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The ontogeny of mammalian sleep: a response to Frank and Heller (2003)

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SUMMARY In a recent review, Frank and Heller (2003) provided support for their 'presleep theory' of sleep development. According to this theory, rapid eye movement (REM) and non-rapid eye movement (Non-REM) sleep in rats emerge from a common 'dissociated' state only when the neocortical EEG differentiates at 12 days of age (P12). Among the assumptions and inferences associated with this theory is that sleep before EEG differentiation is only 'sleep-like' and can only be characterized using behavioral measures; that the neural mechanisms governing presleep are distinct from those governing REM and Non-REM sleep; and that the presleep theory is the only theory that can account for developmental periods when REM and Non-REM sleep components appear to overlap. Evidence from our laboratory and others, however, refutes or casts doubt on these and other assertions. For example, infant sleep in rats is not 'sleep-like' in that it satisfies nearly every criterion used to characterize sleep across species. In addition, beginning as early as P2 in rats, myoclonic twitching occurs only against a background of muscle atonia, indicating that infant sleep is not dissociated and that electrographic measures are available for sleep characterization. Finally, improved techniques are leading to new insights concerning the neural substrates of sleep during early infancy. Thus, while many important developmental questions remain, the presleep theory, at least in its present form, does not accurately reflect the phenomenology of infant sleep.

KEYWORDS active sleep, atonia, myoclonic twitching, rat, rem sleep, slow-wave sleep

INTRODUCTION

Beginning with the revitalization of interest in the embryonic origins of behavior in the 1960s, sleep in early life has been viewed as a diffuse collection of phasic and cyclic motor events that gradually coalesce with other sleep components to form the complex, differentiated forms of sleep that are most easily recognized in adults (Corner, 1977, 1985). Based on numerous studies of fetuses and infants in a variety of mammalian species, it is widely believed that the earliest form of sleep is properly characterized as active sleep, that is, an immature form of REM sleep (Jouvet-Mounier *et al.*, 1970; Parmelee *et al.*, 1967; Roffwarg *et al.*, 1966; Ruckebusch *et al.*, 1977; Shimizu and Himwich, 1968; Szeto and Hinman, 1985). Accordingly, it is thought that quiet sleep, an immature form of slow-wave sleep (SWS), emerges or becomes more prominent as REM sleep's predominance diminishes during ontogeny.

In a recent review, Frank and Heller (2003) present evidence and argument to support an alternative theory of sleep development, which they call the 'presleep theory'. According to their theory, infant presleep is comprised of 'spontaneous, dissociated activity' that can be characterized as neither REM nor non-REM sleep. Accordingly, any resemblance between the components of presleep and the components of mature forms of sleep is misleading. Moreover, they argue that the transformation of presleep into REM and non-REM sleep does not occur until the neocortical EEG exhibits state-dependent differentiated activity.

To their credit, Frank and Heller explicitly delineate the assumptions and inferences that they believe differentiate the presleep theory from other perspectives. Specifically, they

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argue (a) that sleep prior to EEG differentiation (i.e. presleep) is only 'sleep-like'; (b) that only behavioral measures are available for characterizing sleep in rats during early infancy; (c) that the spontaneous motor activity that characterizes presleep may outwardly resemble REM sleep but is, in fact, 'fundamentally distinct from this EEG-defined sleep state' (p. 31); (d) that the 'central executive mechanisms' that govern adult sleep are distinct from the mechanisms that function during presleep; and (e) that evidence of overlapping REM and NREM sleep components during development demands a reconceptualization of sleep along the lines of their presleep theory.

We applaud Frank and Heller for helping to reinvigorate interest in sleep development. With them, we believe that this area of sleep research has been neglected for far too long and that we have much to learn about sleep and its neural substrates by studying rats and other species that give birth to altricial young. With them, we bemoan the 'maddeningly imprecise range of criteria' (p. 30) that are used to define sleep states in infants and that make steady progress in this area so difficult. And with them, we believe that investigators interested in the origins of sleep 'should begin their experiments as early in development as possible and not restrict them to a single time-point' (p. 30). Despite these common goals and attitudes, however, recent findings from our laboratory lead us to doubt each of the assumptions and inferences of the presleep theory outlined above. In this response, we review the basis for this doubt and, in the process, describe the conceptual perspective that underlies our approach.

SLEEP, NOT PRESLEEP

Infant sleep presents a challenge to sleep researchers because it differs from adult sleep on a number of important dimensions. Perhaps most critically, infant sleep is difficult to categorize because some sleep components are absent or intermittently expressed early in ontogeny. For example, the neocortical EEG does not exhibit state-dependent differentiation, including slow wave activity, until 115–120 days postconception in sheep (Clewlow *et al*., 1983; Szeto and Hinman, 1985), 50 days postconception in guinea pigs (Umans *et al*., 1985), approximately 32 weeks postconception in preterm human infants (Dreyfus-Brisac, 1975), and until 12 days of age (P12) in rats (Frank and Heller, 1997; Gramsbergen, 1976; Mirmiran and Corner, 1982). When attempting to describe and quantify sleep at ages before EEG differentiation or when measurements of EEG are not possible or are considered unreliable (e.g. in human fetuses and preterm infants), investigators have relied on other measures of state, including body movements, respiration, heart rate, and muscle tone (Gramsbergen *et al*., 1970; Nijhuis *et al*., 1984; Parmelee *et al*., 1967). Perhaps inevitably, disagreement and confusion have emerged as different investigators have relied on different measures and adopted different criteria for categorizing sleep at ages prior to EEG differentiation (Dreyfus-Brisac, 1970; Prechtl, 1974).

