Avian Sleep Homeostasis:

Electrophysiological, Molecular and Evolutionary Approaches

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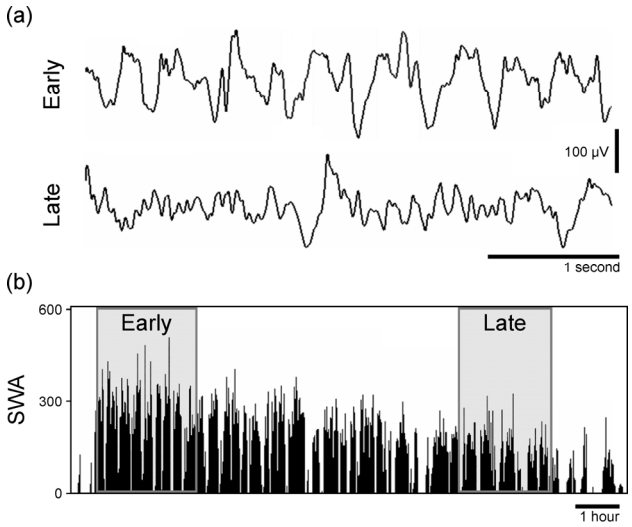
Summary

The function of slow wave sleep (SWS) and rapid eye movement (REM) sleep in mammals is an unanswered question in neuroscience. Aside from mammals, only birds engage in these states. Because birds independently evolved SWS and REM sleep, the study of sleeping birds may help identify shared traits related to the function of these states. Throughout this dissertation, we apply such a bird's perspective to the sleeping brain. We begin with a review on knowledge gained through the study of sleep in animals (Chapter 1). Next, we present results from the first electrophysiological study of sleep in the most basal group of living birds by studying ostriches (Chapter 2). Although ostriches engage in unequivocal SWS, their REM sleep electrophysiology is unique and resembles features of REM sleep present only in basal mammals. Thus, the evolution REM sleep may have followed a recurring sequence of steps in mammals and birds. The remaining chapters deal with the regulation of sleep (or *sleep homeostasis*). Sleep homeostasis refers to an increase in the intensity of sleep (typically quantified as slow wave activity, SWA) following an extended period of wakefulness. Although such a response has long been known to occur in mammals, it has been unclear whether birds are capable of similar changes in SWA following sleep loss. We provide the first experimental evidence for a mammalian-like increase in SWA following enforced wakefulness in birds (Chapter 3). In mammals, SWA increases locally in brain regions used more during prior wakefulness. To see if SWS is regulated locally in birds, we stimulated one part of the pigeon brain during enforced wakefulness and observed a local increase in SWA during subsequent sleep (Chapter 4). Brain regions not stimulated asymmetrically during wakefulness showed a symmetric increase in SWA. These patterns of a/symmetry may reflect changes in the strength of synapses, as they do in mammals, because they are mirrored by changes in the slope of slow waves during SWS – a purported marker of synaptic strength. Lastly, we investigate whether local increases in SWA in birds are mediated by similar molecular mechanisms to those of mammals (Chapter 5). Surprisingly, mRNA levels of such proteins did not respond to unilateral visual stimulation during enforced wakefulness in the manner predicted based on work derived from mammals, but further study is needed to resolve the meaning of this difference. Overall, this dissertation presents several novel findings on the evolution and regulation of avian sleep.

General Introduction

The function of sleep is unknown, but not for lack of study (Stickgold 2005, Tononi and Cirelli 2006, Krueger et al. 2008, Rector et al. 2009, Diekelmann and Born 2010). Most research aimed at answering this question focuses on mammals, and has yielded a wealth of information on changes in brain activity between sleep and wakefulness (Loomis et al. 1937, Aserinsky and Kleitman 1953, Kaufmann et al. 2006), changes in sleep across ontogeny (Kurth et al. 2010a,b), across species (Lesku et al. 2006, 2009, Rattenborg 2007), in the sick (Imeri and Opp 2009), with different waking activities (Tononi and Cirelli 2006), etc. Most basically, we have learned that while asleep the brains of all marsupial and eutherian mammals, with the possible exception of cetaceans (Lyamin et al. 2008), alternate between two states, slow wave sleep (SWS) and rapid eye movement (REM) sleep. The electroencephalogram (EEG) during REM sleep resembles the low amplitude fast waves (or activation) of an alert animal, but with reduced muscle tone and rapid eye movements, while SWS is characterized by high amplitude slow waves (< 4 Hz) in the EEG. During SWS, the level of slow wave activity (SWA, typically defined as 0.5 – 4.5 Hz power density) increases with prior time spent awake and decreases with sleep (Figure 1), a phenomenon that is thought to reflect homeostatically regulated sleep processes (Riedner et al. 2007, Tobler 2011). One approach to understanding the function of SWS and REM sleep is to prevent an animal from entering these states and quantify the effect on subsequent 'recovery' sleep. Such studies identified the homeostatically regulated increase of SWA following sleep loss (Figure 2, reviewed in Tober 2011). Not only does SWA increase with prior time spent awake, but it also increases locally in brain regions used more extensively during prior wakefulness (e.g., Kattler et al. 1994, Vyazovskiy et al. 2000, Huber et al. 2004, Hanlon et al. 2009). For example, rapidly vibrating the right hand of awake humans causes a local increase in SWA in the somatosensory cortex receiving projections from the stimulated hand (Kattler et al. 1994). Conversely, unilateral arm immobilization during wakefulness causes a local decrease in SWA in the corresponding region of the somatosensory cortex (Huber et al. 2006). These studies on local sleep homeostasis reveal that SWA increases as a function of the magnitude of prior waking brain use.

Figure 1. Homeostatic changes in the sleep of the rat. (a) EEG during early and late SWS. (b) SWA (0.5 – 4.0 Hz power density, % of baseline SWS mean) during SWS across a baseline 12 hr sleep period. Note the decline in wave amplitude (a) and SWA (b) with time spent in SWS. Modified from Vyazovskiy et al. (2009) Cortical firing and sleep homeostasis. *Neuron* 63:865-87.



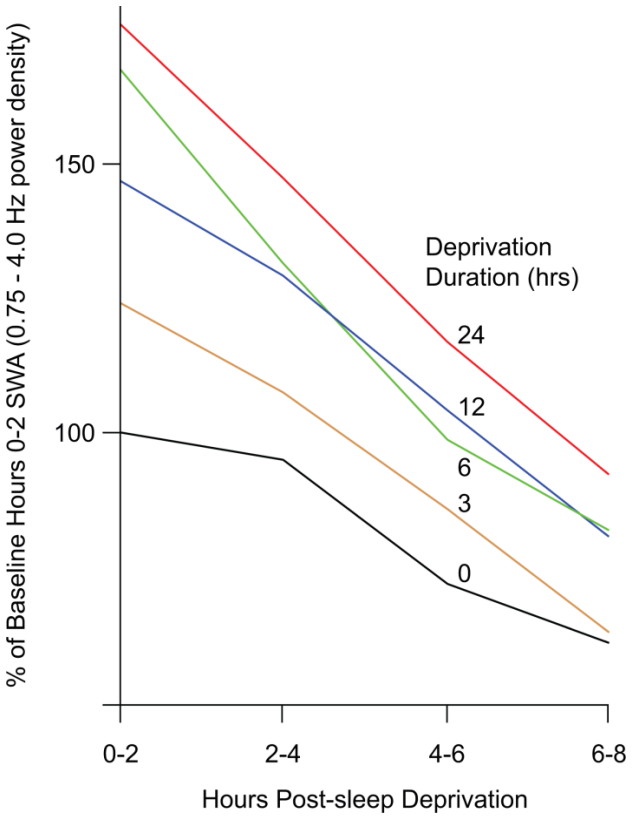


Figure 2. Time course of SWA across a baseline night (0 hrs of deprivation) and following 3, 6, 12 or 24 hrs of sleep deprivation in the rat. Note that the level of SWA during early SWS (0-2) increases with increasing durations of sleep deprivation in a dose-dependent manner, and that SWA decreases during recovery sleep, indicative of a sleep homeostatic response to sleep loss. Modified from Tobler and Borbély (1986) Sleep EEG in the rat as a function of prior waking. *Electroencephalogr Clin Neurophysiol* 64:74-76.

Local sleep homeostasis forms the basis for several, prominent hypotheses for the function of SWS (Tononi and Cirelli 2006, Krueger et al. 2008, Scharf et al. 2008). For instance, waking brain use could deplete brain stores of glycogen, increasing extracellular adenosine, which in turn may increase SWA (Scharf et al. 2008). Complementary to this hypothesis, brain use could increase the production of sleep regulatory substances (Krueger et al. 2008) and the strength and number of synapses in a use-dependent manner (Figure 3, Tononi and Cirelli 2006). Consistent with the latter, several lines of evidence support the idea that wakefulness promotes synaptic strengthening (or potentiation) (Vyazovskiy et al. 2007, 2008, 2009, Bersagliere and Achermann 2010, Kurth et al. 2010a, Leemburg et al. 2010, Liu et al. 2010). However, due to energetic and space constraints in the brain, potentiation is not sustainable in the long-term (Tononi and Cirelli 2006). Moreover, in the absence of a mechanism to check wake-dependent potentiation, connections in the brain would saturate reducing the ability to assimilate new information in the future (Tononi and Cirelli 2006, Hill et al. 2008). The synchrony of the slow oscillation of neuronal membrane potentials underlying SWS (Steriade 2006) has been hypothesized to be the mechanism by which SWS reduces synaptic strength (Figure 3). During early SWS, synapses are stronger and may increase SWA (Esser et al. 2007), because stronger connections are better able to synchronize the slow oscillation (Figure 3, Vyazovskiy et al. 2009). The synchrony of the slow oscillation itself may promote net synaptic downscaling, as stimulation at a low frequency similar to the slow oscillation induces long-term depression (Tononi and Cirelli 2006, Collingridge et al. 2010). Moreover, the neuromodulatory milieu during SWS is also conducive to synaptic depression and genes involved in depression are expressed during sleep (Cirelli et al. 2004, 2005). Regardless of the specific mechanism(s) by which SWS facilitates downscaling, molecular (Vyazovskiy et al. 2008) and electrophysiological (Riedner et al. 2007, Vyazovskiy et al. 2007, 2009, Kurth et al. 2010a, Leemburg et al. 2010, Liu et al. 2010) data and computer simulations (Esser et al. 2007, Olcese et al. 2010) suggest that synapses weaken during SWS. Because the slow oscillation (and SWA) appears to reflect the level of connectivity and may also serve as the mechanism for downscaling, slow oscillation-mediated downscaling could be a self limiting process (Figure 3), terminating when there is insufficient connectivity to maintain the oscillation. The removal of

weak synapses during SWS can account for some of the improvements observed in cognitive tasks by increasing the signal-to-noise ratio of neural circuits (Hill et al. 2008). Despite being well-supported from work derived from mammals, it is unclear if the 'synaptic homeostasis hypothesis' applies only to mammals or to other animals that also show the slow oscillation during sleep.

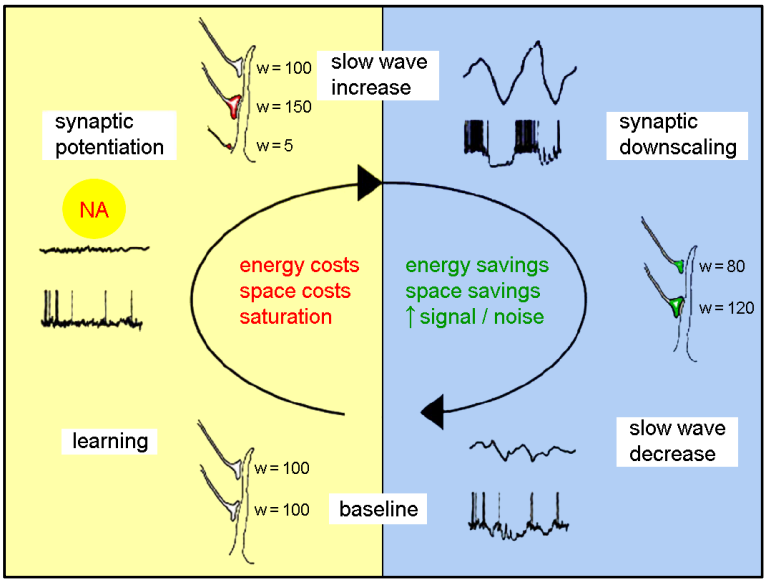


Figure 3. The dynamics of SWA may be explained by concomitant changes in the strength and number of synapses according to this 'synaptic homeostasis hypothesis' (see text for details; noradrenaline, NA). Modified from Tononi and Cirelli (2006) Sleep function and synaptic homeostasis. *Sleep Med Rev* 10:49-62.

Why Study Sleeping Birds?

One paradigm for the study of sleep is the comparative approach, wherein sleep is compared across species in an effort to explain differences or similarities in sleep behavior or physiology. For example, the daily amount of time nearly 100 mammalian species spend in SWS and REM sleep, as determined by laboratory-based electrophysiological methods, is available for phylogenetic comparative analysis (reviewed in Lesku et al. 2009). The most obvious feature of this dataset is that sleep quotas vary greatly across species; e.g., a horse (*Equus caballus*) may sleep for only 12% of the 24 hr day (Ruckebusch 1972), but the large hairy armadillo (*Chaetophractus villosus*) can sleep more than 7 times that amount (Affanni et al. 2001). Assuming that such interspecific differences reflect differences in sleep need, then identifying the factors responsible for maintaining such variation should provide insight into the function of sleep. Many studies have sought relationships between sleep quotas and various traits

thought to be functionally relevant for sleep (e.g., Zepelin and Rechtschaffen 1974, Elgar et al. 1988, Siegel 2005, Lesku et al. 2006). Notably, the relationships between (i) the amount of SWS and lifespan has been quantified to assess sleep's possible role in longevity (Zepelin and Rechtschaffen 1974), (ii) SWS with metabolic rate to assess comparative support for an energy conservation role for sleep (Elgar et al. 1988) and (iii) SWS and REM sleep with encephalization – a potential measure of interspecific cognitive abilities – to determine if sleep-dependent memory consolidation could apply across mammals (Lesku et al. 2006). By identifying evolutionary patterns that are unobservable at other levels of analysis, phylogenetic analyses can provide unique support for hypotheses for the function of sleep. Below we demonstrate the usefulness of the comparative approach using another evolutionary system.

Aside from mammals, only birds engage in SWS and REM sleep. Like mammals, SWS in birds is characterized by high amplitude, low frequency waves in the EEG and intermittent, fast oscillations of the eye (Figure 4). REM sleep in birds is characterized by a mammal-like EEG of low amplitude fast activity (but without a hippocampal theta rhythm like that observed in mammals), bilateral eye closure and rapid eye movements (Figure 4). Birds engaged in REM sleep also show behavioral signs of reduced muscle tone, such as head drooping or sliding of the wings off the sides of the body, but muscle tone rarely reaches the low (atonic) levels observed in mammals engaged in REM sleep (e.g., Klein et al. 1964, Ookawa and Gotoh 1964, Rattenborg et al. 2001). Because of the electrophysiological similarities between sleeping mammals and birds, an obvious question is: *Did SWS and REM sleep arise once in the common ancestor to mammals and birds or twice in the respective ancestor to both groups?*

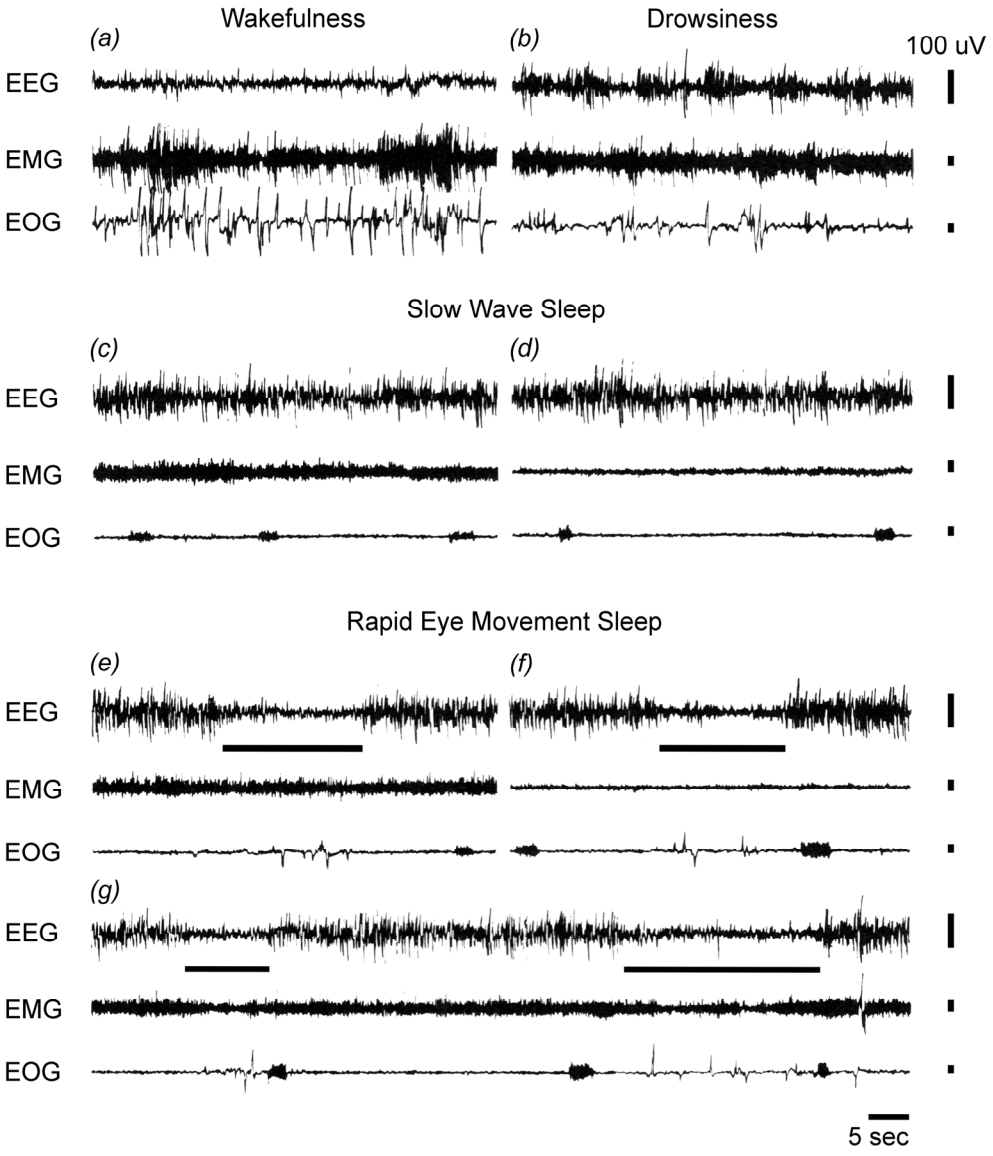


Figure 4. Typical avian electroencephalogram (EEG), electromyogram (EMG) and electrooculogram (EOG) activity, from an emperor penguin (*Aptenodytes forsteri*), during wakefulness (a), drowsiness (b), slow wave sleep (SWS, c-d) and REM sleep (e-g). During drowsiness, the EEG alternates between patterns of wakefulness and SWS. SWS is characterized by high amplitude, slow waves with (c) or without (d) a tonic EMG; the phasic EOG activity reflects brief, rapid oscillations of the eye, unlike that seen during REM sleep. REM sleep (horizontal bar) can likewise occur with (e) or without (f-g) muscle tone and REMs are common. Modified from Buchet et al. (1986) An electrophysiological and behavioral study of sleep in emperor penguins under natural ambient conditions. *Physiol Behav* 38:331-335.

Mammals and birds last shared a common ancestor in the Carboniferous Period, some 300 million years ago (Reisz and Müller 2004, Benton and Donoghue 2007). Unfortunately, the brain activity that defines SWS and REM sleep does not fossilize, hence, we are unable to directly determine if the common ancestor to mammals and birds engaged in similar states during sleep. Instead, we can only infer the evolutionary history of sleep by studying sleep in living animals (see Siegel et al. 1998). For example, assuming that extant animals sleep in the same manner as their ancestors, sleeping reptiles and amphibians may provide insight into how the most recent common ancestor to mammals, birds and reptiles slept.

Reptiles living today are classified into one of four Orders: the Crocodilians (crocodiles and their allies), Squamata (lizards and snakes), Chelonians (turtles and tortoises) and Rhynchocephalia (tuataras). Sleep has been studied electrophysiologically in species from all Orders, with the exception of the threatened tuataras. Because Crocodilians are the closest living relatives to birds one might expect crocodilians to show sleep states similar to that observed in birds; however, a study on sleeping caiman (*Caiman sclerops*) revealed only wake-like, low voltage activity with intermittent high voltage sharp spikes in the EEG (Flanigan et al. 1973). The incidence of spikes correlated with arousal thresholds and increased following sleep deprivation, suggesting that spikes might be a marker of sleep intensity (see also Hartse 1994, Rattenborg 2006, 2007). A similar sleep EEG has been described also in squamate lizards (desert iguana, *Dipsosaurus dorsalis*, Huntley 1987; green iguana, *Iguana iguana*, Flanigan 1973; spiny-tailed iguana, *Ctenosaura pectinata*, Flanigan 1973; chameleons, *Chameleo jacksoni* and *C. melleri*, Tauber et al. 1966), turtles (box turtles, *Terrapene Carolina*, Flanigan et al. 1974) and tortoises (red-footed tortoise, *Geochelone carbonaria*, Flanigan 1974). Interestingly, administration of atropine sulfate – a cholinergic blocking agent that increases mammalian ventral hippocampal spikes that occur during SWS in mammals – increased spiking activity in the red-footed tortoise (Hartse and Rechtschaffen 1974), and administration of parachlorophenylalanine – a serotonin synthesis inhibitor that suppresses hippocampal spikes in mammals – reduced spiking activity (Hartse and Rechtschaffen 1982), suggesting that these spikes occur in a state homologous to mammalian SWS (Hartse 1994, Rattenborg 2006, 2007).

Recent studies have shown that these spikes originate in the reptilian hippocampus (Gaztelu et al. 1991, Lorenzo et al. 1999), thereby corroborating the pharmacological evidence indicating that reptilian spikes are comparable to similar spikes occurring in the mammalian hippocampus during SWS (Hartse and Rechtschaffen 1974, 1982). Despite this similarity at the level of the hippocampus, the reptilian dorsal cortex does not generate concurrent high amplitude slow waves typical of SWS in mammals and birds. REM sleep has been reported in reptiles based on the occurrence of eye and limb movements during sleep (e.g., Tauber et al. 1966), but it remains unclear whether such behaviors reflect REM sleep-related twitching similar to that observed in mammals and birds, or simply brief arousals from sleep. Moreover, sleeping turtles do not show patterns of neuronal activity in the brainstem comparable to that observed during REM sleep in mammals (Eiland et al. 2001). Consequently, reptiles do not appear to exhibit REM sleep or the high amplitude, slow waves that characterize SWS in mammals and birds.

The EEG correlates of sleep behavior in amphibians is poorly studied. In general, the EEG of quiescent frogs is of equal or lower amplitude relative to the EEG of frogs more conspicuously awake (Hobson et al. 1968). Thus, the available evidence from frogs suggests that, like reptiles, amphibians lack mammal (or bird)-like SWS and REM sleep (see also Hobson 1967), such that these states may have evolved twice, once in the most recent common ancestor to mammals, and again in ancestral birds.

