulness in newborns (Karlsson *et al*., 2004; Karlsson & Blumberg, 2005; Karlsson *et al*., 2005; Mohns *et al*., 2006). These hypothalamic mechanisms likely include the DMH, the SCN, and the ventrolateral preoptic area (Aston-Jones *et al*., 2001; Saper *et al*., 2001; Saper *et al*., 2005). In particular, based on work in adult animals and the evidence from individuals with narcolepsy, it has been suggested that the orexinergic system plays a critical role in the stabilization of the sleep-wake 'flip-flop' switch (Saper *et al*., 2001). One impression given by this model is that stabilization of states does not occur in the absence of orexin. The present results, however, indicate that a functioning orexinergic system is not necessary for infants to consolidate sleep and wakefulness over levels seen in newborns. Nor is the orexinergic system in mice necessary for the development of the power-law behaviour that characterizes wake bouts in all adult mammalian species studied thus far (Lo *et al*., 2002; Lo *et al*., 2004). Thus, the interpretation that is most consistent with the available information, including that from the present study, is that orexin is an important modulator of sleep and wakefulness, enhancing stability beyond that attained during early infancy.

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Abbreviations

EMG, electromyogram; P, postnatal day.

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