Interestingly, a similar problem of categorization has been confronted by those investigating sleep in invertebrates, such as the fruit fly, that do not possess a neocortex (Hendricks *et al*., 2000; Shaw *et al*., 2000). Categorizing sleep in such 'non-traditional' species is relevant to the present discussion because, as already mentioned, neonatal rats do not exhibit state-dependent neocortical EEG activity. Accordingly, if the neocortical EEG is considered the *sine qua non* of sleep, then we are confronted with the odd juxtaposition whereby sleep in fruit flies is gaining acceptability even as sleep in infant rats is being relegated to the category of 'presleep.'

We will return to the significance of the EEG for infant sleep later. First, however, it is important to address the fundamental question of whether sleep during the 'pre-EEG period' is more properly categorized as 'sleep-like,' as Frank and Heller suggest. We address this question below by determining whether sleep in infant rats conforms to standard criteria used by other researchers to assess the existence of sleep in a variety of vertebrate and invertebrate species (Campbell and Tobler, 1984; Hendricks *et al*., 2000).

Sleep is characterized by an absence of voluntary movements

Behaviorally, an infant rat housed in a thermoneutral, humidified environment exhibits behavioral activation that entails high-amplitude movements of the limbs, such as stretching, locomoting, yawning, and kicking (Blumberg and Stolba, 1996; Gramsbergen *et al*., 1970); such movements are often designated as voluntary, coordinated, or purposeful (although each of these terms has limitations) and are typically considered to indicate periods of wakefulness. After brief bursts of awake activity, a period of quiet ensues, followed by the onset of myoclonic twitching of the limbs and tail. Such bursts of twitching are typically considered to indicate periods of *active sleep*. Periods of twitching are almost always followed by the abrupt onset of high-amplitude awake behaviors, thus completing the cycle. Experienced observers can reliably distinguish twitches from wake-related movements, especially when pups are observed in a supine position so that the limbs are unloaded and their movements are easily visualized (Robinson *et al*., 2000). Clearly, twitching does not fall into the category of voluntary movements. Therefore, infant rats satisfy this criterion.

Sleep is spontaneous, occurring with a circadian rhythm

Spontaneous rhythms occur in the absence of an external trigger, that is, when exogenous conditions remain constant. Sleep in infant rats satisfies this criterion in that the provisioning of a thermoneutral, humidified environment permits ultradian cycling between sleep and wakefulness (Gramsbergen *et al*., 1970; Jouvet-Mounier *et al*., 1970; Karlsson *et al*., 2004). There is currently little information concerning the onset of circadian sleep–wake rhythms in infant rats.

Sleep is reversible

During periods when infant rats are twitching, sensory stimulation is sufficient to produce arousal (Seelke and Blumberg, 2004), thus distinguishing this state from coma and other irreversible pathological states.

Sleep is characterized by a species-specific posture and/or resting place that minimizes sensory stimulation

In the wild, infant rats are reared in species-typical nests or burrows in which the combined influences of the shelter, mother, and littermates ensures a warm, humid environment that is conducive to sleep.

Sensory and/or arousal thresholds increase during sleep

It was recently shown that P8 rats exhibit an increased olfactory threshold during periods of myoclonic twitching relative to periods of wakefulness (Seelke and Blumberg, 2004). In addition, in a recent sleep-deprivation study using P5 rats (see below), it was shown that arousal threshold increases as sleep pressure intensifies (Blumberg *et al*., in press).

Sleep is regulated by a homeostatic mechanism

Very few sleep deprivation studies have been conducted in infant rats (Feng *et al*., 2001; Frank *et al*., 1998; Mirmiran *et al*., 1981, 1983), and in none of these previous studies has the effect of short-term sleep deprivation been examined before P12. We conducted such an experiment at P5 using electric shock applied to the flank during periods of sleep (Blumberg et al., in press). Over the course of a 30-min deprivation period, it was necessary to increase the intensity of the shock to maintain arousal, an indication of increased sleep pressure. Surprisingly, during the first 5 min of recovery sleep, we also found a significant rebound in myoclonic twitching (although there was no rebound in sleep duration). This study indicates that some aspects of sleep are regulated homeostatically in early infancy in rats.

Sleep exhibits state-related changes in neural function, including those leading to decreased sensory input to the CNS

Few studies have been conducted to examine state-dependent neural activity in infant rats. Nonetheless, in two studies (Corner and Bour, 1984; Tamásy *et al*., 1980), it was demonstrated that neurons in the pontine and mesencephalic reticular formation exhibited state-dependent activity at P8 and earlier. More recently, hippocampal theta and gamma rhythms were found to exhibit state dependency at P2–5 (Karlsson and Blumberg, 2003; Lahtinen *et al*., 2001). We have also found neurons within the ventromedial medulla that fire selectively during sleep at P8 (Karlsson and Blumberg, in press). To our knowledge, however, there have been no studies that address the issue of neuronal mediation of decreased sensory input during sleep in infants, as has been shown in adults (Soja *et al*., 2001). Thus, although there has been a flurry of recent progress, we agree with Frank and Heller that 'more work needs to be done characterizing neuronal activity [during sleep] in the perinatal period' (p. 30).