The evolutionary convergence of SWS and REM sleep between mammals and birds is key to the importance of birds for sleep research. While sharing similar sleep states, the mammalian neocortex is laminar and largely unlike that of the developmentally homologous nuclear avian pallium (Jarvis et al. 2005). That is not to say that mammalian and avian brains are entirely dissimilar. Indeed, the neocortex and hyperpallium have independently evolved a high degree of connectivity that may be unique among animals (Medina and Reiner 2000). We can use such similarities and differences in brain organization to reduce the number of shared traits potentially linked to the function of sleep (Rattenborg et al. 2009). For instance, the absence of SWS-like high amplitude slow waves in reptiles was historically thought to be related

to their absence of a thick (mammal-like) six-layered neocortex (reviewed in Rattenborg 2006). However, because birds also lack a neocortex and yet show unequivocal SWS, a neocortex *per se* can be rejected as a requisite structure for the genesis of this state.

Organization of the Dissertation

Chapter 1 serves as an introduction to sleep that is more detailed that the one given here. We review what is known about sleeping animals in general, from amniotes to fish and invertebrates, and highlight particularly salient 'non-human / non-rat' contributions to our understanding of the function of sleep. Notably, the study of sleeping cetaceans in the 1970s identified unihemispheric sleep, wherein one hemisphere shows SWS-like waves in the electroencephalogram and the other shows the low amplitude, high frequency pattern characteristic of wakefulness (reviewed in Lyamin et al. 2008). This early discovery would later shift views about sleep and sleep homeostasis from whole-brain to local (Krueger and Obál 1993, Kattler et al. 1994). Importantly, such local sleep is now thought to be intimately linked to the function of SWS in mammals (Tononi and Cirelli 2006, Krueger et al. 2008, Rector et al. 2009). Chapter 1 also serves as a concise introduction into the basics of avian sleep and bird-brain organization.

Chapter 2 examines the electrophysiology of sleeping ostriches. The cortex of the most basal group of mammals, the monotremes, shows only slow waves during sleep (Allison et al. 1972, Manger et al. 2002), but the brainstem shows intermittent signs of eutherian-like REM sleep (Siegel et al. 1996, 1999). This suggests a pattern of REM sleep evolution in which REM sleep in the brainstem and SWS in the cortex were present in the most recent common ancestor to all mammals, and that the alternation of SWS and REM sleep in the cortex is a derived feature present only in marsupials and eutherians (Siegel et al. 1998). To determine if this pattern reflects requisite stages in the evolution of 'classical' REM sleep or one specific to mammals, we studied sleep in basal (Palaeognathae) birds. Indeed, until now, electrophysiologically-based studies of avian sleep have been conducted only on members of the more derived Neognathae, and all show unequivocal SWS and REM sleep (Roth et al. 2006,

Lesku et al. 2009). Ostriches were found to engage in 'typical' SWS. Interestingly, REM sleep, characterized by Neognathae-like reduced muscle tone, head and eye movements, combines aspects of the REM sleep EEG observed in other birds, marsupials and eutherians (i.e., forebrain activation) with features present only in monotremes (i.e., slow waves). These findings reveal a general pattern of REM sleep evolution shared by mammals and birds alike.

Chapters 3 – 5 focus on sleep homeostasis. An increase in SWA following an extended period of wakefulness is a well described homeostatic response to sleep loss in mammals (Tobler 2011), but early studies in birds failed to find a compensatory increase in SWA following ≥ 24 hrs enforced wakefulness (Tobler and Borbély 1988, Berger and Phillips 1994). This puzzling result suggested that, despite sharing similar sleep states, sleep in mammals and birds were regulated differently. Thus, hypotheses for the function of sleep in mammals that relied on such a homeostatic response might not apply to birds. However, in mammals there is a dichotomy in the response of SWA to sleep loss: in some rodents, short-term sleep loss (< 24 hrs) results in increased SWA, but long term does not (Tobler and Jaggi 1987). In Chapter 3, we re-investigate avian sleep homeostasis using a shorter (8 hr) deprivation. By doing so, we demonstrate for the first time that extended periods of wakefulness leads to a mammal-like increase in SWA. This response was symmetric between the hemispheres.

In Chapter 4, we examine whether birds show local sleep homeostasis. In mammals, brain regions used more extensively during prior wakefulness show a local increase in SWA during subsequent sleep (e.g., Kattler et al. 1994, Vyazovskiy et al. 2000, Huber et al. 2004), suggesting that SWS maintains or restores some brain parameter in a use-dependent manner. One possible parameter, proposed for mammals, is the strength of synapses, which increases during wakefulness and decreases with sleep (Tononi and Cirelli 2006). Here, we stimulate one part of the pigeon brain during enforced wakefulness and observe a local increase in SWA during subsequent sleep in the brain region stimulated during the deprivation procedure. In addition, we provide evidence that this local effect might reflect an increase in synaptic strength accrued during wakefulness. Because sleep is regulated locally in the avian brain,

hypotheses for the function of sleep that rely on such regulation may represent a *core* or fundamental function of SWA common to mammals and birds.

Lastly, in Chapter 5, we sought to determine if the molecular mechanisms responsible for increasing SWA in mammals, mediate the increase of SWA in birds as well. Using a similar experimental design to that of Chapter 4, we quantify the levels of brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc) mRNA in stimulated and non-stimulated brain regions. In mammals, BDNF increases the level of SWA (Faraguna et al. 2008). Although the unilateral visual stimulation treatment was successful at eliciting an asymmetry in BDNF in the (visual-processing) hyperpallium of pigeons, BDNF was greater in the visually-deprived hyperpallium. Moreover, an asymmetry in BDNF was identified in the (non-visual) mesopallium, which showed a symmetric increase in SWA in the previous study (Chapter 4). These unexpected findings, coupled with the non-mammal-like observation of lower BDNF mRNA at the end of the day (when pigeons have spent more time awake), suggests that BDNF is not involved in increasing SWA in birds, although further study is clearly needed. Overall, this dissertation provides several novel findings on Avian Sleep Homeostasis using Electrophysiological, Molecular and Evolutionary Approaches.

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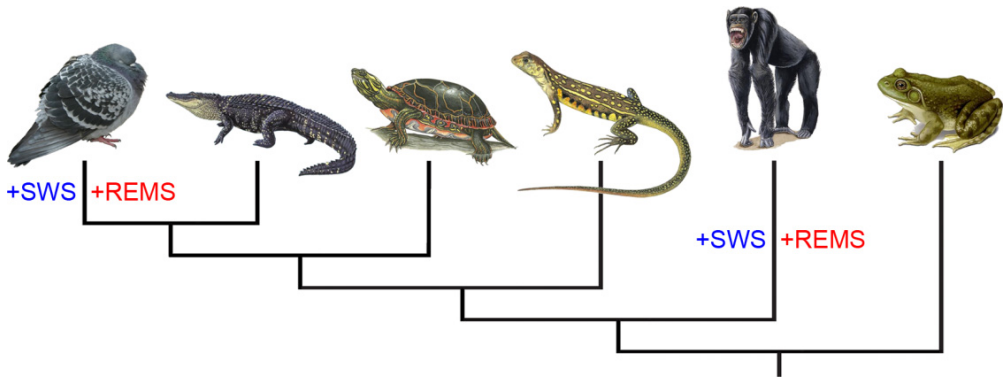
CHAPTER 1

Sleep and Sleep States: Evolution and Ontogeny



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Abstract

The functions of sleep remain elusive. Here, we review our current understanding of the evolution and ontogeny of sleep and demonstrate how comparative sleep research has contributed to our greater understanding of why we sleep. For instance, the occurrence of SWS and REM sleep exclusively in mammals and birds may be related to the convergent evolution of heavily interconnected brains and associated cognitive abilities. We discuss results from comparative analyses on mammalian and avian sleep and highlight promising areas for future research. Lastly, we show how changes in sleep across early ontogeny can provide insight into the functions of sleep.

Introduction

Sleep is a prominent yet enigmatic animal behavior. Despite the apparent ubiquity of sleep and associated vulnerability that (in part) defines sleep, the functions of sleep remain elusive (Stickgold 2005, Tononi and Cirelli 2006, Krueger et al. 2008, Rector et al. 2009, Diekelmann and Born 2010). The seemingly simplest approach to determining sleep's functions would be to compare animals that sleep with those that do not, and then determine whether a relationship exists between various traits thought to be functionally involved in sleep and the presence or absence of sleep. However, all animals adequately studied sleep in one form or another making such comparisons currently impossible (Cirelli and Tononi 2008). Nevertheless, of the 30 or more animal Phyla, detailed sleep information is known for only two: Chordata (includes vertebrates) and Arthropoda (includes insects). Thus, there is an opportunity for truly sleepless animals to be discovered in the future (Siegel 2008). For example, sponges (Phylum: Porifera) are nearest to the base of the Metazoan phylogenetic tree and lack a nervous system. Based on the generally accepted belief that a plastic nervous system is the biological target benefiting from sleep (Tononi and Cirelli 2006, Krueger et al. 2008, Diekelmann and Born 2010), sponges should not sleep.

Determining whether sleep evolved many independent times or only once early in the evolution of animals is of fundamental importance in contemporary comparative sleep

research. With the application of genetic approaches to the study of sleep (Cirelli et al. 2004, Jones et al. 2008), it has become important to establish homology between sleep in mammals and sleep in invertebrates, such as the fruit fly (*Drosophila melanogaster*, Hendricks et al. 2000, Shaw et al. 2000), where genomes generally have less redundancy and are amenable to manipulation (Cirelli et al. 2005, Ganguly-Fitzgerald et al. 2006). Establishing homology between sleep in fruit flies and sleep in mammals would reinforce the usefulness of invertebrate models for determining the molecular correlates of human sleep. Even without homology at the molecular level, comparative studies can enhance our understanding of sleep. For instance, evolutionary convergence (i.e., distantly related species that independently evolved similar sleep-related traits) can provide insight into sleep by revealing overriding principles otherwise obscured by nonessential traits specific to one lineage (see Corticocortical Connectivity and Slow Wave Sleep as an example of convergent evolution).

The lack of a highly-resolved cladogram of sleep across Animalia has not impeded comparative analyses of sleep. Indeed, many studies have tried to determine the relationships between the time species spend asleep and various constitutive, physiological, and ecological traits (Lesku et al. 2006, 2008a, reviewed in Lesku et al. 2009). Historically, this approach has been applied only in mammals where the electrophysiological correlates of sleep have been identified and quantified in a large number of species; however, a recent study provides insight into the correlates of sleep in birds as well (Roth et al. 2006, Lesku et al. 2009). Although the strengths of functional hypotheses derived from comparative analyses are necessarily limited given the correlational nature of the data, the evolutionary patterns gleaned from such analyses, especially those that employ modern phylogenetic statistical techniques (e.g., Lesku et al. 2006), provide a framework for the development of experimentally testable hypotheses (see Comparative Perspectives on the Functions of Sleep).

As with comparing sleep among taxonomic groups, changes in sleep occurring during early development can provide insight into the functions of sleep. For example, changes in sleep duration, intensity, or in the relative proportion of the two sleep states in mammals and

birds over early ontogeny might be functionally linked to the concurrent development of the central nervous system (see Early Ontogeny of Mammalian Sleep and Early Ontogeny of Avian Sleep). In this chapter, we review current knowledge of sleep in various taxonomic groups and over early ontogeny, and relate important patterns to existing hypotheses for the functions of sleep. By doing so, we demonstrate the contribution that comparative sleep research has made to our greater understanding of why we sleep.

Behavioral Definition of Sleep

Sleep is foremost an animal behavior broadly characterized by quiescence with reduced responsiveness to stimuli (Lima et al. 2005). Sleep can be distinguished from other quiescent states (e.g., hibernation) by rapid reversibility to wakefulness with sufficient stimulation and homeostatic regulation; i.e., sleep shows a compensatory increase in intensity or time following sleep loss (Tobler 2011). Sleeping animals often retreat to a species-specific location and assume a characteristic posture (Shaw et al. 2000). Although the occurrence of concurrent sleep and swimming in some aquatic mammals (see Aquatic Mammals) is an exception to the quiescence criterion, these criteria apply to the vast majority of animals studied, ranging from insects to mammals and birds.

Mammalian Sleep

Behavioral sleep is associated with specific changes in brain activity. Eutherian ("placental") and marsupial mammals exhibit two basic states of sleep: slow wave sleep (SWS), also known as non-rapid eye movement (non-REM) sleep, and REM sleep (Siegel 2005). In nonhuman animals, the term SWS usually refers to all non-REM sleep, whereas SWS refers only to stages 3 and 4 of non-REM sleep in humans. SWS is characterized by an electroencephalogram (EEG) of low-frequency, high-amplitude activity arising from the large-scale synchronous slow oscillations of neurons in the neocortex (Steriade et al. 1993, Vyazovskiy et al. 2009). SWS is homeostatically-regulated with SWS-related slow wave activity (SWA) (typically 0.5 – 4.5 Hz EEG spectral power, Riedner et al. 2007) reflecting the intensity of SWS, as arousal thresholds are correlated with the amount of SWA (Neckelmann and Ursin 1993), and SWA increases as a

function of prior time awake (Tobler 2011). REM sleep is characterized by an EEG of high-frequency, low-amplitude activity similar to wakefulness, with a hippocampal theta rhythm (between 4 – 9 Hz or higher depending on the species) observed in some mammals, and an atonic electromyogram (Siegel 2011). Heart and respiratory rate are irregular during REM sleep, and thermoregulatory mechanisms that rely on motor control are diminished (Siegel 2011). REM sleep does not appear to have an intensity component, although the time spent in REM sleep can increase following sleep loss (Tobler 2011). In mammals that engage in long periods of wakefulness, the time spent in SWS is the greatest and SWA is the highest early in the subsequent sleep bout, while the proportion of time devoted to REM sleep increases towards the end of the bout (Tobler 2011).

Monotremes

The study of sleep in monotremes, an egg-laying Order of mammals that are most basal (or "ancient") among extant mammals (Figure 1), may provide insight into sleep in the most recent common ancestor to eutherians and marsupials. An early study of the short-beaked echidna (*Tachyglossus aculeatus*) found only EEG activity indicative of SWS during sleep (Allison et al. 1972), thereby suggesting that REM sleep evolved after the appearance of the eutherian + marsupial lineage. The electrophysiological correlates of sleep in the echidna were recently re-examined using a combination of EEG and brainstem neuronal recordings (Siegel et al. 1996). As in the earlier study, REM sleep with cortical activation was not observed; however, during sleep with cortical slow waves, brainstem reticular neurons fired in an irregular pattern similar to that observed in eutherians during REM sleep (Siegel et al. 1996). This suggests that aspects of REM sleep in the brainstem and SWS in the cortex occur concurrently (Siegel et al. 1998, Manger et al. 2002).

Moreover, an EEG-based study of the duck-billed platypus (*Ornithorhynchus anatinus*), suggests that this mixed sleep state is typical of monotremes (Figure 1, Siegel et al. 1999). Although neuronal activity in the brainstem was not recorded, the platypus showed frequent rapid eye movements and twitching of the head and bill, similar to that associated with REM

sleep in eutherians and marsupials, while the cortex exhibited an EEG pattern indicative of SWS (Siegel et al. 1999). Based on the incidence of twitches, the time spent in REM sleep was estimated at up to 8 hours per day, the highest in any animal. A comparison of sleep times with eutherians and marsupials is difficult to interpret, however, given the heterogeneous nature of sleep and the absence of cortical correlates of REM sleep in monotremes (Lesku et al. 2009).

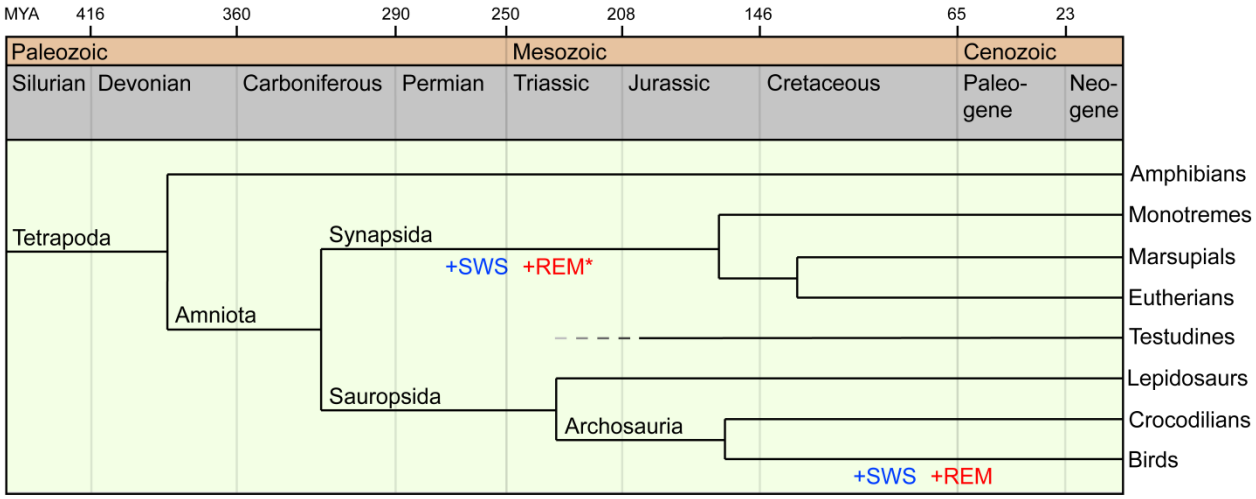

Figure 1. An evolutionary tree for tetrapods showing the evolutionary appearance of SWS and REM sleep. Here, SWS and REM sleep arose independently in the most recent common ancestor to mammals and the most recent common ancestor to birds, although the most basal birds (e.g., ostriches) have not been studied electrophysiologically (but see Chapter 2). However, in mammals, REM sleep first appeared in the common ancestor to all mammals as a heterogeneous state with neuronal activity in the brainstem indicative of REM sleep (+REM*) occurring concurrently with EEG activity indicative of SWS. In the most recent common ancestor to eutherians and marsupials, REM sleep and SWS became segregated into two distinct states with EEG activation occurring in conjunction with REM sleep-related brainstem activity. Time (millions of years ago, MYA) is given at the top of the plot above geological era (dark gray) and period (light gray). The phylogenetic relationships are well established for all groups except testudines (turtles). As a result, the root of this lineage is not depicted. Lepidosaurs include lizards and snakes, and the tuatara.

Aquatic Mammals

Sleeping in the water poses a significant challenge for air breathing mammals. Among Cetaceans, the electrophysiological correlates of sleep have been recorded only in the Odontocetes (dolphins and porpoises). Dolphins and porpoises exhibit unihemispheric SWS (USWS), a mixed state in which one cerebral hemisphere shows EEG activity characteristic of deep SWS while the other shows a pattern indistinguishable from wakefulness (Figure 2, reviewed in Lyamin et al. 2008).

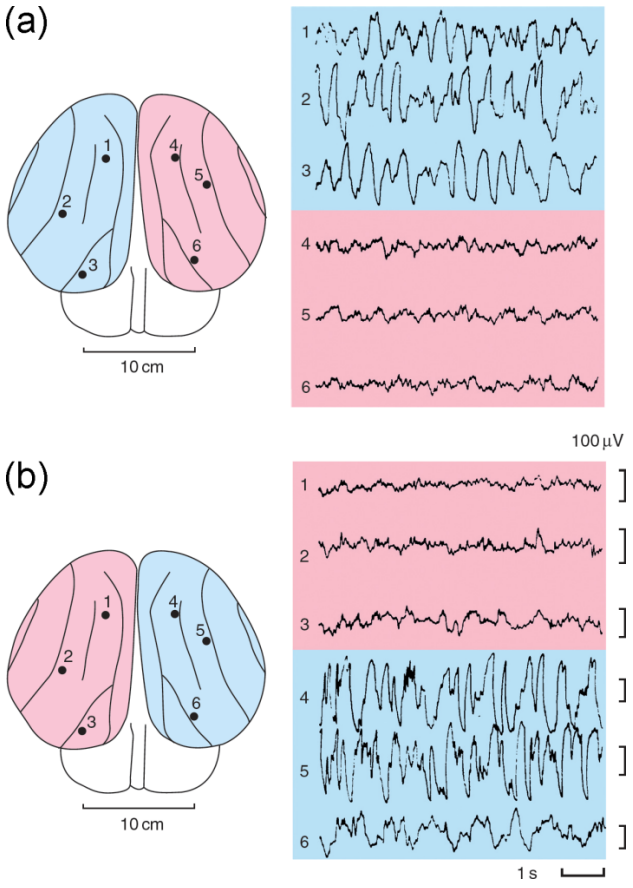

Figure 2. Examples of unihemispheric slow wave sleep in the bottlenosed dolphin (*Tursiops truncatus*). The electroencephalogram was recorded from the anterior, medial, and posterior neocortex. Note the high amplitude, low-frequency activity indicative of slow wave sleep (blue) in only the left (a) or right (b) hemisphere concurrent with low-amplitude, high-frequency activity indicative of wakefulness (red) in the other hemisphere. Modified from Mukhametov et al. (1977) Interhemispheric asymmetry of electroencephloagraphic sleep patterns in dolphins. *Brain Res* 134:581-584.

Although the lighter stages of SWS can occur concurrently in both hemispheres, deep SWS occurs only unihemispherically (Lyamin et al. 2008). Some evidence suggests that SWS is homeostatically-regulated independently in the two hemispheres (Oleksenko et al. 1992), an example of local use-dependent SWS homeostasis, a phenomenon discovered only recently in terrestrial mammals, including humans (Kattler et al. 1994, Vyazovskiy et al. 2000). Dolphins

and porpoises swim and surface to breath during USWS, but can also float at the surface or rest motionless under water. Given that the eye opposite the awake hemisphere remains open, USWS may allow dolphins and porpoises to monitor the environment for conspecifics and predators during sleep (Goley 1999, reviewed in Rattenborg et al. 2000). Although behavioral signs of REM sleep (e.g., twitching and rapid eye movements) have been observed infrequently in quiescent Cetaceans, unequivocal REM sleep has not been recorded electrophysiologically, suggesting that either Cetaceans have lost REM sleep secondarily or REM sleep occurs in very small amounts or in a modified form in Cetaceans (Lyamin et al. 2008). Interestingly, manatees (Order: Sirenia) also exhibit USWS and small amounts of REM sleep (Mukhametov et al. 1992). Unlike Cetaceans and manatees, seals can sleep in the water and on the land (Lyamin 1993). Seals in the Family Phocidae hold their breath during periods of bilateral SWS and REM sleep under water, whereas seals in the Family Otariidae show interhemispheric asymmetries in the intensity of SWS while sleeping on the surface of the water (Lyamin et al. 2008).

Early Ontogeny of Mammalian Sleep

The time spent asleep changes greatly over early development. In mammals, newborns generally sleep the longest. This high amount of sleep declines over early ontogeny until it stabilizes at a species-specific level similar to that seen in adults. The proportion of total sleep time devoted to REM sleep (%REM sleep) and SWS also changes greatly over the first postnatal weeks. Interestingly, the magnitude of these changes appears to be influenced by the degree of precociality at birth (Figure 3).

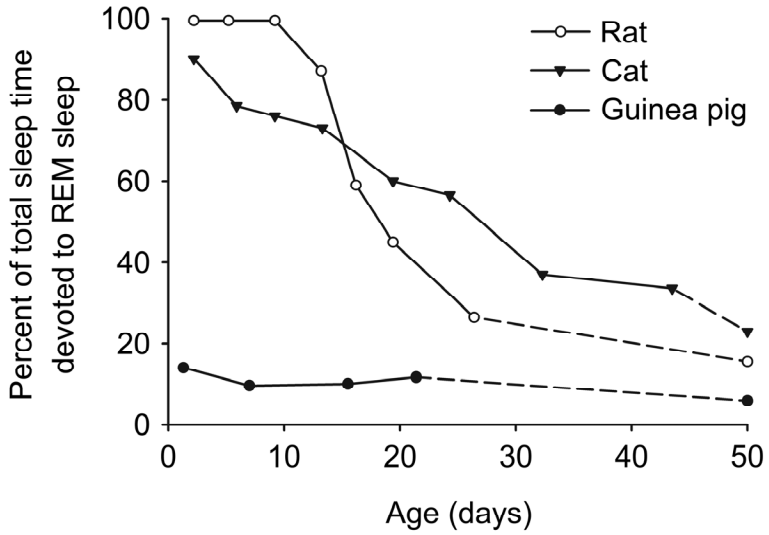

Figure 3. Changes in the percent of total sleep time devoted to rapid eye movement (%REM) sleep across early development in the altricial rat and cat, and in the precocial guinea pig. Note, however, that even as adults, altricial species have higher %REM sleep than more precocial species. Reprinted from Jouvet-Mounier et al. (1970) Ontogenesis of the states of sleep in rat, cat, and guinea pig during the first postnatal month. *Dev Psychobiol* 2:216-239.

Altricial mammals (those that are relatively more dependent on their parents at birth for food, warmth, and protection; e.g., rats and cats) show a marked reduction in %REM sleep through the first postnatal weeks (Figure 3), and show more exaggerated behavioral characteristics of REM sleep, such as rapid eye movements and twitches. Conversely, newborn precocial mammals (e.g., guinea pigs) are more developed relative to altricial mammals, and exhibit a %REM sleep comparable with that of adults (Figure 3). These observations led (in part) to the hypothesis that REM sleep provides endogenous stimulation necessary for the early development of the central nervous system, particularly that related to the visual system (Roffwarg et al. 1966). Indeed, experimental evidence implicates REM sleep in maturational processes of the visual system during early ontogeny (Marks et al. 1995, Shaffery et al. 2002). As discussed below, phylogenetic comparative analyses also support the idea that REM sleep facilitates early brain development (see Comparative Perspectives on the Functions of Sleep).

Unlike newborn terrestrial mammals, newborn Cetacean calves are continuously active, swimming along side their mothers during the first few weeks postpartum (Lyamin et al. 2005). Although electrophysiological recordings have not been obtained during this period of activity, the calves close one eye intermittently while swimming under water and, therefore, might be engaging in USWS. However, calves are unlikely to be engaging in REM sleep if REM sleep is incompatible with swimming. The possible absence of REM sleep in newborn calves during their first weeks of life seemingly challenges theories for a functional role of REM sleep in brain development; however, given that Cetacean calves are extremely precocial, REM sleep could nonetheless play a role in brain development *in utero*.

Avian Sleep

Birds are a particularly interesting taxonomic group in which to study sleep. Birds have brains comparable in relative size to mammals (Jerison 1985), and cognitive abilities that include vocal learning and tool making (Emery and Clayton 2004, 2005, Bolhuis and Gahr 2006), and yet much of the avian forebrain is organized in a manner markedly different from mammals (Jarvis et al. 2005, Kirsch et al. 2008). Although the dorsal two-thirds of the avian forebrain (a region formerly thought to be primarily striatal) is derived from the same embryonic neural tissue (the pallium) that gives rise to the mammalian neocortex, the avian pallium is arranged in a nuclear manner that largely lacks the true laminar organization of the neocortex (Figure 4). Interestingly, despite this difference in pallial organization, birds are the only nonmammalian taxonomic group to show unequivocal SWS and REM sleep (Figure 1, Rattenborg et al. 2009).

Like mammalian SWS, avian SWS is characterized by an EEG of low-frequency, high-amplitude activity. Birds that sleep predominantly at night, such as European blackbirds (*Turdus merula*, Szymczak et al. 1996) and non-migrating white-crowned sparrows (*Zonotrichia leucophrys gambelii*, Rattenborg et al. 2004), show SWA that is greatest early in the night, and gradually declines thereafter in a manner suggestive of mammalian-like SWS homeostasis (Rattenborg et al. 2009). Indeed, we recently demonstrated that pigeons (*Columba livia*) show a compensatory increase in SWA following short-term sleep deprivation (Martinez-Gonzalez et

al. 2008), suggesting that mammalian and avian sleep is regulated in a similar manner (see also Lesku et al. 2011).

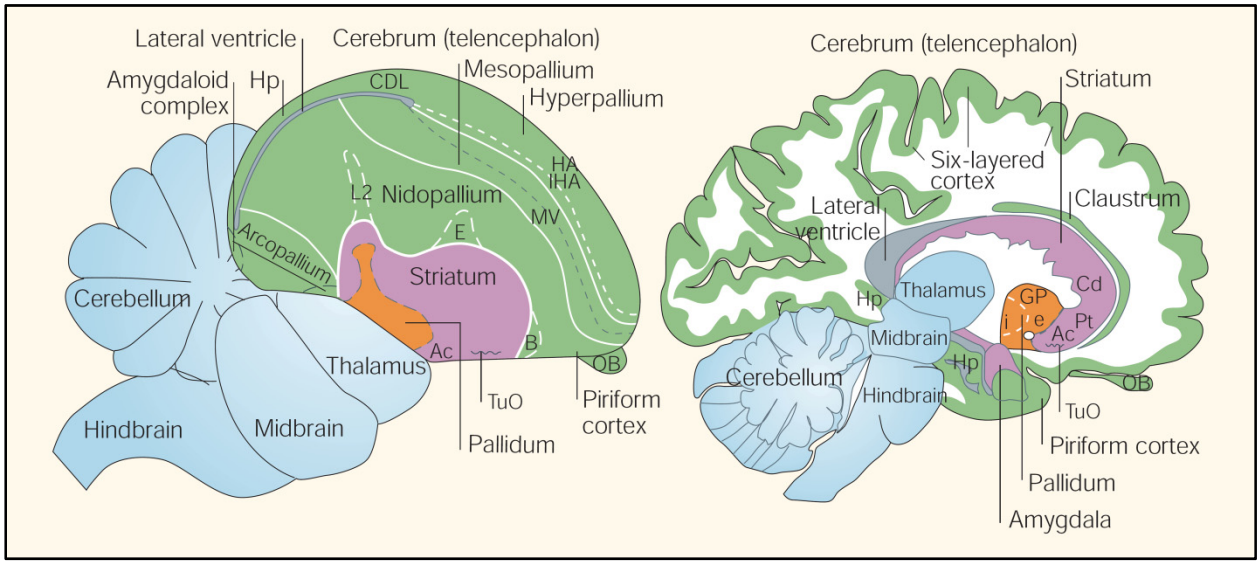

Figure 4. Modern consensus view of avian (left) and mammalian (right) brain relationships. Historically, much of the avian telencephalon was thought to be homologous to the mammalian striatum. However, converging lines of evidence demonstrate that the dorsal two-thirds of the avian telencephalon is actually derived from the pallium (green), the same neural tissue that gives rise to the mammalian neocortex. The new nomenclature reflecting this fundamental change in our understanding of brain evolution is depicted in the sagittal view of a zebra finch (left) and human (right) brain: Ac, accumbens; B, basorostralis; Cd, caudate nucleus; CDL, dorsal lateral corticoid area; E, entopallium; GP, globus pallidus (i, internal segment; e, external segment); HA, hyperpallium apicale; Hp, hippocampus; MV, mesopallium ventrale; IHA, interstitial hyperpallium apicale; L2, field L2; LPO, lobus parolfactorius; OB, olfactory bulb; Pt, putamen; TuO, olfactory tubercle. The several, large, white regions are axon pathways (i.e., white matter) in the cerebrum. Lamina (cell-sparse zones separating brain subdivisions) are marked as solid white lines, primary sensory neuron populations are distinguished from neighboring regions by dashed white lines, and regions differing in cell density or size are demarcated by dashed grey lines. Reprinted from Jarvis et al. (2005) Avian brains and a new understanding of vertebrate brain evolution. *Nature Rev Neurosci* 6:151-159.

Avian REM sleep also shares several features in common with mammals. REM sleep is associated with closure of both eyes, rapid eye movements, occasional bill movements and behavioral signs of reduced muscle tone, such as dropping of the head (Klein et al. 1964, Ookawa and Gotoh 1964, Berger and Walker 1972, Šušić and Kovačević 1973). However, muscle atonia has been observed only in birds that can securely rest their head on the back (Dewasmes et al. 1985). As in mammals, thermoregulatory responses are diminished during REM sleep (Heller et al. 1983). During REM sleep, the EEG reverts to a pattern similar to wakefulness, but often with lower amplitude (Klein et al. 1964, Ookawa and Gotoh 1964). Unlike mammalian REM sleep, however, a hippocampal theta rhythm has not been found in birds (reviewed in Rattenborg et al. 2010). Episodes of REM sleep typically last 2 – 10 seconds and occur in clusters (e.g., Buchet et al. 1986, Martinez-Gonzalez et al. 2008). The short duration of REM sleep episodes does not appear to be related to a need to maintain balance, because REM sleep episodes are equally short when birds are sitting with their heads supported on their backs. In many birds, REM sleep increases across the night, in a manner similar to humans (Szymczak et al. 1993, Rattenborg et al. 2004, Low et al. 2008, Martinez-Gonzalez et al. 2008). In pigeons, this reflects an increase in the incidence and the duration of REM sleep episodes across the night (Martinez-Gonzalez et al. 2008). The proportion of total sleep time devoted to REM sleep appears to be lower in bird species (mean = 8%, Roth et al. 2006) than in mammalian species (mean = 17%, Lesku et al. 2008a). As in mammals, the time spent in REM sleep increases following sleep deprivation (Tobler and Borbély 1988, Martinez-Gonzalez et al. 2008, Lesku et al. 2011). Finally, because REM sleep and SWS have been recorded in every avian species investigated, representing diverse Orders, both states were likely present in the most recent common Dinosaur ancestor to birds (Figure 1).

Like aquatic mammals, birds often sleep with one eye open, a behavioral state associated with SWS in the hemisphere opposite the closed eye and EEG activity intermediate between wakefulness and SWS in the hemisphere opposite the open eye (reviewed in Rattenborg et al. 2000). Birds have the ability to switch from sleeping with both eyes closed to sleeping with one eye open in response to a perceived increase in the risk of predation

(Rattenborg et al. 1999). Here, birds direct the open eye toward the potential threat and are able to respond to threatening stimuli presented to the open eye (Rattenborg et al. 1999, 2001). In contrast to mammals, where unihemispheric SWS occurs only in aquatic mammals, such interhemispheric asymmetries in SWA are common in birds and may be an ancestral trait (Rattenborg et al. 2000). As in Cetaceans that swim in a coordinated manner during unihemispheric SWS, sleeping with one eye open and half the brain awake may allow birds to sleep during flight (Rattenborg 2006a). Although there is strong evidence showing that birds, such as common swifts (*Apus apus*) and frigatebirds (*Fregata* sp.), spend periods lasting days to weeks or longer in constant flight, sleep in flight has not been confirmed with electrophysiological recordings (Rattenborg 2006a).

Early Ontogeny of Avian Sleep

The ontogenetic development of avian sleep has been studied in relatively few species. In precocial chickens (*Gallus gallus*), the EEG correlates of SWS and REM sleep can be distinguished one day before hatching (Garcia-Austt 1954), whereas in the altricial pigeon, EEG activity is absent at hatching and does not resemble that of adults until 14 days later (Tuge et al. 1960). Although it is unclear whether chicken chicks show more or less REM sleep than adults shortly after hatching, the proportion of sleep allocated to REM sleep seems to reach adult levels within the first week post-hatch (Schlehuber et al. 1974). Among altricial birds, the ontogeny of SWS and REM sleep has only been examined in magpies (*Pica pica*), where juveniles sleep longer and have more REM sleep than adults (Szymczak 1987). Additional studies on early sleep ontogenesis in species of greater taxonomic diversity are needed to determine whether the ontogenetic changes in sleep in altricial and precocial birds parallel those observed in mammals. Sleep appears to play a role in filial imprinting in chicks and song learning in juvenile zebra finches (*Taeniopygia guttata*, reviewed in Rattenborg et al. 2010).

Sleep in Reptiles

The presence of SWS and REM sleep in all mammalian and avian species investigated suggests that either these states were present in the most recent common ancestor to extant mammals,

birds and reptiles, or that they evolved independently in mammals and birds. Several studies have attempted to distinguish between these alternatives by examining the electrophysiological correlates of sleep behavior in reptiles, and to a lesser extent, amphibians and fishes (reviewed in Harse et al. 1994, Rattenborg 2006b, 2007). Unlike mammals and birds, however, where largely similar results have been obtained across species and laboratories (e.g., Lesku et al. 2008a), the results from reptiles have been less consistent, and therefore subject to more diverse interpretations. Although some controversy persists, the EEG during reptilian sleep behavior typically shows intermittent high-voltage spikes arising from a background pattern similar to or slightly reduced in amplitude when compared to quiet wakefulness (Rattenborg 2006b, 2007). Because the incidence of spikes is correlated with arousal thresholds and increases following sleep deprivation, spikes appear to reflect sleep intensity (Hartse et al. 1994, Rattenborg 2006b). Recent studies have shown that these spikes originate in the reptilian hippocampus, thereby corroborating earlier pharmacological evidence indicating that reptilian spikes are comparable to similar spikes occurring in the mammalian hippocampus during SWS (Rattenborg 2006b, 2007). Despite this similarity at the level of the hippocampus, however, the reptilian dorsal cortex does not generate concurrent high-amplitude slow waves typical of SWS in mammals and birds (Rattenborg et al. 2009).

REM sleep has been reported in reptiles and fishes based on the presence of eye, head, and limb movements during sleep (Hartse et al. 1994). However, it remains unclear whether these behaviors truly reflect REM sleep-related twitching similar to that observed in mammals and birds, or partial arousals from sleep. Although the presence of brainstem neural activity suggestive of REM sleep in sleeping echidnas (Siegel et al. 1996, 1998, 1999) raised the possibility that reptiles also exhibit REM sleep at the level of the brainstem, no sign of REM sleep was detected in the brainstem of sleeping turtles, despite the presence of neural structures involved in generating REM sleep in mammals (Eiland et al. 2001). The presence of unequivocal SWS and REM sleep in mammals and birds, but not in reptiles, amphibians, or fishes, suggests that these sleep states arose independently in the mammalian and avian lineages through convergent evolution (Figure 1).

Corticocortical Connectivity and Slow Wave Sleep

Historically, the absence of slow waves in the EEG of sleeping reptiles has been attributed to the lack of a thick cortex similar to that which generates slow waves in mammals (Hartse et al. 1994). However, the presence of slow waves in sleeping birds, despite the absence of a neocortex (Figure 4), demonstrates that the neocortex is not essential for the genesis of EEG slow waves. Instead, the extent of connections within the mammalian neocortex, avian hyperpallium, and reptilian dorsal cortex may explain why slow waves are present during sleep in mammals and birds, but not reptiles (Figure 5). In mammals, corticocortical connections in layers II and III play an integral role in synchronizing the slow oscillation of neurons in a manner sufficient to generate slow waves in the EEG (Rattenborg 2006b). In accord with the absence of slow waves in sleeping reptiles, the three-layered reptilian dorsal cortex lacks layers II and III, and shows limited corticocortical connectivity (Figure 5). Furthermore, although birds lack a true neocortex, the hyperpallium shows extensive interconnectivity (Figure 5). Thus, the occurrence of sleep-related SWA in amniotes seems to be related to the extent of interconnectivity in the neocortex, hyperpallium, and dorsal cortex (Rattenborg 2006b, 2007), although additional factors may play a role.

A persistent question in sleep research is whether the EEG correlates of sleep are involved in the functions of sleep, or simply reflect an epiphenomenon of the state. For example, the presence of slow waves in the EEG of sleeping mammals and birds may simply be an emergent property of a heavily interconnected neocortex and hyperpallium (Rattenborg et al. 2009). Alternatively, as suggested by recent experimental work in mammals, the corticocortical connections that give rise to slow waves may also depend on slow waves to maintain the level of connectivity at an energetically and functionally adaptive level (Tononi and Cirelli 2006). Experimental evidence indicates that slow waves may also be involved in sleep-dependent memory processing and plasticity (Huber et al. 2004, Landsness et al. 2009). Additional studies are needed, however, to determine whether slow waves evolved independently in mammals and birds (and not in reptiles) to maintain their heavily interconnected brains and associated cognitive abilities (Rattenborg et al. 2009, Lesku et al.

2011), or whether similar processes occur during reptilian sleep in a manner undetectable in the EEG. As indicated by the presence of hippocampal spikes during sleep, at least some of the neural correlates of SWS seem to be present in reptiles (Figure 1). A comparison of sleep-related gene expression among mammals, birds, and reptiles may clarify the evolutionary history of SWS and REM sleep.

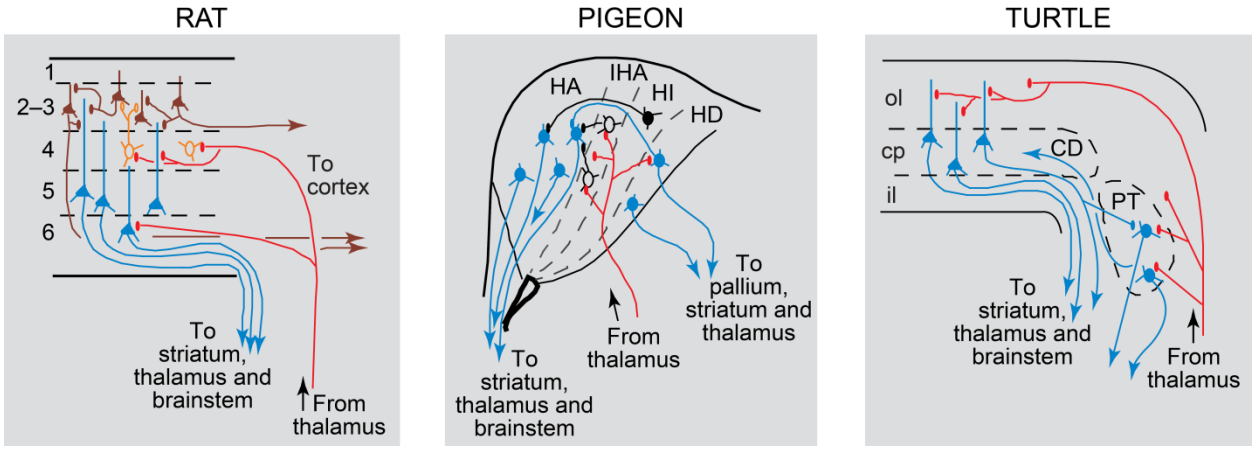

Figure 5. Comparison of the dorsal pallium in representative mammalian, avian, and reptilian species. Note the comparatively high degree of interconnectivity in the rat neocortex and the pigeon Wulst (i.e., hyperpallium), when compared to the turtle dorsal cortex. Abbreviations: hyperpallium apicale (HA), nucleus interstitialis hyperpallii apicalis (IHA), hyperpallium intercalatum (HI), hyperpallium densocellulare (HD), outer layer (ol), cell plate (cp), inner layer (il), dorsal cortex (CD) and pallial thickening (PT). Reprinted from Medina and Reiner (2000) Do birds possess homologues of mammalian primary visual, somatosensory and motor cortices? *Trends Neurosci* 23:1-12.

Comparative Perspectives on the Functions of Sleep

More is known about sleep in mammals than in any other group of animals. Electrophysiological sleep data exist for just less than 100 mammalian species (Siegel 2005, Lesku et al. 2008a). An examination of this comparative dataset reveals that the time spent in SWS and REM sleep varies greatly across Mammalia. Many studies have tried to identify the evolutionary determinants responsible for maintaining such interspecific variation in the

structure of sleep (reviewed in Lesku et al. 2009). The identification of significant predictors of sleep times may help shed light on the functions of SWS and REM sleep. For example, species with a higher mass-specific basal metabolic rate (i.e., basal metabolic rate per gram of body mass) were once thought to engage in more SWS (Zepelin and Rechtschaffen 1974), a relationship that could support an energy conservation role for SWS, because metabolic rates are lower during SWS (Berger and Phillips 1995). Additionally, it was once thought that species with greater encephalization allocated a lower proportion of time spent sleeping to REM sleep, seemingly refuting a neurophysiological role for REM sleep, such as memory processing and plasticity (Siegel 2001). However, excluding our own recent analyses (Lesku et al. 2006, 2008a), all comparative studies of sleep treated each species (or in one case Family) as a statistically independent unit (see Lesku et al. 2009). Independence is a basic assumption of all statistical analyses, but species cannot be considered independent as they are related to one another through common ancestry. Phylogenetic comparative methods (e.g., independent contrasts) were developed by evolutionary biologists to deal with such interspecific relatedness in comparative studies; however until recently, these procedures had not been used in comparative sleep research.

In our recent analyses, we controlled for shared evolutionary history among species using independent contrasts, and analyzed our phylogenetically-controlled data using a multivariate analysis that incorporated hypotheses for the functions of SWS and REM sleep (Lesku et al. 2006, 2009). Many of our results were different from those in previous studies. Unlike all previous studies, we found that species with greater encephalization (i.e., brain mass controlling for body mass using regression) allocate a greater proportion of time asleep to REM sleep, thus providing the first comparative support for a neurophysiological role for REM sleep. Although no relationship was found between encephalization and the time spent in SWS, cumulative SWA may be the more accurate measure of SWS. Contrary to some comparative studies and expectations under the energy conservation hypothesis, we found that species with a higher residual basal metabolic rate (BMR) engage in less SWS and sleep less altogether. These relationships might reflect increased foraging demands associated with higher residual

BMR and thus less time available for sleep (see also Elgar et al. 1988). Nevertheless, mammals with higher residual BMRs may obtain functionally comparable amounts of SWS by engaging in more intense SWS, although such intensity-data are largely unavailable. Alternatively, the restorative processes occurring during sleep could be achieved more quickly with higher residual BMR and thus take less time to accomplish.

Predation risk should be among the strongest selection pressures influencing how to structure sleep and how long to sleep, as sleeping is dangerous (reviewed in Lima et al. 2005). Interestingly, the vulnerability associated with sleep may depend upon the sleep state. For example, due to high arousal thresholds, deep SWS and REM sleep may be particularly dangerous sleep states from an anti-predator point-of-view (Lesku et al. 2008b). Indeed, in the context of our multivariate models, we found that species sleeping at relatively exposed or risky locations in the wild engage in less REM sleep and allocate a lower proportion of sleep time to REM sleep in the laboratory (Lesku et al. 2006, 2009). Although the time spent in SWS was largely independent of predation risk, once again, SWS intensity may respond more strongly to the risk of predation. As per earlier studies, we found that species more precocial at birth engage in less REM sleep as adults, a relationship that was not mediated strongly by predation risk as precocial species did not generally sleep in more vulnerable locations than relatively altricial species. The degree of precociality as a predictor of REM sleep time in adults may reflect an extension of the ontogenetic changes in REM sleep shown in Figure 3, which supports the idea that REM sleep is particularly important in early brain development (Shaffery et al. 2002); however, why high levels of REM sleep during early ontogeny should persist in adults remains enigmatic (Siegel 2005).