The sleep state should be identifiable as a stable species characteristic

Sleep in infant rats clearly satisfies this criterion in that it exhibits stable and predictable characteristics across litters and across time.

Although these eight criteria were devised to help characterize sleep in the adults of diverse species, the extent to which infant rats satisfy them is notable. It seems, then, that Frank and Heller's designation of sleep at these early ages as 'presleep' does not accurately reflect the phenomenology of infant sleep.

ADDING NUCHAL ATONIA AS AN ELECTROGRAPHIC CRITERION OF ACTIVE SLEEP

Frank and Heller view the neocortical EEG as a central element in their theoretical approach. For example, they write that the 'emergence of REM and NREM sleep from presleep occurs approximately at the time of EEG differentiation in both altricial and precocial species' (pp. 29–30). In a tautological rendition of this idea, they write that 'most studies report that states similar to EEG-determined sleep and NREM sleep seem to emerge from [spontaneous fetal activity] approximately at the time of EEG differentiation' (p. 29). Elsewhere, they state: 'Although mechanisms governing EEG differentiation do not necessarily drive the organization of other sleep phenomena, the appearance of the EEG is a consistent hallmark of organized sleep behavior in these species,' and that the concordance of sleep parameters into 'recognizable sleep states … invariably occurs near the time of EEG differentiation' (p. 29). These and other similar comments leave little doubt that Frank and Heller view the EEG as an essential component for assessing sleep in infants (as indeed it has been for many other investigators). Our question, however, is whether this single component plays an inordinate role in their conceptualization, committing an error akin to (in their words) 'restricting the definition of REM sleep to the presence of a single behavior' (p. 30).

Sleep researchers have long cautioned against the overgeneralization of sleep scoring methods established in one species or age to other species and ages. For example, in their manual for scoring sleep, Rechtschaffen and Kales (1968) were clear in stating that 'it is well known that human infants show combinations of polygraphic features which defy classification by the criteria proposed [in this manual]. A strict adherence to the proposed system would not yield an adequate description of infant sleep' (p. 1). Thus, at those ages where the EEG does not provide useful information, we must rely on other measures for characterizing sleep.

In contrast, Frank and Heller seem troubled that 'precursor sleep states are identified based solely on their behavioral similarities to EEG-determined sleep' (pp. 25–26). It is widely acknowledged, however, that the neocortical EEG is not causal to sleep, but rather is a non-causal correlate of sleep. Although Frank and Heller come close to making this point ('mechanisms governing EEG differentiation do not *necessarily* drive the organization of other sleep phenomena,' p. 29), Siegel (1999) is more clear: 'Both active sleep in the neonate and REM sleep in the adult can be defined by purely behavioral criteria. We must remember that the EEG derives its value because of its correlation with behavioral measures of sleep. If animals are responsive and locomoting, we say they are awake, even if their EEG is high in voltage, a condition that can be created by certain brain lesions and by administration of the muscarinic receptor blocker atropine' (p. 89). Conversely, patients exhibiting a condition called alpha coma are behaviorally non-responsive despite exhibiting a wake-like EEG (Jones, 2000).

To emphasize the reliance on behavioral (as opposed to electrographic) characterizations of sleep in infants at ages where the EEG is not a reliable indicator of sleep, Frank and Heller introduce a novel nomenclature: bAS and bQS for 'behavioral active sleep' and 'behavioral quiet sleep,' respectively. We contend, however, that this nomenclature is not warranted in light of recent studies showing that infant rats as young as P2 (a) cycle rapidly between periods of high nuchal muscle tone and atonia, and (b) exhibit myoclonic twitching only against a background of atonia (Karlsson and Blumberg, 2002; Karlsson *et al*., 2004). In our view, this early concordance between twitching and atonia is not a coincidence, but rather indicates a state that is closely related to REM sleep, as others have concluded on the basis of less definitive evidence (Siegel, 1999).

We can now revisit the sleep–wake cycle of infant rats, already described above, but now add information derived from the measurement of the nuchal EMG (Karlsson and Blumberg, 2002). During high-amplitude awake behaviors, nuchal tone is high and remains high for several seconds after the movements cease; then, pups remain behaviorally quiet as nuchal tone decreases (this decrease in tone is often abrupt); finally, after a brief period in which pups exhibit behavioral quiescence against a background of muscle atonia, myoclonic twitching begins and continues until one observes the simultaneous expression of high-amplitude awake behaviors and the abrupt increase in nuchal muscle tone, thus completing the cycle.

SPONTANEOUS MOTOR ACTIVITY AND CENTRAL EXECUTIVE MECHANISMS

Spontaneous motor activity in the form of myoclonic twitching plays a central role in Frank and Heller's presleep hypothesis: it is viewed as 'dissociated' motor activity that is 'merely a form of [spontaneous fetal activity] that continues to be expressed *ex utero* in altricial species' (p. 30). The notion that myoclonic twitching represents the postnatal expression of fetal motor activity was championed by Corner (1977) and it is clear that the two forms of behavior – prenatal and postnatal – are closely related (Robinson *et al*., 2000). Regardless, the finding that twitching is tightly coupled with nuchal atonia at P2, as discussed above, belies the notion that twitching in newborn rats is 'dissociated' from other indicators of sleep.

Frank and Heller consider myoclonic twitching during presleep in infant rats to be the product of spinal mechanisms alone. Although our own work supports the notion that spinal mechanisms contribute to spontaneous movements in fetuses (Robinson *et al*., 2000) and neonates (Blumberg and Lucas, 1994), Frank and Heller go further to claim that the 'normal cycling of high and low periods of spontaneous motility … is not controlled by executive sleep centers' (p. 30), by which they apparently mean brain mechanisms implicated in adult REM sleep. In an earlier paper (Frank *et al*., 1997), they stated this idea even more clearly: 'Brainstem-midbrain nuclei important in mediating REM sleep expression do not mediate the expression of AS, or AS myoclonia' (p. 64).

While we continue to actively explore the neural substrates of infant sleep, there is already compelling evidence that supraspinal mechanisms are involved, including mechanisms typically associated with adult sleep. First, our finding of a tight link between twitching and nuchal atonia argues for coordination of these two sleep components within the brain. This inference gains perhaps its strongest support from evidence that, as early as P7, activation of the ventromedial medulla produces nuchal atonia (Karlsson and Blumberg, in press), just as it does in adults during REM sleep (Hajnik *et al*., 2000). It follows, then, that coordination of nuchal atonia and twitching must involve mechanisms within the brain, including at least one neural mechanism that appears functionally identical to that involved in REM sleep in adults.

Secondly, numerous additional findings support the notion of central coordination of sleep states during the first postnatal week. For example, sleep-related expression of hippocampal theta (Karlsson and Blumberg, 2003) and eye muscle activity (A.M.H. Seelke and M.S. Blumberg, unpublished observations), sleep-related modulation of olfactory threshold (Seelke and Blumberg, 2004), and homeostatic regulation of sleep (Blumberg et al., in press) all imply more complex central organization of sleep than Frank and Heller's conceptualization allows.

Thirdly, we have reported substantial decreases in twitching by P8 rats after transections that are caudal, but not rostral, to the mesopontine region (Kreider and Blumberg, 2000). Frank and Heller question these findings, writing that they would 'have been more compelling had younger rats been examined as EEGs begin differentiating very early' (p. 28) in the albino strain of rats used by Kreider and Blumberg. The basis for Frank and Heller's suggestion that albino rats exhibit EEG differentiation approximately four days earlier (i.e. at P8) than hooded rats (i.e. at P12) is a methods paper that reports no comparison of strains and no measures of sleep (Snead and Stephens, 1983). Where such comparisons are available,

however, the evidence indicates that albino rats exhibit state-related EEG differentiation only 1 day earlier than hooded rats (Gramsbergen, 1976).

Additional findings from our laboratory support the view that mesopontine – and even hypothalamic – mechanisms contribute to sleep regulation during early infancy. For example, we have found that P2 rats cycle rapidly (i.e. approximately every 10 s) between periods of high muscle tone and atonia and that these cycles elongate significantly during the first postnatal week (Karlsson *et al*., 2004). We have also observed in P2 rats that transections caudal to the mesopontine area result in animals that exhibit neither atonia nor myoclonic twitching (K.Æ. Karlsson and M.S. Blumberg, unpublished observations); as the transections are moved rostral to the mesopontine region, atonia and twitching are restored. In P8s, transections that lie between the mesopontine area and the rostral hypothalamus produce rapid cycling that is characteristic of P2s (without disrupting the coupling between nuchal atonia and myoclonic twitching), suggesting that rostral hypothalamic structures, perhaps those within the ventrolateral preoptic area (Saper *et al*., 2001), play an increasing role in sleep regulation over the first postnatal week.

Frank and Heller also examine neuropharmacological differences between infants and adults to support their claim of distinct neurophysiological mechanisms. For example, they note that the cholinergic system, well-known to be an important modulator of REM sleep in adults, is 'extremely immature' (p. 28) in infants at an age when active sleep predominates. Frank and Heller support this claim in part by citing evidence concerning neurotransmitter and receptor levels in infants. For example, they cite research in infant mice showing that brainstem and cortical acetylcholine levels are at 10% of adult levels. Evidence from rats, however, tells a somewhat different story. Specifically, in infant rats during the first postnatal week, acetylcholine levels are 40% of adult whole-brain values (cholinergic markers appear sooner in the pons and medulla) and then decrease over the next 2 weeks before increasing to adult values around the sixth postnatal week (Johnston and Silverstein, 1998; Semba, 1992). Moreover, although levels of acetylcholine and muscarinic receptor densities are reduced in fetuses and neonates, there is a compensatory increase in the responsiveness of muscarinic receptors to cholinergic stimulation (Heacock *et al*., 1987; Johnston and Silverstein, 1998). In other words, the cholinergic system of infants may not be functionally immature.