Correlates of Avian SWS and REM Sleep

As discussed above, the electrophysiological correlates of avian sleep are remarkably similar to those observed in mammals. In addition to these similarities, there is also great variation in the time avian species spend in SWS and REM sleep (Roth et al. 2006). Thus, an obvious question is: Do birds share the same evolutionary determinants of SWS and REM sleep as mammals?

Recently, we addressed this question by conducting the first electrophysiologically-based comparative analysis of avian sleep architecture using the same phylogenetically-controlled variables as in our mammalian analysis (Roth et al. 2006, Lesku et al. 2009). Overall, we found that birds that sleep at relatively exposed sites in the wild engage in less SWS in the laboratory, but this was the only significant relationship identified. Thus, if relationships identified in mammals reflect functional aspects of sleep architecture, then the same functions may not apply broadly to birds (but see Lesku et al. 2009, 2011).

Sleep in Invertebrates

More than 97% of all animal life is invertebrates, of which 80% are arthropods, the taxonomic group which includes insects. Surprisingly, however, of the 30 or more animal Phyla, sleep has been studied extensively in only two: Chordata (includes vertebrates) and Arthropoda (includes insects), with much work having been focused on the fruit fly (*Drosophila melanogaster*, Hendricks et al. 2000, Shaw et al. 2000, Nitz et al. 2002, Ganguly-Fitzgerald et al. 2006, Donlea et al. 2009, Gilestro et al. 2009) and the honey bee (*Apis mellifera*, Kaiser and Steiner-Kaiser 1983, Kaiser 1988, Sauer et al. 2003, 2004, Klein et al. 2008). Sleep in honey bees is characterized as a sustained period of quiescence accompanied by increased arousal thresholds, and specific postures (e.g., antennal immobility). Optomotor interneurons in the optic lobes of forager honey bees show lowered sensitivity during the subjective night, when forager bees are often quiescent, than during the subjective day (Kaiser and Steiner-Kaiser 1983). Recently, sleep in honey bees was shown to be homeostatically-regulated as antennal immobility increased following 12 hours of sleep deprivation (Sauer et al. 2004). Sleep has also been demonstrated in scorpions (*Heterometrus* and *Pandinus* spp., Tobler and Stalder 1988), cockroaches (*Blaberus giganteus* and *Leucophaea maderae*, Tobler 1983, Tobler and Neuner-Jehle 1992), and crayfish (*Procambarus clarkii*, Ramón et al. 2004, Mendoza-Angeles et al. 2007, 2010), and preliminary work suggests that sleep is also present in three additional animal Phyla: Nematoda (*Caenorhabditis elegans*, Olofsson and de Bono 2008, Raizen et al. 2008), cephalopods (*Octopus vulgaris* and *Sepia pharaonis*, Duntley and Morrissey 2004, Brown et al.

2006) in the Phylum Mollusca, and box jellyfish (*Chironex fleckeri*, Seymour et al. 2004) in the Phylum Cnidaria.

Conclusions

Our understanding of how and why we sleep has been enhanced by studies examining the evolution and ontogeny of sleep. The presence of SWS and REM sleep in all avian and mammalian species studied, and their apparent absence in reptiles, suggests that these states arose independently twice: once in the ancestor to birds, and once in the ancestor to mammals (Figure 1, Rattenborg et al. 2009). A comparison of neurocytoarchitecture among homeotherms (Medina and Reiner 2000) suggests that the degree of corticocortical (or palliopallial) connectivity is responsible for EEG SWA in mammals and birds (Rattenborg 2006b, 2007). Interestingly, REM sleep-related cortical activation evolved independently in the ancestor to eutherians and marsupials and the ancestor to birds (Figure 1). The similarity in the electrophysiological correlates of behavioral sleep in homeotherms suggests similarities in functions, possibly related to having heavily interconnected brains and associated cognitive abilities (Rattenborg et al. 2009, Lesku et al. 2011). Our understanding of the evolution, ontogeny, and functions of sleep would benefit greatly from studies in species representing broader phylogenetic diversity. Here, the study of species nearest the base of the Metazoan phylogenetic tree would be most revealing. A systematic approach to selecting taxa for study should be employed that takes into consideration neuroanatomy, neurophysiology, and phylogenetic position in the context of current hypotheses for the functions of sleep. Future work should also expand existing genetic work on fruit flies and rats to nonmammalian vertebrate taxa and to nonarthropod invertebrate taxa. The success of these endeavors will depend upon the broad collaboration of animal behaviorists, evolutionary biologists, geneticists, and neurophysiologists, but will do much to aid our greater understanding of sleep.

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CHAPTER 2

Ostriches Sleep Like Platypuses

Lesku JA, Meyer LCR, Fuller A, Maloney SK, Dell'Omo G, Vyssotski AL and Rattenborg NC. *In review*.

Abstract

Mammals and birds engage in two distinct states of sleep, slow wave sleep (SWS) and rapid eye movement (REM) sleep. SWS is characterized by slow, high amplitude brain waves, while REM sleep is characterized by fast, low amplitude waves, known as activation, occurring with rapid eye movements and reduced muscle tone. However, monotremes (platypuses and echidnas), the most basal (or 'ancient') group of living mammals, show only a single sleep state that combines elements of SWS and REM sleep, suggesting that these states became temporally segregated in the common ancestor to marsupial and eutherian mammals. Whether sleep in basal birds resembles that of monotremes or other mammals and birds is unknown. Here, we provide the first description of brain activity during sleep in ostriches (*Struthio camelus*), a member of the most basal group of living birds. We found that the brain activity of sleeping ostriches is unique. Episodes of REM sleep were delineated by rapid eye movements, reduced muscle tone, and head movements, similar to those observed in other birds and mammals. However, during REM sleep, forebrain activity would flip between REM sleep-like activation and SWS-like slow waves, the latter reminiscent of sleep in the platypus. Moreover, the amount of REM sleep in ostriches is greater than in any other bird, just as in platypuses, which have more REM sleep than other mammals. These findings reveal a recurring sequence of steps in the evolution of sleep in which SWS and REM sleep arose from a single heterogeneous state that became temporally segregated into two distinct states. This common trajectory suggests that forebrain activation during REM sleep is an evolutionarily new feature, presumably involved in performing new sleep functions not found in more basal animals.

Introduction

Mammals engage in two types of sleep, slow wave sleep (SWS) and rapid eye movement (REM) sleep. SWS is characterized by slow, high amplitude brain waves (Vyazovskiy et al. 2009), while REM sleep is characterized by fast, low amplitude waves (reflecting brain activation), rapid eye movements, and reduced muscle tone (Siegel 2011). Unlike SWS, which is initiated and maintained by the forebrain, REM sleep-related cortical activation, rapid eye movements, and reduced muscle tone are generated by the brainstem (Jouvet 1962, Siegel 2011). Interestingly,

the cortex of monotremes (platypuses and echidnas), the most basal (or 'ancient') group of living mammals, shows only SWS-like slow waves during sleep (Allison et al. 1972, Siegel et al. 1998, Manger et al. 2002). Furthermore, during sleep in the platypus (*Ornithorhynchus anatinus*), cortical slow waves occur with REM sleep-like rapid eye movements and reduced muscle tone (Siegel et al. 1999, Siegel 2005). This suggests that REM sleep at the level of the brainstem and SWS in the cortex were present in the most recent common ancestor to all mammals, and that REM sleep with cortical activation evolved only after the appearance of the marsupial / eutherian lineage (Siegel et al. 1998, Siegel 2005). Alternatively, the unusual brain activity of sleeping monotremes may reflect an evolutionary loss of REM sleep with cortical activation (Rattenborg et al. in press).

One way to distinguish between these possibilities would be to characterize REM sleep in reptiles, the sister-group to mammals. However, reptiles do not exhibit the neuronal activity observed in the brainstem during REM sleep in mammals (Eiland et al. 2001), including monotremes (Siegel et al. 1996), nor do they show cortical signs of REM sleep and SWS (Hartse 1994, Eiland et al. 2001, Rattenborg 2007). Alternatively, animals that independently evolved SWS and REM sleep may provide insight into the evolution of REM sleep by revealing recurring evolutionary patterns. Because birds are the only animals outside of mammals to engage in SWS and REM sleep, only birds can provide such insight. However, whether basal birds exhibit brain activity during sleep that resembles that of monotremes or other mammals and birds is unknown (Tomo et al. 1973, Ookawa and Yamashita 1982, Amlaner and Ball 1994, Roth et al. 2006, Lesku et al. 2009). Here, we provide the first description of sleep electrophysiology in ostriches (*Struthio camelus*), a member of the most basal group of living birds. We found that the brain activity of ostriches during sleep is unique, and most closely resembles that of the distantly-related monotremes, revealing a recurring sequence of steps in the evolution of REM sleep.

Methods

Six female adult ostriches (82 ± 4 kg, mean ± s.e.m.) were purchased from a farm in Free State, South Africa, and transported to the Lichtenburg Game Breeding Center, South Africa (26°06' S, 26°10' E) for study. The study was conducted in February and March 2009 (southern hemisphere summer). The birds were implanted with electrodes for measuring brain waves (electroencephalogram, EEG), eye movements (electrooculogram, EOG), neck muscle tone (electromyogram, EMG) and a thermistor for brain temperature using standard aseptic techniques by experienced surgeons (see Supplementary Data for details). EEG, EOG and EMG electrodes terminated at a plug housed in a head-mounted aluminum box (length x width x height: 44 x 24 x 32 mm). The plug connected to an upgraded version of a logger (Neurologger) previously used for recording the EEG of birds (Vyssotski et al. 2006, Supplementary Data). A 3-dimensional accelerometer on the Neurologger recorded acceleration as a positive or negative deflection depending on the direction of the movement along each of the three axes; the magnitude of the deflection was proportional to the acceleration. Temperature was recorded via a thermistor in the brain connected to a logger positioned subcutaneously in the neck (Fuller et al. 2003, Supplementary Data). All methods were approved by the National Zoological Gardens of South Africa (P08/22) and the Animal Ethics Screening Committee at the University of the Witwatersrand (2008/45/05), and adhere to the NIH standards regarding the care and use of animals in research.

The recordings were conducted at two locations. First, the ostriches were group-housed in an outdoor enclosure (5 x 5 m) with occasional access to a connecting enclosure of similar size. Grass (*Eragrostis* spp.), alfalfa (*Medicago sativa*), pelleted ostrich food and clean water were available *ad libitum*. The main enclosure was monitored using 8 video cameras equally spaced along the perimeter, and an infrared illuminator in each corner provided light (850 nm) for nighttime recordings. These video recordings were used to establish relationships between specific behaviors and the electrophysiological and accelerometer signals. After 7 – 10 d, the ostriches were moved to a large (51 ha) naturalistic reserve less than 1 km away (Figure 1A). The reserve had a floral assemblage characteristic of South African savannah (or Highveld) and

large herbivores that are sympatric with ostriches in the wild (e.g., blesbok, *Damaliscus pygargus phillipsi*; impala, *Aepyceros melampus*; roan antelope, *Hippotragus equines*). Ostriches occupied the full area of the reserve, as determined by a GPS logger attached to the leg of each bird for their first 10 days in the camp (Figure 1B). These naturalistic recordings continue the recent push for EEG-based sleep research to move into more wild environments (Rattenborg et al. 2008), as some aspects of normal physiology may not be reflected in the laboratory (Fuller et al. 2004, Goldstein and Pinshow 2006, Rattenborg et al. 2008, Calisi and Bentley 2009, Lesku et al. 2009, Daan 2011).

EEG, EOG, EMG and head movements were recorded from all ostriches for between 0.7 to 18.6 d total (9.2 ± 2.8 d, mean ± s.e.m.). Signals were downsampled from 800 Hz to 200 Hz for visualization and analysis in Somnologica Science v. 3.3.1 (Embla®, www.embla.com). One undisturbed 24 h day in the reserve (~ 13L:11D) was visually scored for wakefulness, SWS and REM sleep using 4 s epochs. Epochs that contained more than one state were scored according to the state occupying the majority of that epoch. This undisturbed day was characterized by unexceptional temperatures (black globe temperature, day: 29.7 ± 0.1 °C, night: 14.6 ± 0.7 °C), little-to-no wind (wind speed, day: 0.80 ± 0.29 m/s, night: 0.05 ± 0.03 m/s), and no rain, as measured by a weather station located adjacent to the reserve. Brain temperature was recorded successfully from 5 of the 6 ostriches throughout the entire study. To investigate the relationship between brain state and temperature, we compared brain temperature at night during wakefulness to that during sleep. Because the logger recorded brain temperature instantaneously at the top of every second minute, only bouts of wakefulness and sleep that occupied the entire 2 min period immediately before temperature was recorded were included in this analysis. Brain temperature during REM sleep could not be calculated reliably as episodes of REM sleep rarely met this criterion. Data were analyzed with one-way repeated measures analysis of variance or paired t-tests using SYSTAT 10 (©SPSS, Inc., www.systat.com).

Results

An awake ostrich had both eyes open and was generally walking, feeding or preening. Not surprisingly, during such periods, neck muscle tone was highest and eye movements were common. Sleep followed with the cessation of these waking activities. During SWS, ostriches typically sat motionlessly with their necks held periscopically above the ground; both eyes were always open though without movement (Figure 1C-E). Consequently, an ostrich in SWS did not look like a typical sleeping animal and instead gave the impression of an alert bird. This wake-like sleep posture may explain why sleep is rarely reported in studies on time budgets and activity patterns in wild ostriches (Deeming and Bubier 1999, Cooper et al. 2010). SWS with open eyes has been reported in other avian (Berger and Walker 1972, Šušić and Kovačević 1973, Tobler and Borbély 1988, Rattenborg et al. 2001) and mammalian (Ruckebusch 1972, Pigarev et al. 2011) species, and may allow for visual processing concurrent with sleep (Rattenborg et al. 2001, Lima et al. 2005). During SWS, the EEG showed slow waves (Figure 1E) like those recorded from other birds engaged in SWS (van Twyver and Allison 1972, Buchet et al. 1986, Rattenborg et al. 2004).

The transition from SWS to REM sleep was marked by bilateral eye closure, rapid eye movements, and a forward falling head (Figure 1C). As in owls (Berger and Walker 1972, Šušić and Kovačević 1973) and some ruminating mammals (Ruckebusch 1972), bilateral eye closure was observed only in conjunction with REM sleep. In ostriches, the drooping and swaying head movements that accompanied REM sleep were readily distinguishable from movements occurring during wakefulness (Figure 1D, Figure S1). In extreme cases, the head fell to the ground (see also Immelmann 1959, Sauer and Sauer 1966). This behavioral correlate of REM sleep has been observed in wild ostriches, where it was attributed to drowsiness:

> "Closing its eyes, a tired Ostrich would slowly tilt its head downward and, after a while, jerk it up just to droop it again."
> (Sauer and Sauer 1967)

Interpreting this behavior as belonging to a drowsy animal is understandable given the alert-like sleep posture of an ostrich engaged in SWS. These REM sleep-related head movements have also been described in a close relative of the ostrich, the greater rhea (*Rhea americana*, Amlaner et al. 2001). Concomitant with this REM sleep behavior in ostriches, muscle tone was generally lower than during SWS (Figure 1D,F, Figures S1,S2). The end of an episode of REM sleep was almost always marked by a rapid rise of the head (as described in other birds, van Twyver and Allison 1972), cessation of rapid eye movements, and restoration of wake-like or SWS-like muscle tone (depending on the state entered next) (Figure 1F, Figure S2). Thus, the EOG and accelerometer signals served as well-defined 'bookends' to an episode of REM sleep. Within these 'bookends', the EEG showed SWS-like slow waves that alternated with REM sleep-like activation (Figure 1F, Figure S2). This mixed REM sleep state was identified in all ostriches. REM sleep with activation and REM sleep with slow waves could both occur with rapid eye movements, reduced muscle tone, and head movements; indeed, REM sleep with slow waves could occur with the lowest muscle tone (Figure 1F, Figure S2).

Based on the electrophysiological and accelerometer signals recorded from the animals in the reserve, ostriches spend 88.6 ± 1.7% (mean ± s.e.m.) of the day and 13.8 ± 1.8% of the night awake (Figure 1G). This daytime value is similar to the amount of unequivocal wakefulness (i.e., activity) reported for ostriches in the wild (Bertram 1980, Williams et al. 1993). Such diurnality was reflected in brain temperature, which was significantly higher during the day (39.4 ± 0.1 °C) than during the night (38.3 ± 0.1 °C, $P < 0.001$, Figure 1G). Ostriches spend 9.5 ± 1.5% of the day and 62.2 ± 2.1% of the night in SWS (Figure 1G). The amount of SWS decreased across the night ($F = 2.791$, $df = 10,30$, $P = 0.014$, Figure 1G), a pattern that has been observed in other birds (Tobler and Borbély 1988, Szymczak et al. 1993, Rattenborg et al. 2004). The brain was significantly cooler when in SWS after sunset (38.2 ± 0.1 °C) than when awake after sunset (39.2 ± 0.3 °C, $P = 0.047$); however, circadian effects on brain temperature cannot be discounted, as, long (≥ 2 min) bouts of SWS and wakefulness were rare before and after astronomical twilight, respectively. REM sleep occupied 1.9 ± 0.9% of the day and 24.0 ± 0.9% of the night (or 26.3 ± 1.3% of 24 h total sleep time, Figure 1G), the most reported for any

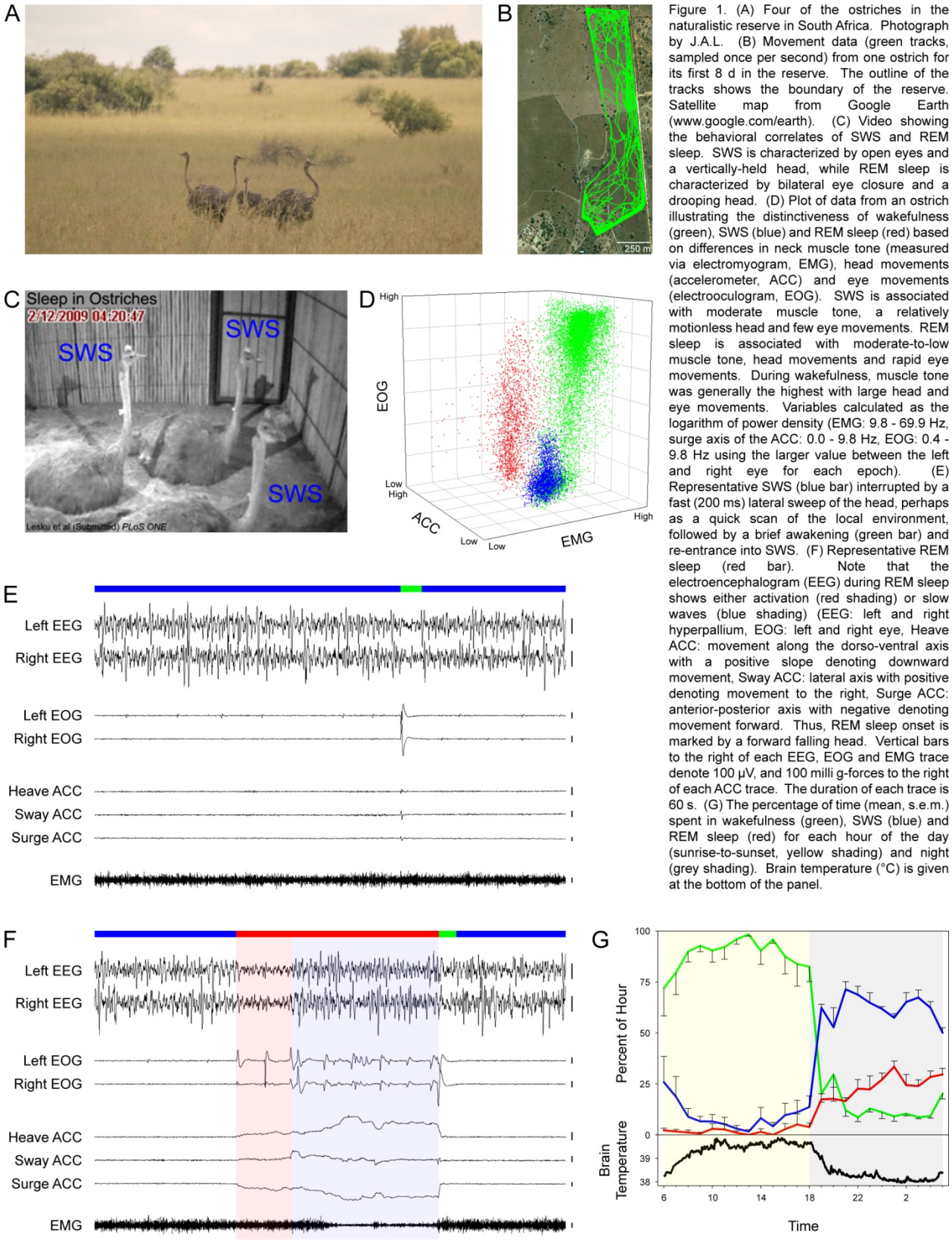

Figure 1. (A) Four of the ostriches in the naturalistic reserve in South Africa. Photograph by J.A.L. (B) Movement data (green tracks, sampled once per second) from one ostrich for its first 8 d in the reserve. The outline of the tracks shows the boundary of the reserve. Satellite map from Google Earth (www.google.com/earth). (C) Video showing the behavioral correlates of SWS and REM sleep. SWS is characterized by open eyes and a vertically-held head, while REM sleep is characterized by bilateral eye closure and a drooping head. (D) Plot of data from an ostrich illustrating the distinctiveness of wakefulness (green), SWS (blue) and REM sleep (red) based on differences in neck muscle tone (measured via electromyogram, EMG), head movements (accelerometer, ACC) and eye movements (electrooculogram, EOG). SWS is associated with moderate muscle tone, a relatively motionless head and few eye movements. REM sleep is associated with moderate-to-low muscle tone, head movements and rapid eye movements. During wakefulness, muscle tone was generally the highest with large head and eye movements. Variables calculated as the logarithm of power density (EMG: 9.8 - 69.9 Hz, surge axis of the ACC: 0.0 - 9.8 Hz, EOG: 0.4 - 9.8 Hz using the larger value between the left and right eye for each epoch). (E) Representative SWS (blue bar) interrupted by a fast (200 ms) lateral sweep of the head, perhaps as a quick scan of the local environment, followed by a brief awakening (green bar) and re-entrance into SWS. (F) Representative REM sleep (red bar). Note that the electroencephalogram (EEG) during REM sleep shows either activation (red shading) or slow waves (blue shading) (EEG: left and right hyperpallium, EOG: left and right eye, Heave ACC: movement along the dorso-ventral axis with a positive slope denoting downward movement, Sway ACC: lateral axis with positive denoting movement to the right, Surge ACC: anterior-posterior axis with negative denoting movement forward. Thus, REM sleep onset is marked by a forward falling head. Vertical bars to the right of each EEG, EOG and EMG trace denote 100 μV, and 100 milli g-forces to the right of each ACC trace. The duration of each trace is 60 s. (G) The percentage of time (mean, s.e.m.) spent in wakefulness (green), SWS (blue) and REM sleep (red) for each hour of the day (sunrise-to-sunset, yellow shading) and night (grey shading). Brain temperature (°C) is given at the bottom of the panel.

bird (Roth et al. 2006, Low et al. 2008, Lesku et al. 2009). Although the amount of REM sleep increases across the night in other birds (Tobler and Borbély 1988, Szymczak et al. 1993, Ayala-Guerrero et al. 2003, Rattenborg et al. 2004), the mean increase in ostriches did not reach statistical significance ($F = 1.757$, $df = 10,30$, $P = 0.113$, Figure 1G) nor did the mean increase in the percentage of sleep time allocated to REM sleep ($F = 1.974$, $df = 10,30$, $P = 0.073$), perhaps due to the small sample size. Episodes of REM sleep, typically less than 10 s in duration in other birds (van Twyver and Allison 1972, Buchet et al. 1986, Ayala-Guerrero et al. 2003, Martinez-Gonzalez et al. 2008), lasted 27 ± 7 s on average in ostriches, and could last up to 5 min (2.3 ± 0.9 min, mean maximum ± s.e.m.), the longest reported for any bird.

Discussion

Ostriches exhibit a heterogeneous REM sleep state characterized by eye closure, rapid eye movements, reduced muscle tone, and a forward falling head, occurring with forebrain activity that flips between REM sleep-like activation and SWS-like slow waves. To our knowledge, such a state has not been reported previously in any animal. Ostriches also have the longest REM sleep episodes, and more REM sleep overall, than any other avian species. The unusual REM sleep state of ostriches is unlikely to be related to their large size *per se*, because the Emperor penguin (*Aptenodytes forsteri*), the next largest species studied (~ 28 kg), shows REM sleep typical of other birds (Buchet et al. 1986). Moreover, REM sleep occupied 13% of sleep time, and the duration of REM sleep episodes was less than 10 s in penguins (Buchet et al. 1986), values typical of small birds (van Twyver and Allison 1972, Ayala-Guerrero et al. 2003, Rattenborg et al. 2004, Roth et al. 2006, Low et al. 2008, Martinez-Gonzalez et al. 2008, Lesku et al. 2009).

How might the ostrich brain initiate this heterogeneous REM sleep state? In mammals, REM sleep-related forebrain activation, rapid eye movements, and reduced muscle tone are generated by the brainstem (Siegel 2011). In mammals (Jouvet 1962, Siegel 2011) and birds (Gusel'nikova 2007), forebrain activation arises via the excitatory action of ascending cholinergic REM sleep-on neurons in the rostral pons of the brainstem. Flipping between

activation and slow waves during REM sleep in ostriches might reflect variation in the strength of signals from ascending REM sleep-on neurons that promote activation (Luppi et al. 2011) and SWS-generating mechanisms of the ventrolateral preoptic nucleus (Komarova et al. 2008, Szymusiak 2010) or those intrinsic to the forebrain (Krueger et al. 2008, Lesku et al. 2011, Nir et al. 2011). If true, then these competing effects appear to occur independently from variation in the strength of descending REM sleep-on neurons that reduce muscle tone (Luppi et al. 2011), because the lowest tone could occur either when the hyperpallium was activated or showed slow waves. An investigation combining EEG and recordings of neuronal activity in the brainstem and ventrolateral preoptic nucleus might reveal the source of the unique REM sleep state in ostriches.

The slow wave component of the REM sleep state described here in ostriches resembles that observed in monotremes. Indeed, monotremes are the only other animals known to engage in slow waves during a state which would otherwise be unequivocally identified as REM sleep (Allison et al. 1972, Siegel et al. 1996, 1998, 1999, Manger et al. 2002, Siegel 2005). Concurrent with slow waves in the cortex, platypuses exhibit REM sleep-like rapid eye movements, reduced muscle tone, and twitches of the head and bill (Siegel et al. 1999). If one calculates the amount of REM sleep as periods with rapid eye movements and reduced muscle tone, then platypuses have more REM sleep than any other mammal (Siegel et al. 1999, Siegel 2005, Lesku et al. 2006, 2008), just as ostriches have more REM sleep than any other bird using similar criteria.

Why might ostriches sleep like platypuses? There appear to be few traits unique to ostriches and monotremes that could explain such an unusual REM sleep state. However, the fact that monotremes and ostriches are both members of the most basal group within their respective lineage (Meyer and Zardoya 2003, Phillips et al. 2010), suggests that this type of REM sleep may reflect an early stage in the evolution of REM sleep. Although other (yet unknown) factors may explain the similarities between ostrich and monotreme REM sleep, it is remarkable that of all the species studied (c. 100 mammals, Siegel 2005, Lesku et al. 2008; c. 30

birds, Tomo et al. 1973, Ookawa and Yamashita 1982, Amlaner and Ball 1994, Roth et al. 2006, Lesku et al. 2009) *only* species of the most basal lineages exhibit such a state. The absence of REM sleep in the brainstem and cortex of turtles (Eiland et al. 2001), suggests that the aspects of REM sleep common to monotremes and ostriches arose independently in the most recent common ancestor to all mammals and again in ancestral birds. In mammals, forebrain activation during REM sleep (or 'classical' REM sleep) evolved in the common ancestor of marsupial and eutherian mammals, as monotremes do not engage in a comparable state. In birds, 'classical' REM sleep was apparently present, at least to some extent, in the ancestor to all living birds, but alternates with the more basal, monotreme-like REM sleep state. It is possible that earlier birds may have slept exclusively like monotremes. This evolutionary scenario suggests a recurring sequence of steps in the evolution of REM sleep shared by mammals and birds in which SWS and REM sleep arose as a single heterogeneous state that became temporally segregated into distinct SWS and REM sleep with forebrain activation. Furthermore, it suggests that, as an evolutionarily new feature of sleep, forebrain activation during 'classical' REM sleep may support shared sleep functions not found in more basal animals. Identifying the functional significance of this evolutionary pattern is an important avenue for future research.

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Supplementary Data

Surgical Details and Logger Specifications

Before surgery, each animal was given a local anesthetic (5 ml 2% lidocaine with adrenaline; Bayer, South Africa) subcutaneously in the surgical field, a non-steroidal anti-inflammatory (I.M. 10 mg / kg meloxicam, MOBIC®; Boehringer Ingelheim, South Africa) and a broad spectrum antibiotic (I.M. 10 mg / kg enrofloxacin, Baytril®; Bayer, South Africa). Throughout the procedure, heart and respiratory rate, oxygen saturation, and colonic temperature were monitored. Animals were anesthetized with isoflurane (induction and maintenance at 8% and 2 – 5%, respectively, vaporized in 100% oxygen) administered initially via facemask then an endotracheal tube. Four holes (0.5 mm diameter) were drilled through the exposed cranium to the level of the dura. Holes were arranged symmetrically over the left and right hyperpallia (comparable to the primary visual cortex of mammals, Medina and Reiner 2000), a particularly prominent brain region in ostriches (Corfield et al. 2008). The holes were located 18 mm and 8 mm anterior to the parieto-occipital suture (lambda) and 6 mm lateral of the midline. The positioning of electrodes on the hyperpallium was facilitated through the examination of dead specimens of similar size. A fifth hole was drilled 13 mm anterior of lambda over the left hemisphere for the ground. Electroencephalogram (EEG) electrodes consisted of gold-plated round-tipped pins (0.5 mm diameter). Stainless steel wire electrodes were glued to the anterior and posterior margin of the supraorbital ridge over both eyes for the electrooculogram (EOG); two wires were sutured to the nuchal (neck) muscle for the electromyogram (EMG). All wires terminated at a plug housed in an aluminum box (length x width x height: 44 x 24 x 32 mm) secured over the center of the cranium with dental acrylic. The plug connected to an upgraded version of a logger (Neurologger) previously used for recording the EEG of birds (Vyssotski et al. 2006, www.vyssotski.ch/neurologger.html). Upgraded features include (i) the ability to record accelerations of the head, (ii) increased maximum recording duration and (iii) lower power consumption. A 3-dimensional accelerometer (MMA7260QT; Freescale Semiconductor Inc., U.S.A.) on the Neurologger recorded acceleration along each axis. To increase maximum recording duration, the previously used 1 GB Secure Digital (SD) memory card was replaced with a lighter, 8 GB microSD card. Voltage on the board was reduced from

3.3 V to 2.7 V, and the frequency of the processor was lowered from 24 MHz to 16 MHz, such that the modified logger consumed only 4.5 mA. Memory card and batteries were renewed every 8 – 10 d. The logger digitized the eight channels (2 EEG, 2 EOG, 1 EMG and 3 accelerometer) at 1600 Hz and stored averaged band-pass filtered (1 – 240 Hz) values at 800 Hz. For hypothalamic brain temperature measurements, one hole (2 mm diameter) was drilled 28 mm anterior of lambda to the level of the dura through which a ruggedized glass-coated bead thermistor (30 mm length, 2 mm outer diameter; Thermometrics, U.S.A.) was inserted, as per our previous study on brain temperature in ostriches (Fuller et al. 2003). Thermistors have been similarly implanted in other studies of avian sleep (Szymczak et al. 1989, Gusel'nikova and Pastukhov 2009). Our thermistor was connected to a logger positioned subcutaneously in the neck. At the end of the study, all equipment was removed from the birds using similar surgical procedures to those outlined above, and the animals were returned to the reserve following post-operative recovery in the outdoor enclosures.

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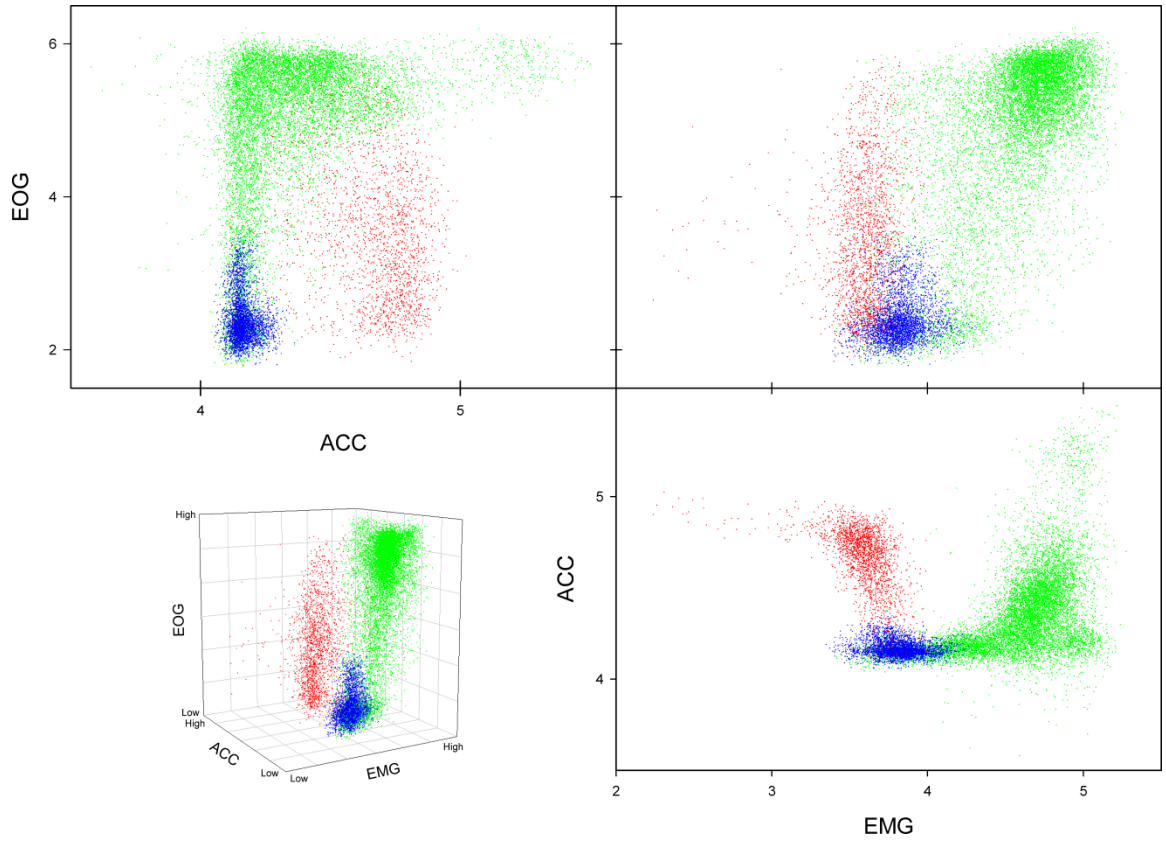
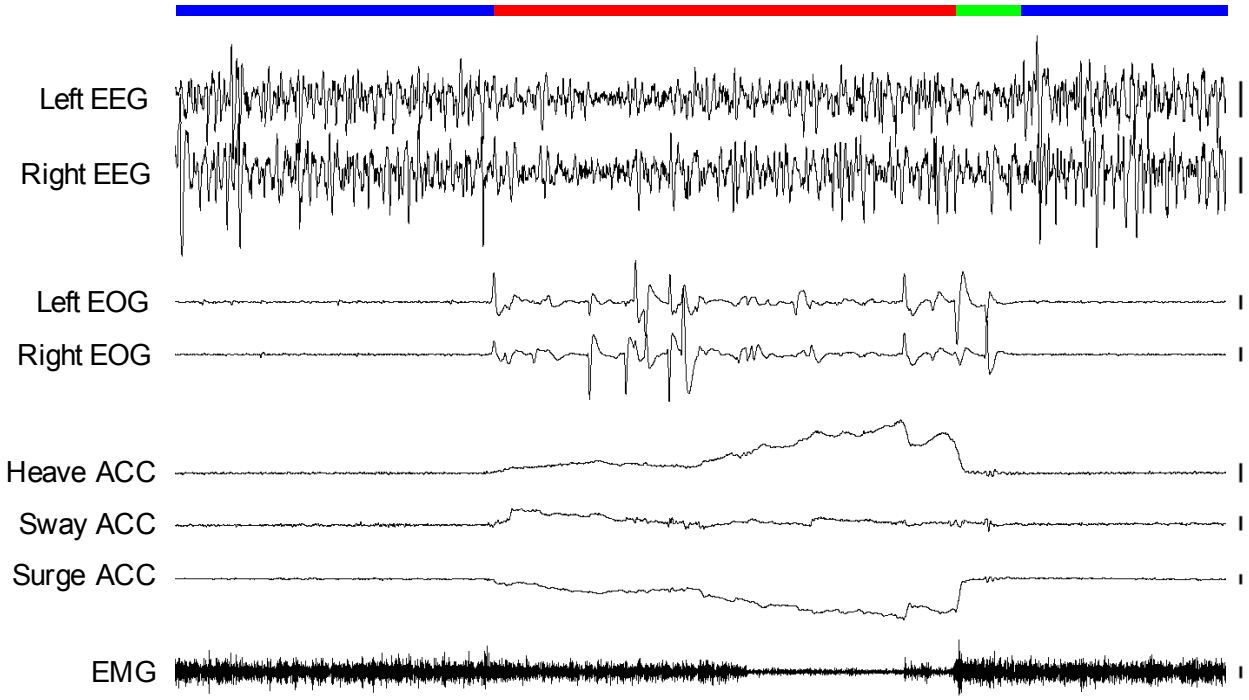


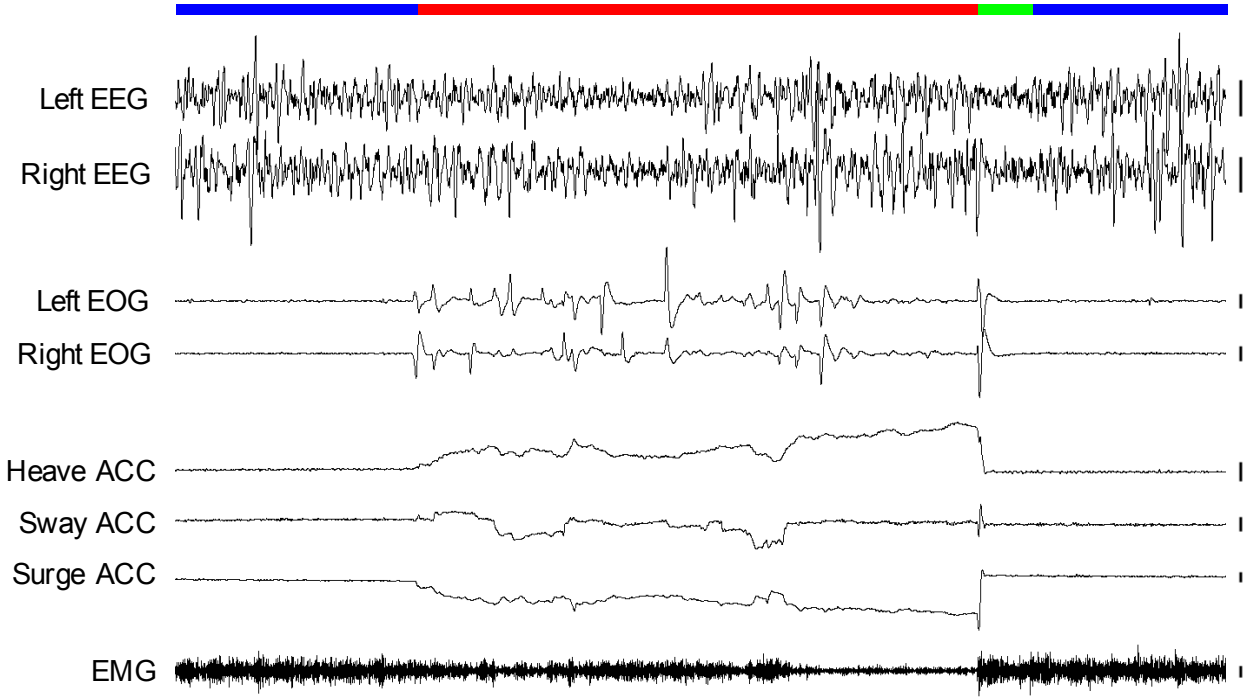
Figure S1. The three 2-dimensional plots that constitute the 3-dimensional Figure 1D in the main article (reprinted here in the bottom left corner). These plots illustrate the distinctiveness of wakefulness (green), SWS (blue) and REM sleep (red) based on differences in eye movements (measured via electrooculogram, EOG), neck muscle tone (electromyogram, EMG), and head movements (surge axis of the accelerometer, ACC). Axes are logarithmic, as detailed in the caption of Figure 1.

Figure S2 *below*. (A-H) Electroencephalogram (EEG) of the left and right hyperpallium, electrooculogram (EOG) of the left and right eye, the three axes (heave, sway and surge) of the head-mounted accelerometer (ACC), and electromyogram (EMG) of the nuchal muscle showing SWS (blue bar), REM sleep (red bar) and wakefulness (green bar) in the ostrich. See main text for a description of each state. These figures illustrate the well-defined nature of an episode of REM sleep, as well as demonstrate the variation in EEG and EMG activity during REM sleep. Vertical bars to the right of each EEG, EOG and EMG trace denote 100 µV, and 100 milli g-forces to the right of each ACC trace. The duration of each trace is 60 s.