Even more significant for the present discussion, however, is that infusions of the cholinergic agonist carbachol into the pontine reticular formation of adult rats do not evoke the powerful and reliable REM-sleep-promoting responses that they do in cats (Boissard *et al*., 2002). This striking species difference does not mean that the cholinergic system plays no role in the activation of REM sleep and its components in rats; indeed, carbachol infusions into the nucleus subcoeruleus of rats activates P waves (Datta *et al*., 1998). But such species differences do highlight the danger of supposing an essential linkage between a complex behavioral process documented across many species and any single neural mechanism documented in one or only a few species. And if this caution is valid for comparisons between species, it should also be valid for comparisons within species at different ages.

For the field of infant sleep research to move forward, we need detailed developmental information concerning the sleep-related functioning of specific nuclei and the role of specific neurotransmitters. Thus, in addition to acetylcholine and the monoamines, which Frank and Heller discuss, there are many other neurotransmitters whose roles in adult – but not infant – sleep have been established, including glutamate, orexin, adenosine, and GABA (Arrigoni *et al*., 2001; Boissard *et al*., 2002, 2003; Datta, 2002; Kiyashchenko *et al*., 2001; Nitz and Siegel, 1997). Of particular importance for our understanding of developmental changes in sleep may be the transition in GABA's effects – from excitatory to inhibitory – during early development (Ben-Ari, 2002). Ultimately, then, our goal should be to understand the developing contributions of these and other transmitter systems to infant sleep regulation, not merely to document differences between infants and adults.

THE SIGNIFICANCE OF OVERLAPPING REM AND NREM SLEEP COMPONENTS

Our reading of Frank and Heller's papers on sleep development suggests to us that their reconceptualization of sleep was inspired by a single observation: Specifically, that with the onset of a differentiated EEG at P12 (the age at which their observations began) they observed episodes where cortical slow waves were accompanied by myoclonic twitches (Frank and Heller, 1997). Such periods of 'half-activated' REM sleep (Jouvet-Mounier *et al*., 1970) were interpreted as a blended state comprising both NREM and REM sleep components (i.e. slow waves and myoclonic twitches, respectively). Although this overlap sometimes occurs at boundaries between states, Frank and Heller contend that the overlap was 'more evenly distributed across periods of sleep' (p. 26). Noting that these periods of overlap diminish as NREM sleep develops, Frank and Heller conclude that they 'represent instances of adult-like NREM sleep emerging from bAS' (p. 26).

The observation of slow waves (or spindles, as has been reported in kittens; Jouvet-Mounier *et al*., 1970) during periods of twitching requires explanation. Before we take these observations at face value, however, consider the following: we have occasionally observed myoclonic twitches in 1-week-old rats that appeared to occur against a background of high nuchal muscle tone, only to find on closer inspection that the nuchal muscle became briefly atonic at the moment when the twitch was observed. Because Frank and Heller (1997) used 10-s epochs to evaluate their sleep data, and because they do not report their method of evaluating twitching (and whether they distinguished twitching from other movements, such as startles), the contention that slow waves and myoclonic twitching overlap at P12 deserves closer scrutiny.

But even if an overlap between some sleep components is a reliable finding, such a finding does not invalidate the milestones in sleep development that have already been reached. In this regard, it is significant that the process of sleep development is orderly and cumulative in that previously integrated components remain integrated as new components are added. Thus, when the differentiated EEG comes 'on-line' at P12, the temporal disintegration of the previously achieved concordance between twitching and atonia is not observed. We repeat: *The possible overlap of sleep components at one age does not negate the processes of sleep development that have already occurred.*

CONCLUDING COMMENTS

Frank and Heller have performed an important service by highlighting the need for a theory of sleep development that accounts for the available data and that makes explicit predictions. At this point in time, however, we believe that those features of the presleep theory that distinguish it from the precursor theory (however construed) are not supported by the available evidence. Regardless, our view is that the challenge of understanding sleep development 'is to explain each of the individual components of active sleep in developmental time and investigate the processes by which these multiple components coalesce, cohere, and self-organize during ontogeny' (p. 4) (Blumberg and Lucas, 1996). Accordingly, any theory of sleep development must account for both the *addition* and *integration* of sleep components, as well as changes in sleep *persistence* during ontogeny (see Dreyfus-Brisac, 1970, and Corner, 1985, for similar perspectives).

Critical gaps remain in our understanding of infant sleep. For example, it remains unclear whether infant sleep is best considered a single state comprising tonic (i.e. atonia) and phasic (e.g. twitching) components, or two states akin to active and quiet sleep. In making this determination, we will want to avoid mere semantic distinctions and focus instead on the organization of multiple components and their neural substrates. Longitudinal assessments may prove extremely valuable for establishing the developmental relations between infant and adult sleep; indeed, a recent longitudinal study in two strains of rats has provided intriguing evidence for a correspondence between active and REM sleep and between quiet and SWS (Dugovic and Turek, 2001).

An obvious additional gap in our knowledge concerns the neural circuitry underlying sleep in newborns and how it changes over the course of development. This is a daunting task that encompasses changes in the neural mechanisms that activate and integrate sleep components, and that alter the temporal regulation of sleep. Although we find little support for Frank and Heller's contention that infant sleep is governed by 'distinct neurophysiologic mechanisms,' there is little doubt that the neural and neuropharmacological substrates of sleep undergo significant changes during ontogeny. Rather than envisioning these substrates as distinct, however, it seems more likely that component circuits are elaborated and integrated over time, similar to the process by which twitches are spinally generated in fetuses and embryos and are gradually brought under the control of more rostral structures during ontogeny in chicks (Corner, 1973; Provine, 1973) as well as rats (Blumberg and Lucas, 1994; Kreider and Blumberg, 2000; Robinson *et al*., 2000). We should be wary, however, of the notion that this process is merely one of 'rostralization;' indeed, the fact that rostral hypothalamic structures are already regulating the expression of sleep in rats during the first postnatal week (Karlsson *et al*., 2004) suggests that neural sleep circuits develop concurrently throughout their rostrocaudal extent.

We end by noting that the field of animal learning made its greatest strides as investigators turned to 'simple' animal models of learning in invertebrates (e.g. *Aplysia*) (Kandel and Schwartz, 1982) and well-defined model systems in adult mammals (e.g. eye-blink conditioning) (Gormezano *et al*., 1983; Thompson, 1986). Similarly, sleep researchers are considering the potential benefits of using 'simple' animal models, including invertebrates (Hendricks *et al*., 2000). We believe that the infants of altricial species, such as rats, also offer uniquely valuable opportunities for making rapid progress in our understanding of the mechanisms and functions of sleep.

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Unresolved issues in sleep ontogeny: a response to Blumberg *et al.*

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In 2003 we presented a review of sleep ontogeny in the hope of stimulating new research in this area (Frank and Heller, 2003). Based on our own work and that of others, we proposed that both REM and NREM sleep develop from a form of spontaneous fetal activity (presleep) that outwardly resembles REM sleep. While our manuscript was in review, Blumberg *et al.* published findings in support of the traditional view that REM sleep is present at birth in altricial species like the rat. Their response to our review is thoughtful and their studies are important because they address basic questions about neonatal sleep. However, the rather strong conclusions in their response are premature. It is difficult to assess their views fully because many of the studies on which they are based are unpublished (see Table 1). We will deal with their peer-reviewed work, respond to specific interpretations of our studies and discuss three main issues raised in their response: the relationship between presleep and sleep, the use of behavior and electrophysiology in neonatal state assignments and subcortical evidence for REM sleep in the pre-EEG (prior to EEG differentiation) period.

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PRESLEEP AND SLEEP

In a classic straw man argument, Blumberg *et al.* present a distorted version of our ideas which they then attack in a rather lengthy discussion. We have never stated that presleep is not a type of sleep. Nor have we claimed that presleep bears no relation to later developing REM and NREM sleep. Our argument is that presleep is not a homolog of REM sleep and instead represents a common precursor to REM and NREM sleep. By analogy, embryonic limb buds are still limbs, but they are not hands, fingers or feet. As discussed in our review, the precise relationship between presleep and adult sleep states is unclear, but it is quite possible that events in presleep are incorporated into later appearing REM and NREM sleep. To what extent this primordial state satisfies general criteria for sleep is an interesting question, but this is largely irrelevant to our main points.

EEG, —TWITCHES AND SLEEP

An old debate in the field of neonatal sleep research concerns the choice of criteria in assigning vigilance states. Our position on this matter (as discussed in our review) is straightforward: EEG differentiation is a consistent hallmark of the appearance of states that satisfy multiple behavioral and neurophysiological criteria for sleep. This suggests that organized sleep states appear

Table 1 Critical findings cited by Blumberg *et al.* in their response to our review

Claim	*Status at time of our letter*
Myoclonia is coupled to 'atonia'	Karlsson and Blumberg (2002)
P2 rat pups exhibit hippocampal theta in AS	Karlsson and Blumberg (2003)
Behavioral sleep deprivation increases arousal threshold	Unpublished
Behavioral sleep deprivation increases myoclonia	Unpublished
State-specific firing of ventromedial medulla	Unpublished
Coupling of hippocampal theta to REMs and homeostatic regulation	Unpublished
Transections caudal to mesopontine area abolish 'atonia' and myoclonia	Unpublished

around this time. This seems reasonable because most scientists agree that mammalian sleep is a *brain* phenomenon and field potentials like the cortical EEG are measurements of brain activity. Could there be states homologous to EEG-defined REM and NREM sleep before the appearance of differentiated cortical EEGs? Yes, but this has not been conclusively demonstrated. Until this happens, we find it useful to classify states as either behaviorally determined (behavioral active sleep-bAS or behavioral quiet sleep-bQS) or electrographically determined (REM and NREM/slow-wave sleep).

In our original study of neonatal rats we used three separate parameters to determine states: video records of behavior, EMG and EEG recordings. The behavioral criteria we used to determine AS and QS were similar to those used by Jouvet-Mounier (Frank and Heller, 1997; Jouvet-Mounier *et al.*, 1970). In contrast, Blumberg *et al.* predominantly use a single measurement (motor activity) to determine vigilance states. Based on the analysis of motor activity, they claim to find evidence of REM sleep (a 'unified' state characterized by myoclonia coupled to 'atonia') before the appearance of differentiated EEGs (Karlsson and Blumberg, 2002). We do not find their data persuasive.