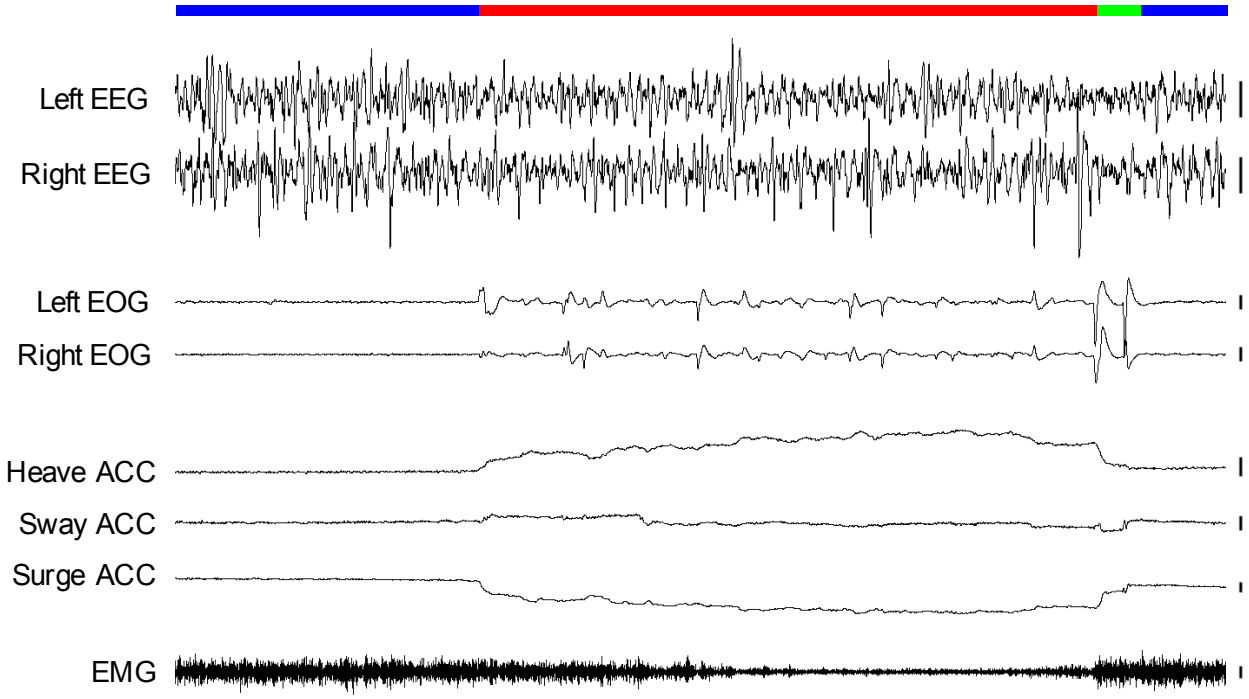
A


Left EEG
Right EEG
Left EOG
Right EOG
Heave ACC
Sway ACC
Surge ACC
EMG

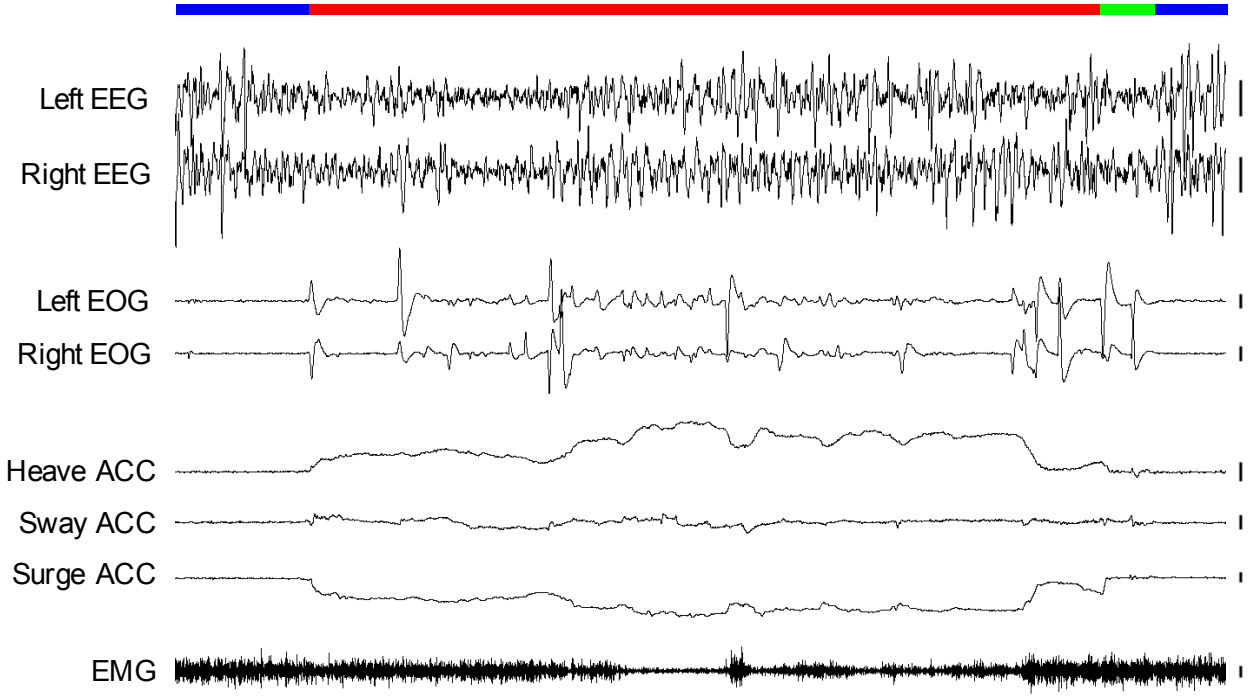
B


Left EEG
Right EEG
Left EOG
Right EOG
Heave ACC
Sway ACC
Surge ACC
EMG

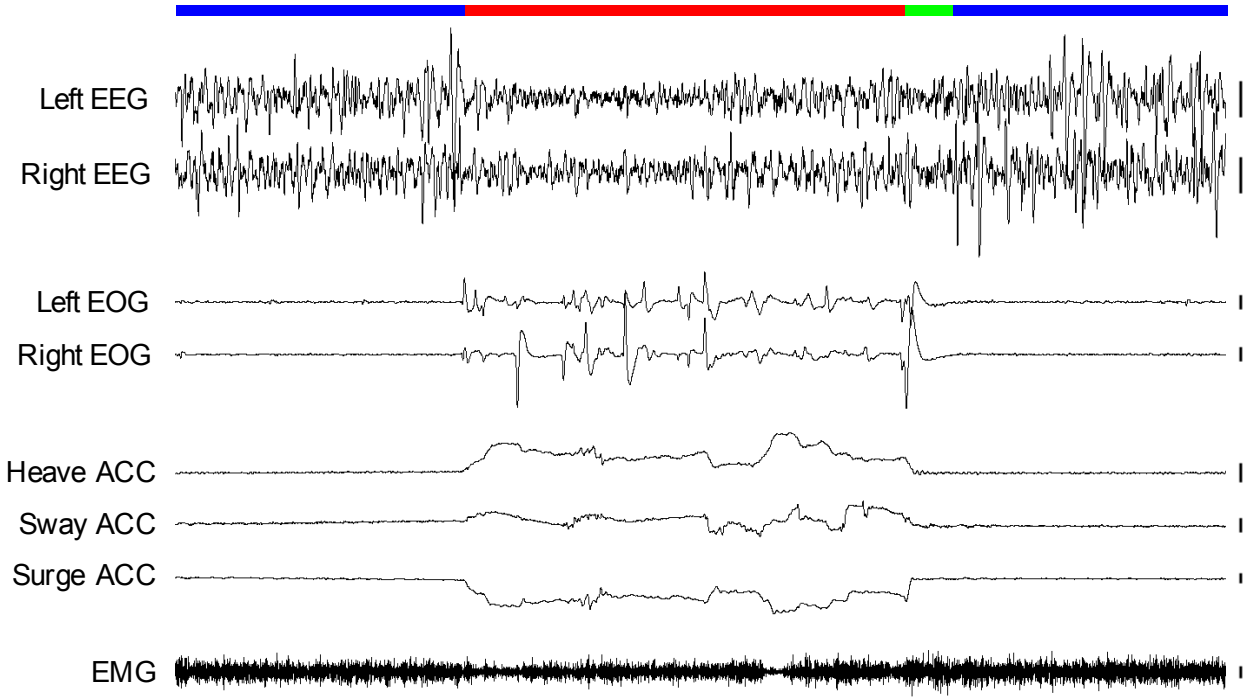
C


Left EEG
Right EEG
Left EOG
Right EOG
Heave ACC
Sway ACC
Surge ACC
EMG

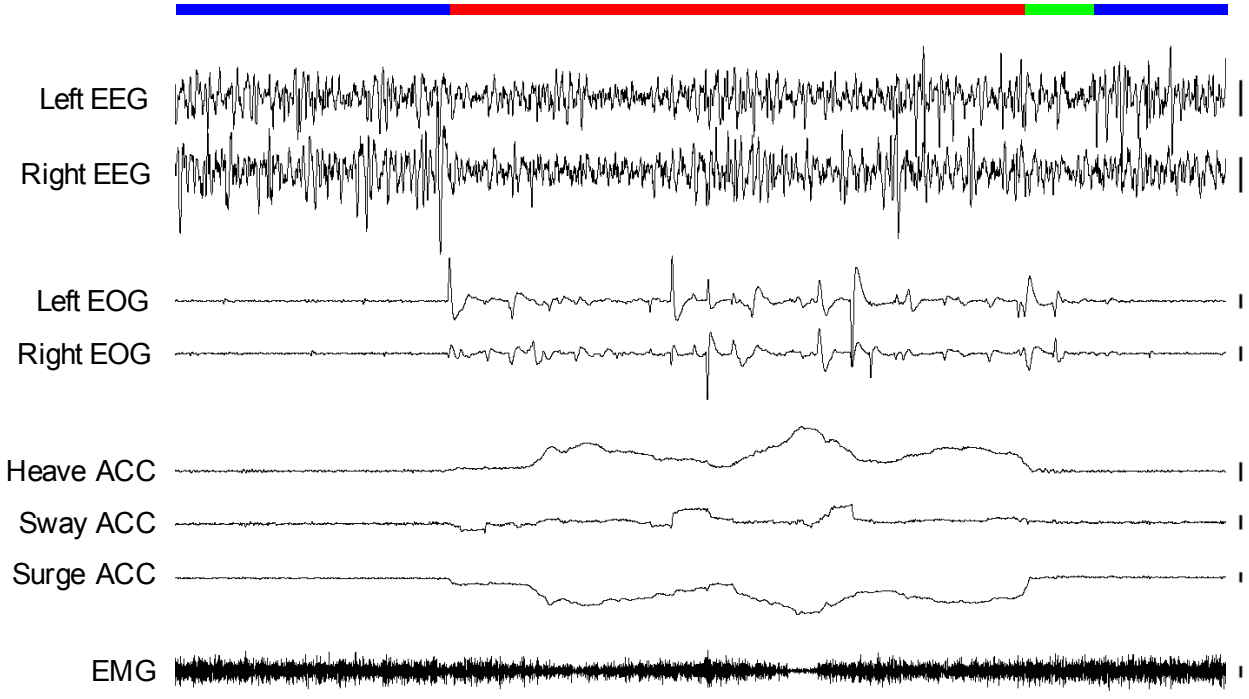
D


Left EEG
Right EEG
Left EOG
Right EOG
Heave ACC
Sway ACC
Surge ACC
EMG

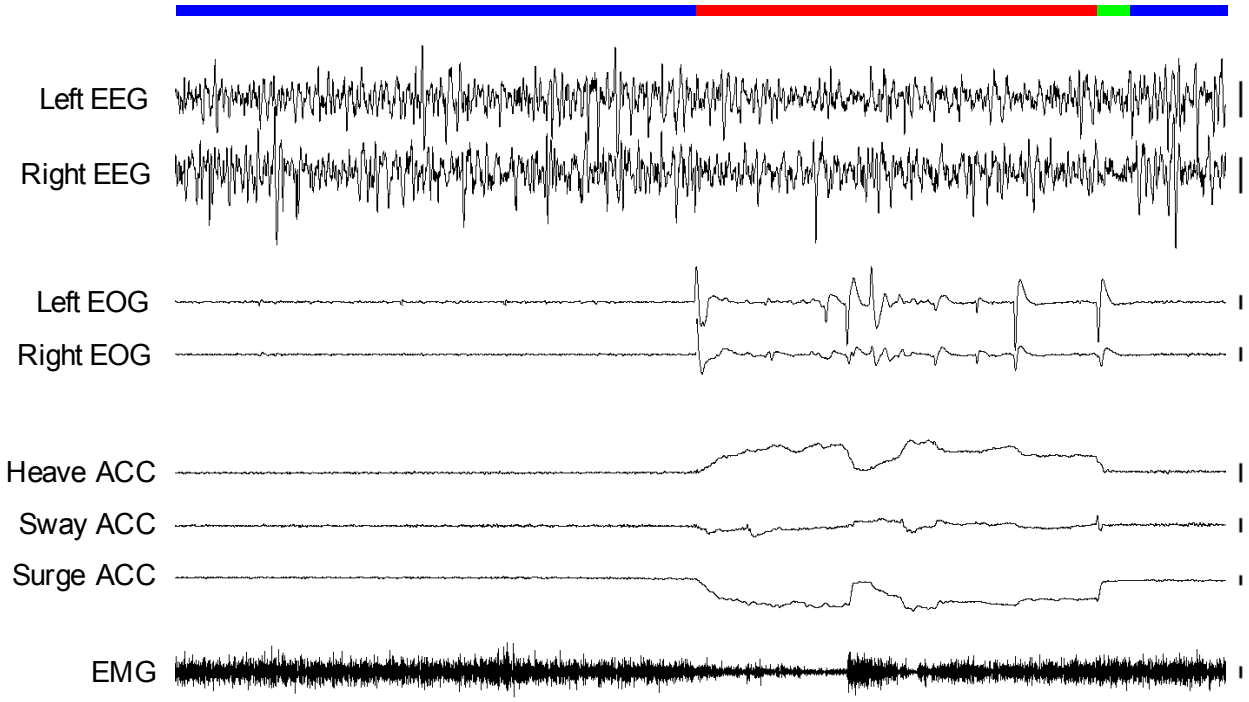
E


Left EEG
Right EEG
Left EOG
Right EOG
Heave ACC
Sway ACC
Surge ACC
EMG

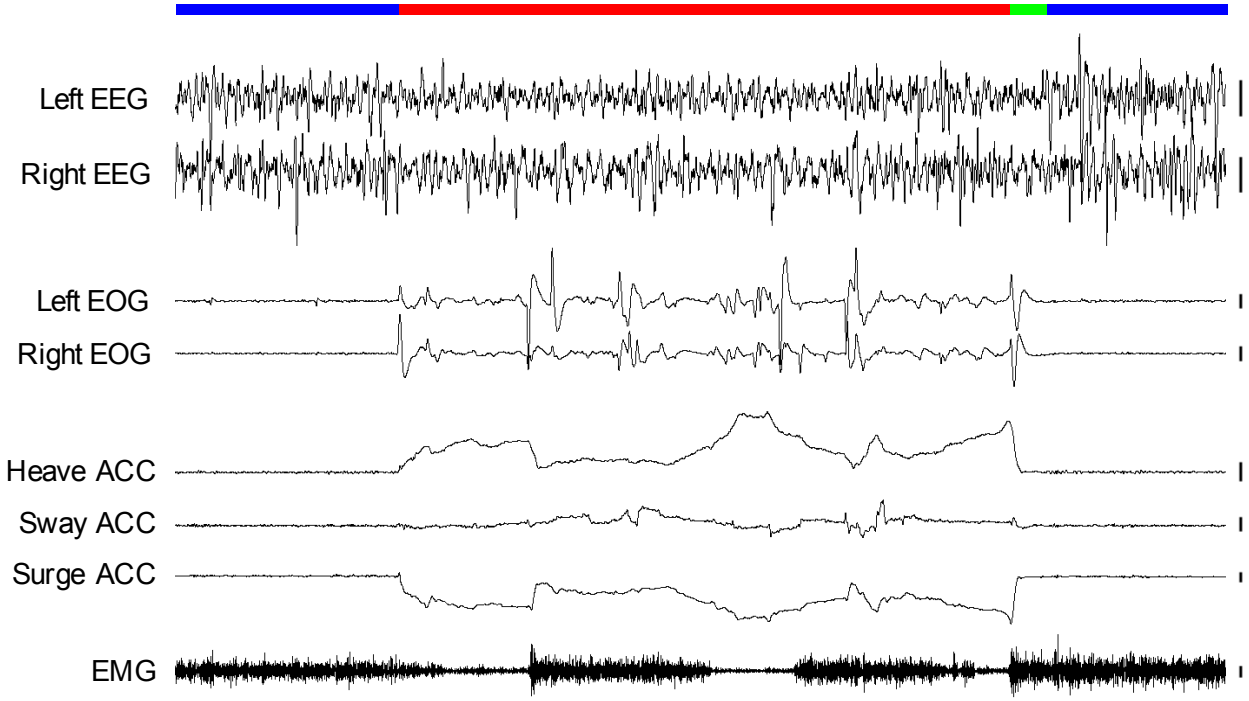
F


Left EEG
Right EEG
Left EOG
Right EOG
Heave ACC
Sway ACC
Surge ACC
EMG

G


Left EEG
Right EEG
Left EOG
Right EOG
Heave ACC
Sway ACC
Surge ACC
EMG

H


Left EEG
Right EEG
Left EOG
Right EOG
Heave ACC
Sway ACC
Surge ACC
EMG

CHAPTER 3

Increased EEG Spectral Power Density during Sleep Following Short-term Sleep Deprivation in Pigeons (*Columba livia*): Evidence for Avian Sleep Homeostasis

Martinez-Gonzalez D, Lesku JA and Rattenborg NC. 2008. Increased EEG spectral power density during sleep following short-term sleep deprivation in pigeons (*Columba livia*): evidence for avian sleep homeostasis. *Journal of Sleep Research* 17:140-153.

Abstract

Birds provide a unique opportunity to evaluate current theories for the function of sleep. Like mammalian sleep, avian sleep is composed of two states, slow wave sleep (SWS) and rapid eye movement (REM) sleep that apparently evolved independently in mammals and birds. Despite this resemblance, however, it has been unclear whether avian SWS shows a compensatory response to sleep loss (i.e., homeostatic regulation), a fundamental aspect of mammalian sleep potentially linked to the function of SWS. Here, we prevented pigeons (*Columba livia*) from taking their normal naps during the last 8 hours of the day. Although time spent in SWS did not change significantly following short-term sleep deprivation, electroencephalogram (EEG) slow wave activity (SWA) (i.e., 0.78 – 2.34 Hz power density) during SWS increased significantly during the first 3 hours of the recovery night when compared to the undisturbed night, and progressively declined thereafter in a manner comparable to that observed in similarly sleep deprived mammals. SWA was also elevated during REM sleep on the recovery night, a response that might reflect increased SWS pressure and the concomitant "spill-over" of SWS-related EEG activity into short episodes of REM sleep. As in rodents, power density during SWS also increased in higher frequencies (9 – 25 Hz) in response to short-term sleep deprivation. Finally, time spent in REM sleep increased following sleep deprivation. The mammalian-like increase in EEG spectral power density across both low and high frequencies, and the increase in time spent in REM sleep following sleep deprivation suggest that some aspects of avian and mammalian sleep are regulated in a similar manner.

Introduction

As in mammals, birds exhibit slow wave sleep (SWS) and rapid eye movement (REM) sleep (Campbell and Tobler 1984, Rattenborg and Amlaner 2002). In both taxonomic groups, the electroencephalogram (EEG) during SWS is characterized by high-amplitude, low-frequency (< 4 Hz) activity, whereas the EEG during REM sleep resembles the low-amplitude, high-frequency activity of wakefulness. In contrast, reptiles do not show high-amplitude, low-frequency EEG activity during sleep, indicating that birds and mammals independently evolved forebrain structures necessary for generating the EEG patterns that characterize mammalian and avian

SWS (Rattenborg 2006). In this respect, comparative studies of sleep in birds and mammals provide a unique opportunity to investigate the still disputed function(s) of sleep (Rechtschaffen 1998, Siegel 2005, Lesku et al. 2006, Roth et al. 2006, Tononi and Cirelli 2006, Lima and Rattenborg 2007, Rattenborg et al. 2007, Rial et al. 2007, Stickgold and Walker 2007, Lesku et al. 2008).

Despite the gross similarities in the EEG correlates of SWS between mammals and birds, SWS might be regulated differently in each taxonomic group. In mammals that engage in extended periods of wakefulness, EEG slow wave activity (SWA) (approximately 0.5 – 4.5 Hz power density) is highest during the first bout of SWS and progressively declines thereafter (Borbély and Achermann 2005). Extending wakefulness beyond that normally experienced on a daily basis increases subsequent SWA further. In humans, taking a nap in the day reduces SWA during non-REM sleep at night (Werth et al. 1996). This relationship between time spent awake and SWS-related SWA has been found in every mammalian species investigated (Tobler 2005). The link between prior time awake and SWA, as well as the positive correlation between arousal threshold and SWA (Frederickson and Rechtschaffen 1978, Neckelmann and Ursin 1993), suggest that SWS is homeostatically regulated with SWA reflecting the intensity of SWS and presumed SWS-related functions (Borbély and Achermann 2005, see also Huber et al. 2004).

The homeostatic regulation of SWS is a fundamental aspect of mammalian sleep that forms the foundation for current theories proposed for the function of SWS (e.g., Krueger and Obál 1993, 2003, Benington and Frank 2003, Tononi and Cirelli 2003, 2006). Given that SWS homeostasis is likely to be directly linked to the function of SWS (Benington 2000), an obvious question is: *Do birds, which show similar sleep-related EEG activity, also show an increase in SWA following sleep deprivation?* The only EEG-based studies that examined the effect of extending wakefulness in birds did not detect an increase in SWS-related SWA (0.75 – 4.5 Hz power density: Tobler and Borbély 1988, 0.75 – 4.0 Hz power density: Berger and Phillips 1994) following long-term (≥ 24 hours) sleep deprivation in pigeons (*Columba livia*). The apparent

absence of a compensatory response to sleep deprivation suggested that the avian forebrain lacks the neural cytoarchitecture necessary for SWS homeostasis, as present in the mammalian neocortex (Zepelin et al. 2005). Although the avian pallium and pallial mammalian neocortex are derived from homologous embryonic neural tissue, and function in a similar manner during wakefulness (Emery and Clayton 2004, Jarvis et al. 2004), the avian pallium is arranged in a nuclear manner that lacks the true laminar organization of the neocortex (Medina and Reiner 2000, Jarvis et al. 2004, Reiner 2005), a possible requisite for the EEG expression of SWS homeostasis.

The apparent absence of a compensatory response to sleep loss in birds would seem to challenge the taxonomic applicability of functional sleep theories that hinge on SWS homeostasis, or at least suggest that SWA is regulated differently in birds, and therefore may be associated with different functions (see Roth et al. 2006). Nevertheless, it may be premature to conclude that SWS is regulated differently in mammals and birds (Tobler 2005). In rodents, the increase in SWA observed following short-term sleep deprivation, is no longer evident following long-term sleep deprivation (Rechtschaffen et al. 1999). This pattern is most evident in the Syrian hamster (*Mesocricetus auratus*) where SWA (0.25 – 4.0 Hz) increased following 3 hours, but not 24 hours of sleep deprivation (Tobler and Jaggi 1987). Given this relationship between the duration of sleep deprivation and subsequent SWA in hamsters, a shorter (i.e., < 24 hours), presumably more ecologically-realistic, period of sleep deprivation might induce a compensatory increase in SWA during recovery SWS in birds as well. To determine whether pigeons show a compensatory increase in SWA following short-term sleep deprivation, we compared SWS-related SWA during a normal night to that occurring immediately following 8 hours of sleep deprivation. To detect any regional differences in recovery sleep similar to that observed in mammals (Vyazovskiy et al. 2002), we recorded the EEG from the dorsal surface of anterior, medial, and posterior pallial regions of each cerebral hemisphere. We expected SWA during recovery SWS at night to, 1) initially increase above baseline levels and, 2) to progressively decline across the recovery night following sleep

deprivation. A preliminary report of our findings was previously published in abstract form (Rattenborg and Martinez-Gonzalez 2007).

Methods

General Experimental Design

The EEG was recorded from pigeons during two consecutive 24-hour periods under a 12:12 light-dark photoperiod with lights on at 0700 and off at 1900. The first 24-hour period started at 0700 and served as a baseline. Starting at 1100 on the second day, the pigeons were kept awake for 8 hours during the day, half of which is normally spent asleep (see Results). At lights out the birds were allowed to sleep undisturbed. The experiment ended at the end of the second night.

Animals and Housing

Five adult tippler pigeons (*Columba livia*; 3 female; 2 male) were purchased from a local breeder. Each bird was housed in an individual wooden enclosure (length = 79 cm, width = 60 cm, height = 60 cm). A rectangular opening on the enclosure door covered with wire mesh provided ventilation. The back wall was made of white translucent Plexiglas. Fluorescent lights placed behind the upper third of the Plexiglas wall and room light entering through the opening in the cage door provided light in the daytime (400 – 500 lux measured at head level in the center of the enclosure). An infrared-sensitive camera was placed in each corner of the enclosure, and an infrared (940 nm) light source hung from the center of the ceiling provided luminance for the cameras at night. The floor of the enclosure was covered with paper and an inverted ceramic dish placed in the center served as a perch. The birds were given mixed grain pigeon feed, grit and water *ad libitum*. Each bird was able to hear, but not see, the other bird in the recording room. The temperature was maintained between 23.5°C and 25.5°C. The experiments were approved by the Government of Upper Bavaria and adhere to the NIH standards for using animals in research.

EEG Electrode Implantation

To detect potential regional differences in the response to sleep deprivation, 6 EEG electrodes were placed over each cerebral hemisphere using standard stereotaxic techniques. In brief, after establishing a suitable surgical anaesthetic plane using isoflurane (1.5 – 2.0 % vaporised in 1.0 LPM O2) 14 holes (0.5 mm diameter) were drilled through the exposed cranium to the level of the dura. The 12 holes for the EEG electrodes were arranged in three rows (i.e., anterior, medial and posterior pallium) with 4 holes per row. The anterior row was positioned at AP +13.0 mm, the medial row at AP +9.25 mm and the posterior row at AP +4.75 mm (Karten and Hodos 1967). Within a row, holes were drilled 2.0 and 6.0 mm lateral (i.e., L 2.0 and L 6.0) of the midline on each side. In the anterior and medial rows, the medial electrodes were positioned over the hyperpallium apicale (L 2.0) and the lateral electrodes (L 6.0) were positioned over the mesopallium (see Reiner 2005 for new avian brain nomenclature). In the posterior row, the medial electrodes (L 2.0) were positioned over the area parahippocampalis and the lateral electrodes (L 6.0) were positioned over the area corticoidea dorsolateralis, thin contiguous structures overlying the nidopallium caudale and separated from it by a narrow ventricular space. An additional hole for the reference electrode was placed over the center of the cerebellum, and a hole for the ground electrode was placed 2 mm anterior of the most anterior-medial hole on the right hemisphere. All electrodes were made from gold-plated pins (0.5 mm diameter) with rounded tips. Each electrode was placed on the dura and glued in position using cyanoacrylic adhesive and wired to a connector that was mounted on the head with Paladur® dental acrylic (Heraeus Kulzer, www.heraeus-kulzer.com). The birds were connected to the recording cable after at least five days of post-operative recovery in the recording enclosure. The recording cable was connected to a swivel (Plastics One®, Inc., www.plastics1.com) mounted on the ceiling of the enclosure. Baseline recordings started after at least one week of adaptation to the recording cable.