In the latter study, 'atonia' is measured with nuchal EMG recordings. Strictly speaking, such measurements do *not* reveal REM sleep atonia *per se* – only gross changes in motor activity (Karlsson and Blumberg, 2002). Sleeping reptiles also have 'atonia' based on EMG recordings, but there is little evidence that reptiles have REM sleep (Frank, 1999). To prove the presence of REM sleep atonia during AS, they must show that REM sleep inhibitory mechanisms are active in newborn rats (Chase and Morales, 2000; Fenik *et al.*, 2004). This is highly unlikely because GABA and glycine are *excitatory*, not inhibitory in the CNS in the first postnatal week (Leinekugel *et al.*, 1999; Singer and Berger, 2000). Indeed, the absence of glycinergic inhibition in newborn animals may explain the unusual finding that reflexes normally suppressed during REM sleep are *enhanced* during pre-EEG (prior to EEG differentiation) AS (Chase, 1971). In short, the significance of this study has been overstated. The 'unified' state they report is simply a period of hypotonia coupled with spontaneous twitches – a condition noted decades ago and just as easily explained as a form of spontaneous fetal activity.

A surprising oversight by Blumberg *et al.* is that they have never shown that cortical EEGs are undifferentiated in their neonatal rats. As shown in Table 2, there is some variability regarding the age when differentiated EEGs are first reported. As noted by Gramsbergen (1976), the variability in the timing of EEG development may be due to differences in rat strains. For example, EEG patterns similar to human 'trace alternant' are detected during sleep in the Lister strain around P9–P10 and transform into SWS EEG patterns by P11 (Gramsbergen, 1976). We have observed a similar developmental pattern in the Long–Evans strain. Other studies report differentiated EEGs at much younger ages *including ages assumed by Blumberg to be pre-EEG*. The Snead and Stephens (1983) study is informative because it shows that differentiated EEGs in alert rat pups are reported very early in the strain used by Blumberg. Sleep-specific EEGs were not examined, but in other studies sleep EEGs usually appear near the time wake EEGs are detected (Aristakesyan and Vataev, 1993; Gramsbergen, 1976; Jouvet-Mounier *et al.*, 1970). In light of these findings, Blumberg *et al.* should verify that their rats are pre-EEG at the ages they claim.

Although Blumberg *et al.* do not measure cortical EEGs in their studies, they suggest that our observation of EEG slow-waves coupled with low EMG activity and REMs and twitches is because of rapid cycling of 'micro' REM sleep and NREM sleep episodes. This was not the case. The epochs in question were continuous periods of delta waves, with no intervening periods of EEG 'flattening' as might be expected if there were smaller cycles of fast, low amplitude waves interposed between slower waves. Spectral analysis of the EEG in these epochs confirmed that these epochs were not mixtures of synchronized and desynchronized EEG states (Frank and Heller, 1997).

Table 2 Ontogenetic appearance of state-specific EEGs varies by strain

	EEG type	*EEGs reported in*	*Age at differentiation*
Wistar	CTX	AS, QS and wake	P5-7 (Aristakesyan and Vataev, 1993)
Wistar	CTX	Not reported	P8 (Dux *et al.*, 1992)
Sprague–Dawley	CTX, HPP	Wake	P1-2 (Snead and Stephens, 1983)
Lister (black & white)	CTX	AS, QS and wake	P9 (Gramsbergen, 1976)
Jouvet-Mounier (1976)	CTX	AS, QS and wake	P6–P8 (AS, wake) P11(QS)

Postnatal age at birth has been set to 0 (P0) for all studies.
Jouvet-Mounier did not report the strain used.
CTX, cerebral cortex; HPP, hippocampus.

SUBCORTICAL SIGNS OF REM SLEEP

As discussed in our original review, the evidence for subcortical signs of REM sleep prior to cortical EEG differentiation is equivocal. Many studies do not find evidence of REM sleep and those cited by Blumberg *et al.* (which were included in our review) are open to interpretation. For example, Corner and Bour (1984) recorded from the FTG area in Wistar rat pups; an area now known to be more important in *movement* than REM sleep (Siegel, 2000). In light of what we now know about FTG neurons, heightened firing in the FTG during AS in pre-EEG rats is more likely related to heightened 'twitchiness' and not the activity of REM sleep mechanisms. A very interesting finding from this study is that cells more active in AS than wake appear 'abruptly' in the second postnatal week (Corner and Bour, 1984) – which is precisely the time when differentiated EEGs are commonly detected in the Wistar strain. The Lahtinen *et al.* (2002) study used very small numbers of rats so the results should be cautiously interpreted. The Tamasy *et al.* (1980) study currently provides the best evidence for state-specific activity in the pre-EEG period. In this study, increases in medial reticular formation and basal forebrain multi-unit activity were reported during AS in newborn rats. However, the identity of these neurons is not known nor was the correspondence between behavioral measurements and multi-unit activity quantitatively assessed.

The situation has not improved much in the last few years. Karlsson and Blumberg (2003) report hippocampal theta in P2 rats but these findings have not been confirmed in another study (Leinekugel *et al.*, 2002). It is also unclear if the theta recorded by Karlsson and Blumberg is identical to theta normally generated in REM sleep. First, they find that 78.6% of theta bouts in newborn rats occur during a state that sounds like *quiet sleep or quiet wake* ('still' periods with low-medium motor tone *without* myoclonia) and only 21.4% during AS as defined by low motor tone and 'sporadic twitching' (Karlsson and Blumberg, 2003). Secondly, the theta frequencies they report are much faster than REM sleep theta in older rat pups with differentiated EEGs. We and others have shown that at ages when the determination of state is not in doubt, theta in REM sleep centers around 4.5–5.0 Hz and shifts towards faster frequencies during later development (Bronzino *et al.*, 1987; Cavoy and Delacour, 1981; Frank and Heller, 1997). Karlsson and Blumberg, on the contrary, report 'theta' centered at 8.0 Hz in P2 rats. Are they proposing that REM sleep theta is first fast, then slow, and then fast again with later development? Considering that the majority of theta bouts do *not* occur in a state characterized by 'atonia' and myoclonia, is it possible that they are recording something else entirely? There are different types of hippocampal theta – even a form triggered by rotating restrained rats onto their backs; a situation remarkably similar to the restrained supine recording position used by Karlsson and Blumberg (Buzsaki, 2002; Gavrilov *et al.*, 1995; Karlsson and Blumberg, 2003). Moreover, it is unlikely that REM sleep theta *could* be present at the ages they claim. The inhibitory neurotransmitter GABA, which is critical in the generation of REM sleep theta, is excitatory in newborn rats (Buzsaki, 2002; Leinekugel *et al.*, 1999).

Their discussion of cholinergic maturation is misleading. Reports of heightened muscarinic receptor sensitivity in the neonatal rat were primarily based on assays that use the neuronal uptake of labeled substrates such as [^{3}H] inositol. Immature neurons tend to be more permeable to these substances. Consequently, cholinergic receptor-mediated metabolism of the labeled substrate appears higher in immature neurons than in mature neurons. When this is corrected for, no such 'super-sensitivity' in muscarinic receptors is detected (Lee *et al.*, 1990). More recent studies of cholinergic nuclei important in REM sleep also cast doubt on assertions made by Blumberg *et al.* As discussed in our original review, levels of the synthetic enzyme for acetylcholine (ChAT) in the rat LDT are extremely low in the first postnatal week (Ninomiya *et al.*, 2001). Neurons in the rat PPN are also quite immature in terms of their bursting properties and their inhibition by 5-HT agonists until the third-fourth postnatal week (Kobayashi *et al.*, 2003).

Blumberg *et al.* then suggest that as carbachol infusions do not enhance REM sleep in adult rats, this somehow diminishes the importance of cholinergic maturation in REM sleep ontogeny. The carbachol issue is a red herring and adds little to the discussion. As acknowledged by Blumberg *et al.* acetylcholine is a critical component of mammalian REM sleep. Even if it were true that carbachol does not increase REM sleep amounts in adult rats, this would not invalidate previous findings showing the importance of acetylcholine in rat REM sleep. In fact, carbachol *has* been shown to increase REM sleep amounts in adult rats (Kubin, 2001; Kumar and Raju, 2001; Wetzel *et al.*, 2003). The variable effects of carbachol in rats, as is true in cats, is likely due to methodological differences between studies (e.g. location, volume of injection, diffusion from site) (Kubin, 2001). The relevant facts are that the cholinergic system is crucial for rat REM sleep and its immaturity in newborn rats poses a major problem for these investigators.

CONCLUDING REMARKS

In conclusion, it is worthwhile to remind the reader why we championed the presleep theory. Blumberg *et al.* are correct to point out that our interest in this idea was partly motivated by our finding of states that looked like NREM sleep based on the EEG but were behaviorally indistinguishable from REM sleep. We were also motivated by the fact that upon close inspection of the available literature we found little evidence to support the traditional view that neonatal behavioral sleep is homologous to EEG-defined states. In our opinion this was a view founded on assumptions as much it was on empirical findings. The studies by Blumberg *et al.* are promising, but in our opinion Blumberg *et al.* have not demonstrated an 'orderly and cumulative' addition of REM sleep components to a pre-existing REM sleep state.

Whatever the ultimate outcome of these different ideas about sleep ontogeny, there is no disagreement over the

importance of studying sleep during infancy. SIDS is the most obvious stimulant for further research, but there is much more to be learned by examining sleep ontogenesis. Developmental changes in circadian and homeostatic sleep mechanisms present an important challenge to current views about how and why sleep is regulated. No theory of sleep function is complete without accounting for the dramatic changes in sleep during perinatal development. Indeed, the examination of neonatal sleep may yield important clues about sleep function across the lifespan. We now know that many behavioral systems exhibit critical developmental periods when changes in neurochemistry or stimulation lead to profound and irreversible changes in adult behavior. Are there similar critical periods for sleep? Might not some of the childhood and adult sleep disorders so prevalent in society have their beginnings in some insult during infancy? We thank Blumberg *et al.* for challenging our ideas about sleep ontogenesis. It is only through critical examination of assumptions and evidence that a deeper understanding will emerge.

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