EEG Recordings

Each EEG signal was referenced to the cerebellum and digitally recorded at 200 Hz using commercially available amplifiers (Embla® A10) and software (Embla®, Somnologica Science v.

3.3.1, www.embla.com). The low-cut finite impulse response (FIR) filter was set at 0.5 Hz (-6 dB at 0.5 Hz and 0 dB at 0.78 Hz) and the high-cut anti-aliasing FIR filter was set at 100 Hz (-20 dB at 100 Hz and 0 dB at 80 Hz). The Embla® amplifiers automatically apply the anti-ailiasing filter after first sampling the data at 2000 Hz. The data is then down-sampled to 200 Hz. A 50 Hz notch filter eliminated potential electrical interference. No additional filtering was applied after data acquisition. Bipolar EEG recordings were created offline for the anterior (AP +13.0, L 6.0 – L 2.0), medial (AP +9.25, L 6.0 – L 2.0) and posterior (AP +4.75, L 6.0 – L 2.0) pallia of each hemisphere and used for sleep staging and spectral power density analysis. Monopolar recordings with each pallial electrode referenced to the cerebellum showed results similar to these bipolar derivations (data not shown), as did a bipolar derivation (AP +4.5, L 2.0 – AP +13.0, L 2.0) that most closely approximated that used in an earlier sleep deprivation experiment on pigeons (AP +6.0, L 2.0 – AP +13.0, L 2.0, Tobler and Borbély 1988).

Sleep Deprivation

As in previous short-term sleep deprivation studies in mammals, the pigeons were kept awake using the "gentle handling" technique. Whenever high-amplitude, low-frequency activity appeared in any EEG recording, we stimulated the pigeon by tapping or moving the floor of their enclosure, making a noise or, towards the end of the deprivation, gently touching the bird.

Sleep Staging

The state of the pigeons was visually determined for each 4-second epoch using a combination of EEG and video recordings. Three states were scored: wakefulness, SWS and REM sleep. Wakefulness was characterized by relatively low-amplitude, high-frequency EEG activity. SWS was scored when more than half of an epoch showed low-frequency activity with an amplitude approximately twice that of alert wakefulness. In each case, the onset of scored SWS typically corresponded with the onset of sleep behavior (e.g., immobility, head drawn in to the chest and one or both eyes closed). Because pigeons show interhemispheric asymmetries in low-frequency activity during SWS (Rattenborg et al. 2001), SWS was scored whenever at least one EEG recording met SWS criteria. In practice, as in a previous study (Rattenborg et al. 2001),

both hemispheres usually showed some level of SWS-related EEG activity simultaneously. REM sleep was characterized by periods of EEG activation (> 2 seconds) occurring in association with bilateral eye closure and behavioral signs of reduced muscle tone (e.g., head dropping, swaying, and sliding of the wings off the side of the body). Finally, we calculated the duration of each episode of each sleep state by summing the number of consecutive epochs scored as the same state.

Spectral Power Density Analysis

Fast Fourier transforms were performed on EEG recordings to calculate power density in 0.39 Hz bins between 0.78 and 25 Hz during each 4-second scoring epoch (Embla®, Somnologica Science v. 3.3.1, www.embla.com). EEG artifacts were visually detected and omitted from the analysis. Most artifacts were associated with gross movements during wakefulness (e.g., preening, feeding, walking). Because the pigeons were rarely awake and motionless, the majority (> 80 %) of the EEG during wakefulness was contaminated with artifacts. Consequently, wakefulness was not included in the spectral analysis. In contrast to wakefulness, on average > 95 % of the sleeping epochs were artifact free on the baseline and recovery nights and therefore included in the spectral analysis (98.67 ± 0.69 % (mean ± s.e.m.) and 95.41 ± 2.25 % during baseline and recovery SWS, respectively; 97.75 ± 0.99 % and 97.73 ± 0.80 % during baseline and recovery REM sleep, respectively). For each sleep state and frequency bin, we expressed the average spectral power density for each quarter of the baseline and recovery nights as a percent of the entire baseline night average for that state.

A previous study in pigeons found that SWA during SWS was lower when the birds had at least one eye open (Tobler and Borbély 1988). To detect potential changes in spectral power density during recovery SWS related to changes in the proportion of SWS occurring with eyes open or closed, the state of each eye was determined at the start of each minute during the first three hours of the baseline and recovery nights, when changes in spectral power density were expected to be the greatest. For each eye, we calculated the proportion of corresponding epochs scored as SWS during which that eye was open or closed.

Statistics

We used two-way or three-way repeated-measures analysis of variance (rmANOVA) and two-tailed paired t-tests when comparing various aspects of wakefulness and sleep between the baseline and recovery conditions. In instances where the overall rmANOVA model was significant, we conducted paired t-tests to identify specific comparisons for which significance was reached. To characterize the time course of SWA on the baseline and recovery nights, one-way rmANOVAs were conducted on the data for each night separately. Variables were transformed (when necessary) to meet the assumption of normality of residuals. All statistical analyses were conducted in either Systat 10 (SPSS 2000) or SPSS 15 (SPSS 2006).

Results

Efficacy of Sleep Deprivation

The gentle handling technique was successful in reducing sleep (Figure 1). During the 8-hour period of sleep deprivation, the proportion of time spent awake (Figure 1a) increased significantly relative to baseline (baseline, 48.74 ± 1.43 % vs. deprivation, 91.58 ± 3.32 %; $F = 183.043$, $df = 1,60$, $P < 0.001$). Likewise, the time spent in SWS (Figure 1b) was significantly reduced (baseline, 45.19 ± 1.82 % vs. deprivation, 8.42 ± 3.32 %; $F = 160.562$, $df = 1,60$, $P < 0.001$) and REM sleep (Figure 1c) did not occur at all during this period (baseline, 6.07 ± 0.86 % vs. deprivation, 0.00 ± 0.00 %; $F = 134.640$, $df = 1,60$, $P < 0.001$). The small amount of residual SWS during sleep deprivation occurred in brief episodes lasting 2 – 4 seconds, the time required to detect sleep and stimulate the bird.

Baseline and Recovery Sleep Architecture

During the 12-hour night of recovery, the time spent awake did not differ significantly from the baseline night (Figure 1a; baseline, 20.17 ± 4.28 % vs. recovery, 20.21 ± 2.36 %; $F < 0.001$, $df = 1,92$, $P = 0.988$), nor did the time spent in SWS (Figure 1b; baseline, 67.08 ± 2.95 % vs. recovery, 64.84 ± 1.68 %; $F = 0.943$, $df = 1,92$, $P = 0.334$). In contrast to SWS, time spent in REM sleep increased following sleep deprivation (baseline, 12.75 ± 1.64 % vs. recovery, 14.95 ± 1.79 %; $F = 9.190$, $df = 1,92$, $P = 0.003$) (Figure 1c).

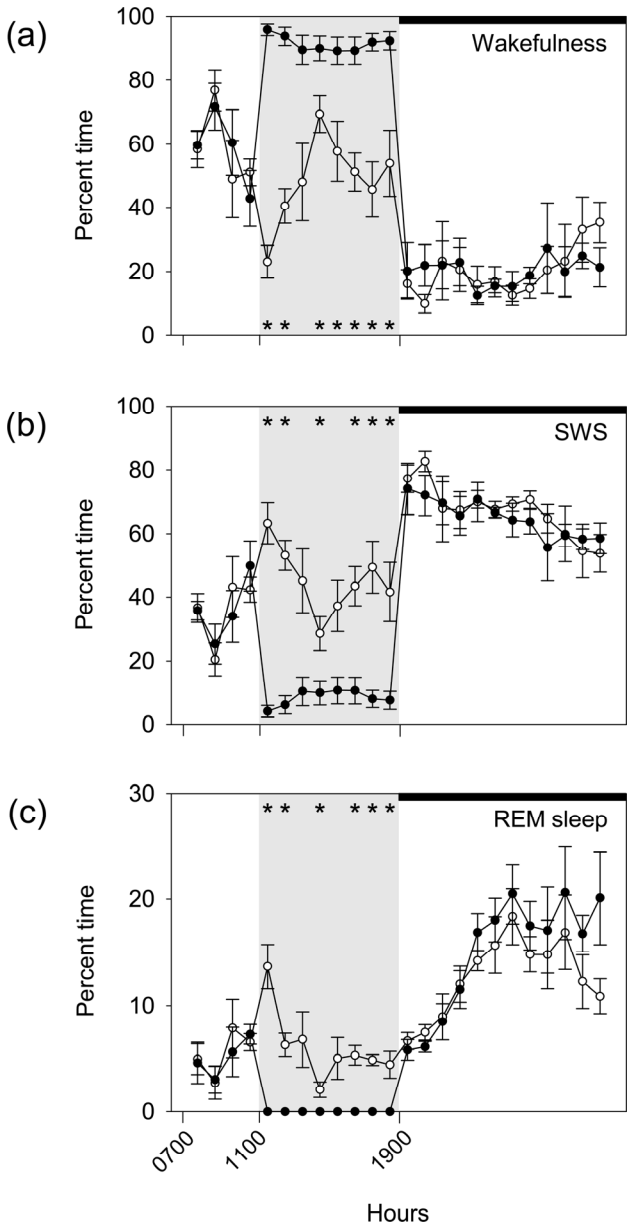


Figure 1. Effect of sleep deprivation on time spent in wakefulness (a), SWS (b) and REM sleep (c). The percent time (mean ± s.e.m.) spent in each state for each hour of the first and second 24-hour periods is plotted at the middle of each hour. The first 24-hour period served as an undisturbed baseline (open circles). The second 24-hour period (filled circles) is divided into an additional 4-hour period of baseline prior to the start of sleep deprivation (0700 – 1100), 8 hours of sleep deprivation (1100 – 1900) shaded in grey, and 12 hours of recovery following sleep deprivation (1900 – 0700). The black bar at the top, right of each plot indicates night. Statistical differences (two-tailed, paired t-test) between the baseline and recovery nights are indicated by an asterisk. The rmANOVA revealed an effect of treatment on REM sleep at night, but none of the hourly comparisons were significantly different between the baseline and recovery nights.

Sleep Duration

Figure 2 shows the duration of SWS (a) and REM sleep (b) episodes across the baseline and recovery nights. The duration of episodes of SWS decreased ($F = 17.021$, $df = 11,92$, $P < 0.001$) and the duration of REM sleep episodes increased ($F = 21.337$, $df = 11,92$, $P < 0.001$) across both nights. However, the duration of both SWS and REM sleep episodes decreased on the recovery night when compared to the baseline night ($F = 18.569$, $df = 1,92$, $P < 0.001$ and $F =$

4.303, df = 1,92, P = 0.041, respectively). This seems to reflect more frequent switching between SWS and REM sleep on the recovery night, rather than shorter episodes of sleep (SWS and REM sleep combined), as the duration of sleep episodes (Figure 2c) was not different between the baseline and recovery nights (F = 0.005, df = 1,92, P = 0.942).

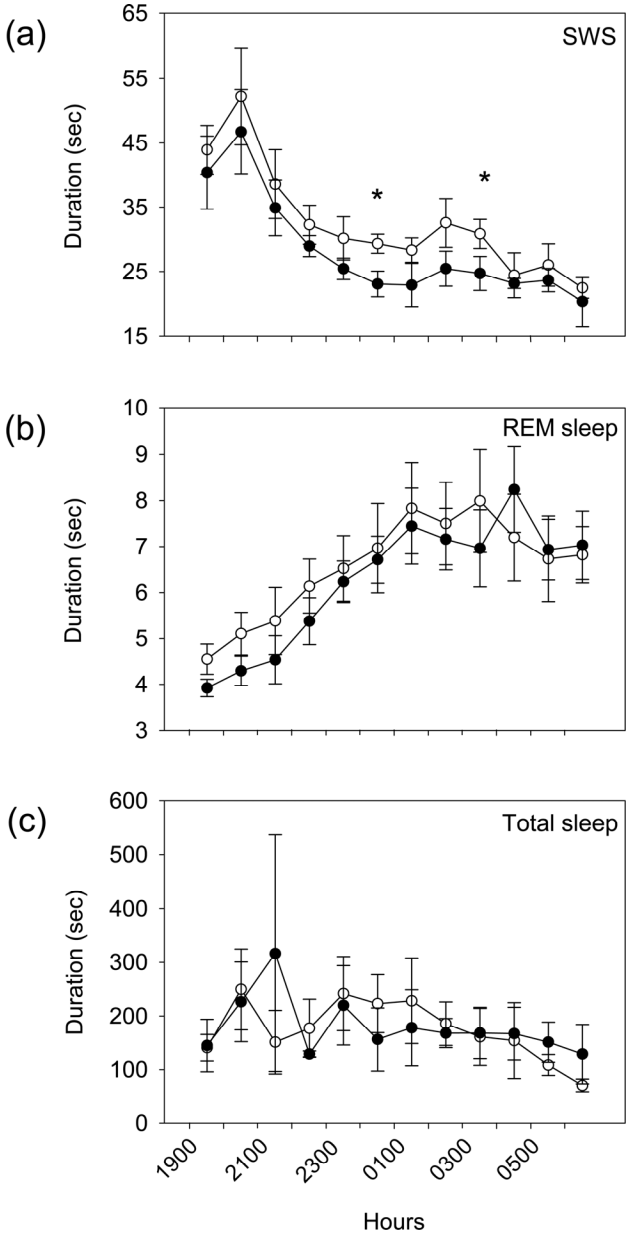


Figure 2. The effect of sleep deprivation on the duration of sleep episodes. The duration of slow wave sleep (a), REM sleep (b) and total sleep (slow wave sleep and REM sleep combined) (c) episodes across each hour of the baseline (open circles) and recovery (filled circles) nights. The mean (± s.e.m.) duration is plotted in seconds at the middle of each hour. Data underlying this figure was log-transformed for analysis, but is presented here untransformed so that the units are more easily interpretable. Statistical differences (P < 0.05, two-tailed, paired t-test) between the baseline and recovery nights are indicated by an asterisk. Although the rmANOVA revealed an effect of treatment on the duration of SWS and REM sleep episodes at night, none of the hourly post-hoc comparisons were significantly different between the baseline and recovery nights for REM sleep.

Spectral Power Density

Slow-wave sleep: Figure 3 shows spectral power density (0.78 – 25 Hz) during SWS on the baseline night for each region of the pallium. In general, spectral power density below 2.5 Hz was highest during the first or second quarter of the night and lowest during the last in all regions except the left and right posterior pallia. The relative power density of high frequencies (approximately 5 – 25 Hz) were lowest during the first and highest during the last quarter of the baseline night in the posterior pallia. Components of this pattern were also evident to a lesser extent in the medial and anterior pallia (Figure 3a). Although the amount of time spent in SWS did not increase during recovery (Figure 1b), power density was significantly affected by prior sleep deprivation (Figure 3). Notably, power density below 2.5 Hz was greatest during the first quarter of the night and then decreased across each successive quarter in the left and right, anterior and medial pallia. In the left and right anterior pallia, this effect extended out to 5.08 Hz. In addition to the increase in low frequencies, high-frequency (approximately, 9 – 25 Hz) power density also increased significantly during the first quarter in the left anterior pallia, and to varying degrees across all quarters of the recovery night in the right anterior and left medial pallia (approximately 8 – 15 Hz).

The posterior pallia showed a response different from the other regions. Power density in the left posterior pallium was largely unaffected by prior sleep deprivation, whereas the right posterior pallium showed a pronounced increase in mean power density below 2.5 Hz. Unlike the anterior and medial pallia where the results were largely consistent across all birds, however, the increase in low-frequency power in the right posterior pallium was present in only three birds; the remaining two showed a much smaller response, and as a result, although the increase in mean power density was greatest in this region (note the different scale on the plots for the posterior pallia), it only reached statistical significance for the first frequency bin (0.78 – 1.17 Hz) during the first quarter of the recovery night.

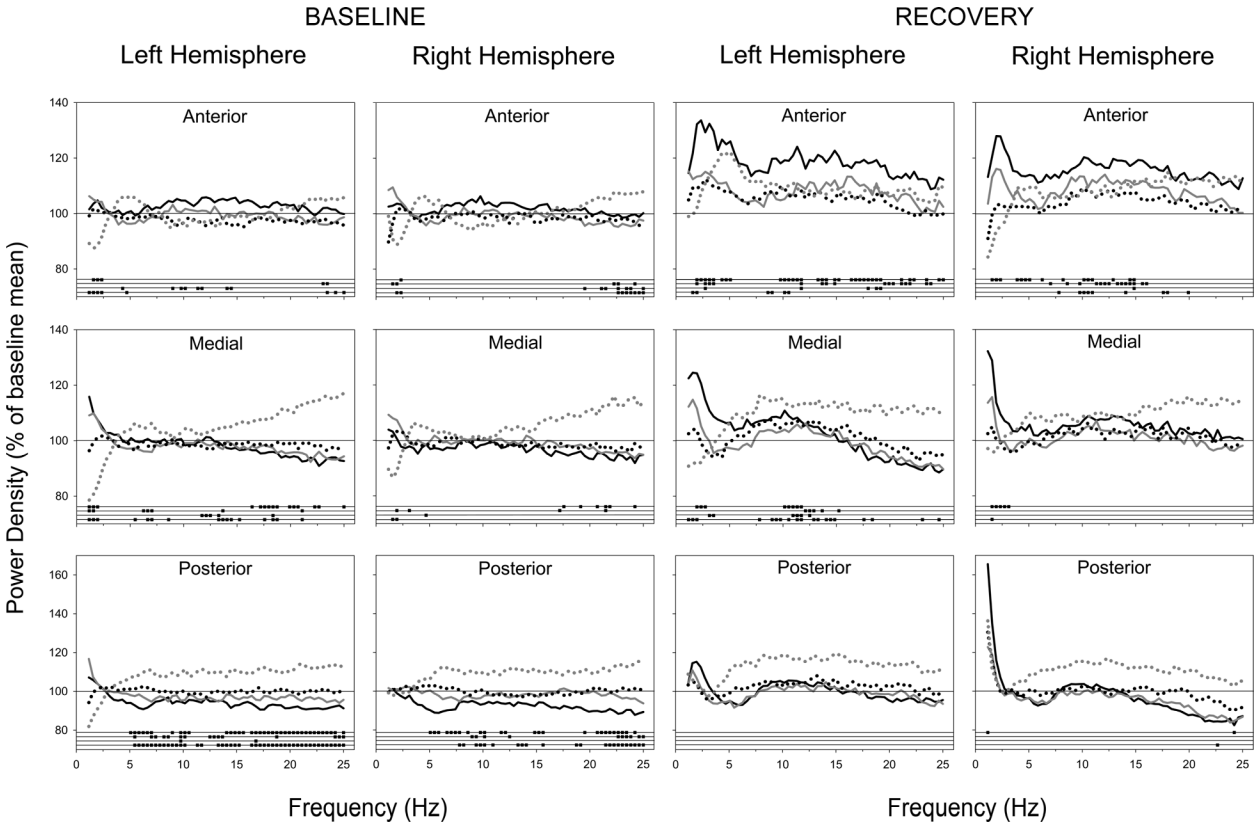


Figure 3. EEG spectral power density (0.78 – 25 Hz) during slow wave sleep (SWS) on the baseline and recovery nights. The power density for each quarter (1st, solid black line; 2nd, solid grey line; 3rd, dotted black line; 4th, dotted grey line) of each night is expressed as a percent of the entire baseline night SWS mean (i.e., the 100 % line) for each frequency bin and brain region (left and right, anterior, medial and posterior pallia) in each pigeon. The mean percent is plotted at the end of each frequency bin. For the baseline night, values for each quarter and frequency bin were compared to the baseline night average. Significant differences ($P < 0.05$, two-tailed paired t-test after significant rmANOVA) are indicated by filled squares on the lines at the bottom of each plot; statistical data for the 1st through 4th quarters is presented on the 1st (top) though 4th (bottom) lines, respectively. For the recovery night, values for each quarter and frequency bin were compared to the corresponding quarter of the baseline night, with significant differences similarly indicated at the bottom of each plot.

Figure 4 summarizes the changes in SWA (i.e., 0.78 - 2.34 Hz) during SWS across the baseline and recovery nights for the left and right, anterior and medial pallia. Sleep deprivation significantly increased SWA during recovery in the left and right anterior pallia (left, $F = 40.959$, $df = 1,28$, $P < 0.001$; right, $F = 4.424$, $df = 1,28$, $P = 0.045$) and the left and right medial pallia (left, $F = 9.170$, $df = 1,28$, $P = 0.005$; right, $F = 6.604$, $df = 1,28$, $P = 0.016$). SWS-related SWA during the first quarter was significantly greater during recovery when compared to baseline for the left ($t = 4.704$, $df = 4$, $P = 0.009$) and right ($t = 4.897$, $df = 4$, $P = 0.008$) anterior pallia, and the right medial pallium ($t = 3.496$, $df = 4$, $P = 0.025$); the left medial pallium showed a similar trend ($t = 2.475$, $df = 4$, $P = 0.069$) (Figure 4). SWA was also greater during the last quarter in the left anterior and medial pallia (anterior, $t = 3.536$, $df = 4$, $P = 0.0241$; medial, $t = 5.916$, $df = 4$, $P = 0.004$), and the right medial pallium showed a similar trend ($t = 2.693$, $df = 4$, $P = 0.055$). None of the post-hoc tests for the other quarters were significant. One-way rmANOVAs showed that SWA decreased in all regions across the recovery night (left anterior, $F = 8.587$, $df = 3,12$, $P = 0.003$; left medial, $F = 12.348$, $df = 3,12$, $P < 0.001$; right anterior, $F = 19.032$, $df = 3,12$, $P < 0.001$; right medial, $F = 5.993$, $df = 3,12$, $P = 0.010$). One-way rmANOVAs also revealed an effect of time on the baseline night for all regions except the right anterior pallium (left anterior, $F = 11.971$, $df = 3,12$, $P < 0.001$; left medial, $F = 37.995$, $df = 3,12$, $P < 0.001$; right anterior, $F = 2.050$, $df = 3,12$, $P = 0.161$; right medial, $F = 4.882$, $df = 3,12$, $P = 0.019$). Post-hoc t-tests for the baseline night revealed a significant increase in SWA from the baseline night mean during the first quarter for the left anterior ($t = 6.377$, $df = 4$, $P < 0.004$) and medial ($t = 3.477$, $df = 4$, $P < 0.026$) pallia, a significant increase during the second quarter in the left medial pallium ($t = 2.870$, $df = 4$, $P < 0.046$), and a significant decrease during the last quarter in the left anterior ($t = -4.320$, $df = 4$, $P < 0.013$) and medial ($t = -3.307$, $df = 4$, $P < 0.030$) pallia. The right medial pallium also showed a trend for higher SWA during the second quarter ($t = 2.693$, $df = 4$, $P < 0.056$) and lower SWA during the last quarter of the night ($t = -2.677$, $df = 4$, $P < 0.056$). The increase in SWA during the first quarter of the recovery night, when the increase in SWA was the greatest, was similar across the left and right, anterior and medial pallia. Specifically, comparisons between the left and right anterior pallia, left and right medial pallia, left anterior

and left medial pallia, and right anterior and right medial pallia were all non-significant (P > 0.27).

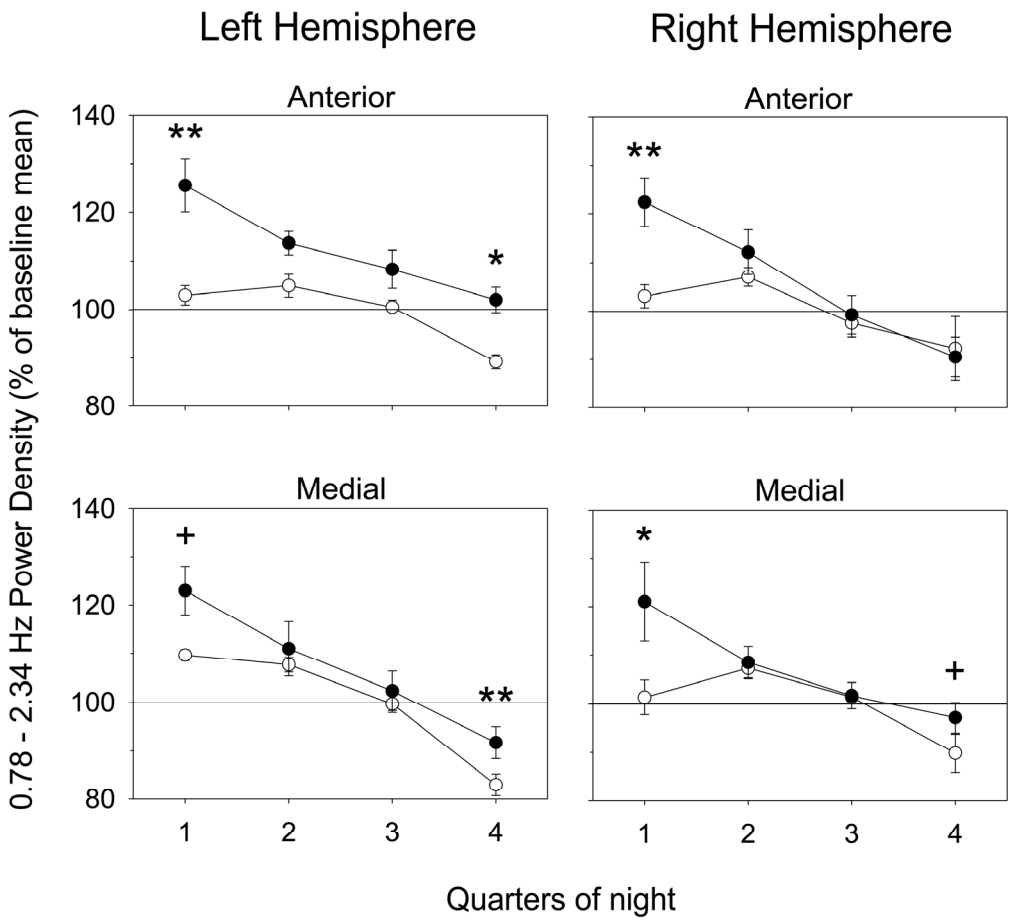


Figure 4. Slow-wave activity during slow wave sleep on the baseline (open circles) and recovery (filled circles) nights expressed as a percent (mean ± s.e.m.) of the entire baseline night mean for the left and right, anterior and medial pallia. Statistical differences between the baseline and recovery nights are indicated as follows: **P < 0.01; *P < 0.05; +P < 0.07 (paired t-test after significant rmANOVA).

Finally, eye closure during SWS occurring in the first quarter of the night (when the increase in SWA was the greatest) did not change between the baseline and recovery nights. During SWS, the left eye was closed 77.53 ± 8.77 % of the time on the baseline night and 77.37 ± 7.64 % of the time on the recovery night (t = -0.013, df = 4, P = 0.990), and the right eye was

closed 86.72 ± 8.50 % of the time on the baseline night and 78.85 ± 10.67 % on the recovery night ($t = -1.409$, $df = 3$, $P = 0.254$). The comparison for the right eye was based on only four birds because one bird obscured this eye on both nights.

REM sleep: Figure 5 shows power density (0.78 – 25 Hz) during REM sleep for each pallial region and quarter of the baseline (a) and recovery (b) nights. For both nights, power density for each frequency bin is expressed as a percent of the entire baseline night average during REM sleep for that specific bin and, thus reflects relative changes in power density during REM sleep. In general, the patterns evident in SWS were also present in REM sleep, particularly in the left anterior and medial pallia. Figure 6 shows the difference in power density during SWS and REM sleep for the first quarter of each night. Although the changes in relative power density during REM sleep and SWS were comparable (Figure 3 and 5), power density, expressed as a percent of average across all frequency bins during SWS occurring during the first quarter of the baseline night, was greater during SWS than REM sleep on both nights for frequencies up to approximately 15 – 22 Hz.

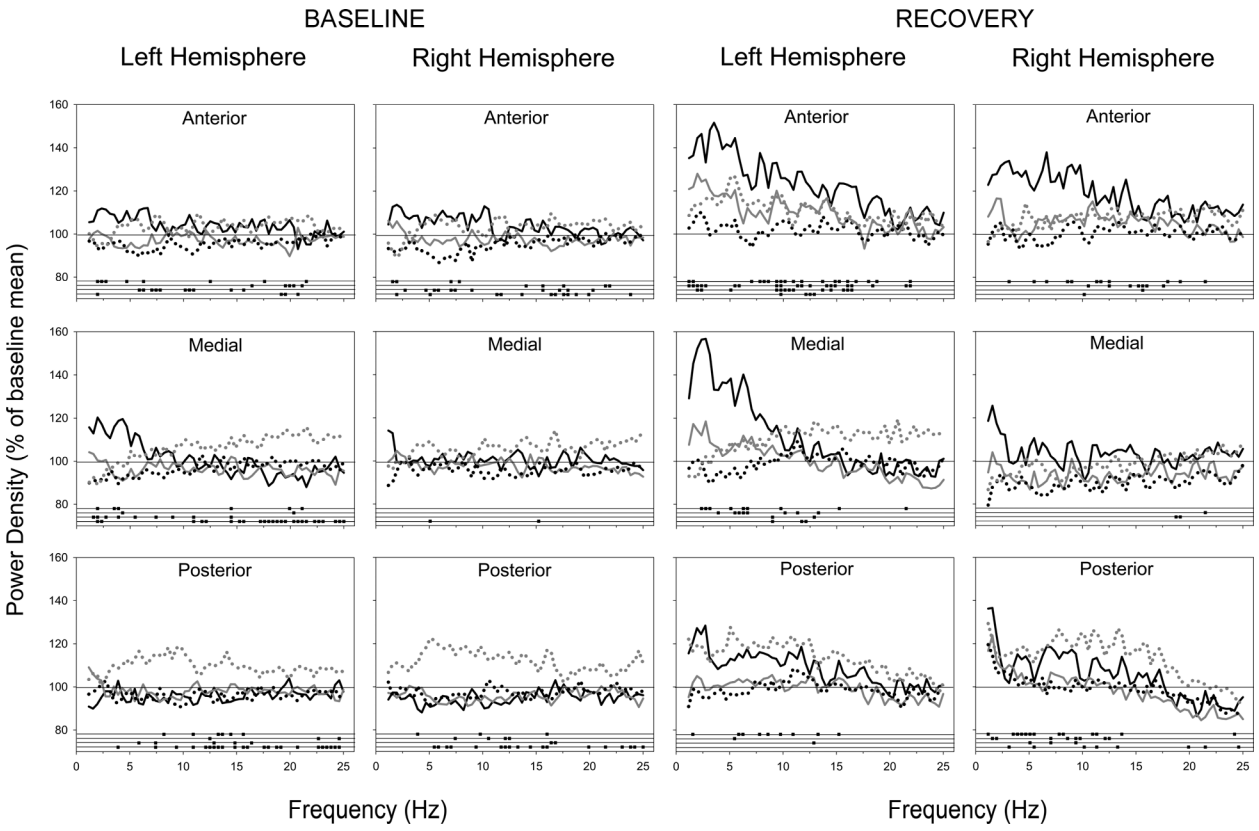


Figure 5. EEG spectral power density (0.78 – 25 Hz) during REM sleep on the baseline and recovery nights. The power density for each quarter (1st, solid black line; 2nd, solid grey line; 3rd, dotted black line; 4th, dotted grey line) of each night is expressed as a percent of the entire baseline night REM sleep mean (i.e., the 100 % line) for each frequency bin and brain region (left and right, anterior, medial and posterior pallia) in each pigeon. The mean percent is plotted at the end of each frequency bin. For the baseline night, values for each quarter and frequency bin were compared to the baseline night average. Significant differences ($P < 0.05$, two-tailed paired t-test after significant rmANOVA) are indicated by filled squares on the lines at the bottom of each plot; statistical data for the 1st through 4th quarters is presented on the 1st (top) though 4th (bottom) lines, respectively. For the recovery night, values for each quarter and frequency bin were compared to the corresponding quarter of the baseline night, with significant differences similarly indicated at the bottom of each plot.

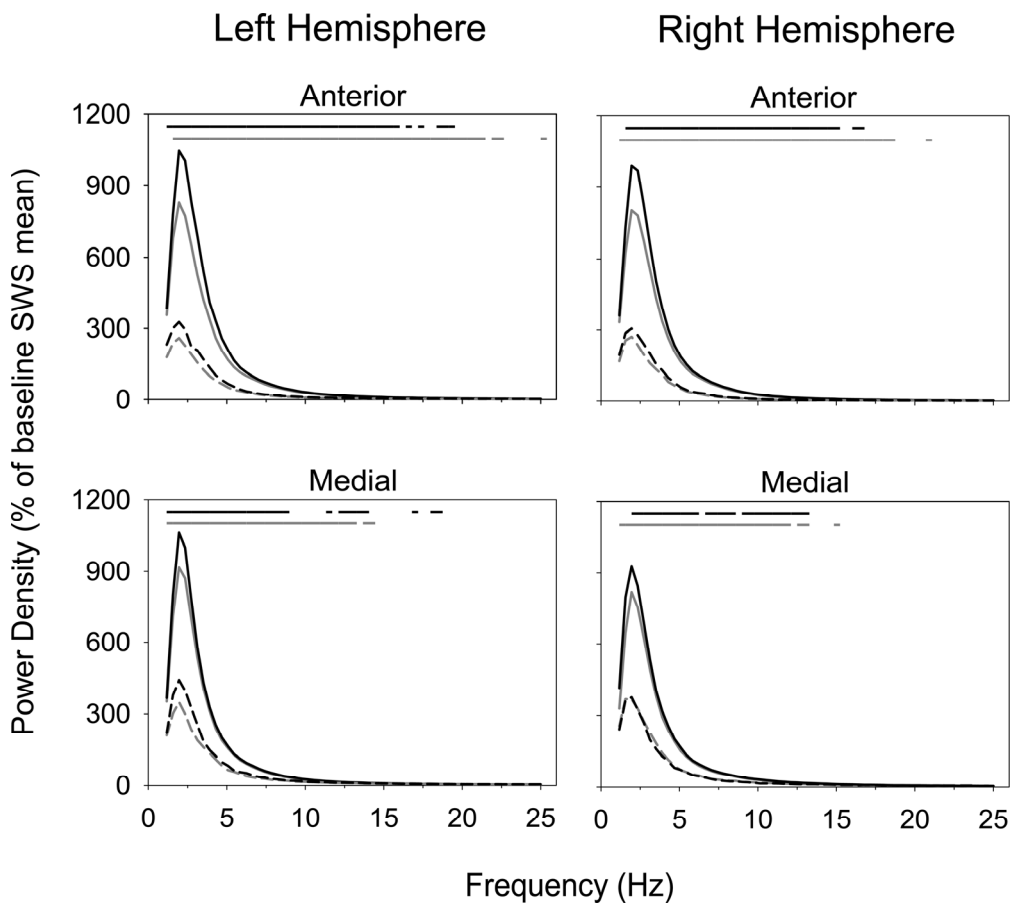


Figure 6. EEG power density (0.78 – 25 Hz) during SWS (solid lines) and REM sleep (dashed lines) during the first quarter of the baseline (grey lines) and recovery (black lines) nights for the left and right, anterior and medial pallia. To reduce variability between individuals, the power density for each frequency bin and state were calculated as a percent of power density averaged across all frequencies (0.78 – 25 Hz) for each bird during SWS occurring during the first quarter of the baseline night. Significant differences ($P < 0.05$, two-tailed paired t-test after significant rmANOVA) between baseline SWS and baseline REM sleep, and recovery SWS and recovery REM sleep are indicated by the grey and black bars, respectively, at the top of each plot.

Discussion

To our knowledge this is the first evidence for an increase in EEG spectral power density following sleep deprivation in birds. As in rodents (rats: Borbély et al. 1984; Syrian hamsters: Tobler and Jaggi 1987; mice: Huber et al. 2000), short-term (8 hours) sleep deprivation caused a significant increase in power density in both low and high frequencies during recovery SWS. Notably, in the left and right, anterior and medial pallia, SWA (i.e., 0.78 – 2.34 Hz) during SWS increased above baseline levels during the first quarter of the recovery night, and progressively decreased across the night in a manner comparable to that observed in similarly sleep deprived mammals. In contrast to these results, Tobler and Borbély (1988) did not detect an increase in SWA (0.75 – 4.5 Hz) following 24 hours of sleep deprivation in pigeons. This discrepancy is not attributable to the different frequency bands used to characterize SWA in the respective studies, because the increase in low-frequency power density in the left and right anterior pallia extended out to 5.08 Hz, thereby encompassing the 0.75 – 4.5 Hz band. Instead, sleep deprivation experiments in rodents suggest that this difference might be due to the duration of sleep deprivation used in the earlier study. In Syrian hamsters, SWS-related SWA (0.25 – 4.0 Hz) increased markedly following 3 hours, but not 24 hours of sleep deprivation (Tobler and Jaggi 1987, see also Rechtschaffen et al. 1999 for a similar pattern in rats). The similarly divergent responses to short- and long-term sleep deprivation in pigeons and Syrian hamsters suggests that differences in the duration of sleep deprivation may explain why an increase in SWA was observed in our study and not the earlier study. Nevertheless, additional experiments that directly compare the effects of 8 and 24 hours of sleep deprivation are needed to rule out other factors (e.g., pigeon strain, electrode placement, efficacy of the deprivation procedure) that might have contributed to the different responses to short- and long-term sleep deprivation in pigeons.

Our results have direct bearing on a previous report of sleep suppression in pigeons. Berger and Phillips (1994) reported that constant light (LL) reduced sleep in pigeons to < 5 % of the recording time for periods lasting several weeks without causing signs of elevated sleep pressure during LL, such as increased drowsiness, SWA (0.75 – 4.0 Hz power density between

50 – 200 μV^2 / Hz), or aspects of the "sleep deprivation syndrome" (i.e., debilitated appearance) observed in rats subjected to long-term sleep deprivation via the disk-over-water method (Rechtschaffen and Bergmann 2002). Moreover, time spent asleep and SWA did not increase above baseline levels during the first 24 hours after the birds (n = 2 for the analysis of SWA) were switched from LL to constant darkness (DD) (i.e., < 3 lux, red incandescent bulb). Based on these findings, Berger and Phillips (1994) concluded that pigeons do not show a mammalian-like compensatory rebound in SWA following sleep deprivation. In the present study, however, pigeons spent 42.1 % of the 12-hour light phase in SWS, an amount similar to the 37.7 % reported by Tobler and Borbély (1988). Differences in light levels do not seem to explain the contradictory results, because the light level used in our study (400 – 500 lux) was greater, and presumably more alerting, than that used in Berger and Phillips' study (200 lux). Although other factors cannot be ruled out, some of the differences seem to rest in the definition of sleep used in the respective studies. Whereas Berger and Phillips included a drowsy category, we followed Tobler and Borbély's (1988) approach and included states presumably comparable to Berger and Phillips' drowsiness in the calculation of SWS time. Even with drowsiness included in SWS time, however, their pigeons only engaged in SWS 28.3 % of the time during the light phase when housed under a 12:12 light-dark (LD) photoperiod, and 26.8 % of the time when exposed to LL. As with the scoring of SWS, the remaining difference may reflect a stricter threshold for scoring drowsiness.

The apparent differences in the scoring of sleep in pigeons may be reflected in the analysis of SWA under LL. Although the time spent in drowsiness and SWS during LL was similar to that during the light phase under LD, SWA (averaged across all states and expressed as a percent of the 24-hour average under LD) actually increased from 87.2 % during the light phase of LD to 94.5 % under LL. Consequently, although time spent in drowsiness and SWS did not increase during LL, SWA did increase (albeit non-significantly given the small sample size) when compared to the light phase of LD, presumably during either drowsiness or wakefulness. Furthermore, if the occurrence of SWA reflects homeostatically regulated SWS-related processes, regardless of the state in which it occurs (Borbély et al. 1984, Finelli et al. 2000,

Vyazovskiy and Tobler 2005), then, given the small decrease (5.5 %) in SWA during LL when compared to the 24-hour LD average, it seems unlikely that LL would cause an increase in SWA following the transition to DD. Consequently, LL may not be an effective means of inducing significant SWA deprivation in pigeons. In contrast, our results demonstrate that pigeons compensate for the loss of SWA and sleep (as defined herein) during the daytime, by increasing SWA during recovery sleep at night, and therefore are consistent with the notion that SWA occurring in the light reflects homeostatically regulated sleep processes.

The increase in SWA following short-term sleep deprivation and the progressive decline across the recovery night in pigeons is consistent with earlier studies describing the time course of SWA during avian sleep. A decline in SWA across the night suggestive of SWS homeostasis has been described in domestic hens (*Gallus domesticus*, number of 2.5 – 5.0 Hz, high-amplitude waves; van Luijtelaar et al. 1987), European blackbirds (*Turdus merula*, 0.5 – 4.0 Hz power density; Szymczak et al. 1996), non-migrating white-crowned sparrows (*Zonotrichia leucophrys gambelii*, 1.5 – 2.5 Hz power density; Rattenborg et al. 2004) and non-migrating Swainson's thrushes (*Catharus ustulatus*, 1.5 – 4.0 Hz power density; Fuchs 2006). In contrast, and perhaps surprisingly given the increase in SWA observed following sleep deprivation, our pigeons did not show a pronounced decline in SWA during SWS on the baseline night. Nevertheless, a significant effect of time was present with SWA being highest during the first or second quarter of the baseline night and lowest during the last. It remains unclear, however, why SWA was not consistently highest during the first three hours of the baseline night in this and previous studies of pigeons (Tobler and Borbély 1988, Berger and Phillips 1994).

In contrast to the left and right, anterior and medial pallia, where the effect of short-term sleep deprivation on SWA was evident and largely similar, the results for the posterior pallia were inconclusive. Whereas the left hemisphere did not show a significant increase in any frequency bin during the first quarter of the recovery night, the right posterior pallium showed a large increase in mean SWA. Due to marked variability between birds, however, this

increase was only significant for the 0.78 – 1.17 Hz frequency bin. Consequently, this finding should be interpreted with caution pending replication with a larger number of birds.

As with SWS, when compared to the baseline night, spectral power density increased across a broad range of frequencies during REM sleep on the recovery night, albeit the absolute magnitude of this effect was clearly lower in REM sleep than SWS. Notably, power density increased most markedly below approximately 5 Hz. Although this finding suggests that the increase in SWA during recovery sleep is not specific to SWS, it may also reflect greater "spill-over" of SWS-related EEG activity into REM sleep on the recovery night resulting from increased SWS pressure. Indeed, early in the baseline night when episodes of REM sleep were shortest (4.5 – 5.5 s), the EEG often did not achieve the fully activated pattern characteristic of longer episodes of REM sleep occurring later in the night (see Fuchs 2006 for a similar pattern in thrushes). As a result, low-frequency power density during REM sleep was greatest during the first quarter of the baseline night. This pattern was even more pronounced on the recovery night due to (1) the increase in SWA during SWS occurring early in the recovery night, and (2) the shortening of REM sleep episodes during the recovery night. A similar increase in SWA during REM sleep following 24 hours of sleep deprivation in humans (Borbély et al. 1981), rats (Franken et al. 1991) and rabbits (Tobler et al. 1990) was also attributed to the spill-over (Tobler et al. 1990) of SWS-related SWA into REM sleep resulting from increased SWS pressure. Nevertheless, it should be noted that SWA during REM sleep did not increase in rats (Tobler and Borbély 1990) or hamsters (Deboer et al. 1994) following shorter (i.e., 3 – 6 hours) periods of sleep deprivation more comparable to that employed in our study. This difference might reflect a greater propensity for SWS-related SWA to spill-over into REM sleep in pigeons, due to the short duration of REM sleep episodes, especially on the recovery night. Alternatively, it may reflect a fundamental difference between mammals and pigeons in the way the EEG during REM sleep responds to sleep deprivation.

During the baseline night high-frequency (5 – 25 Hz) power density during SWS was lowest during the first and highest during the last quarter of the night in the posterior pallium.

This pattern was also partially evident in the medial and anterior pallia. A similar increase in high frequencies during SWS occurring towards the end of the night has been observed previously in pigeons (Tobler and Borbély 1988). Nocturnal Syrian hamsters (Tobler and Jaggi 1987), Djungarian hamsters (Phodopus sungorus: Deboer et al. 1994) and rats (Trachsel et al. 1988) also show an increase in high-frequency activity toward the end of the main sleep period, although rabbits show the opposite pattern (Tobler et al. 1990). As demonstrated in rodents, high-frequency power density in pigeons may be modulated by circadian changes in brain temperature (Deboer 1998).

In addition to SWA, high-frequency (approximately 9 – 25 Hz) power density during SWS also increased following sleep deprivation in the left anterior pallium. Increases in high-frequency (8 – 15 Hz) power density were also evident to varying degrees across the night in the right anterior and left medial pallia. The increase in SWA and high-frequency power density were separated by a distinct dip in the magnitude of the increase around 6 – 8 Hz. Interestingly, sleep deprived rats (Borbély et al. 1984), Syrian hamsters (Tobler and Jaggi 1987), rabbits (Tobler et al. 1990) and mice (Huber et al. 2000) show a similar increase in high-frequency EEG activity, including the dip around 6 – 8 Hz. As suggested for rats and hamsters, the increase in high-frequency EEG activity following sleep deprivation may reflect residual or "covert" activation stemming from the deprivation procedure occurring concurrently with SWA during recovery SWS (Borbély et al. 1984, Tobler and Jaggi 1987). Although the cause or functional significance of the increase in high-frequency power density following sleep deprivation remains unclear, its presence in pigeons, rodents, and rabbits nonetheless further supports the suggestion that the avian pallium and mammalian neocortex respond similarly to sleep loss.

In addition to EEG spectral power density, sleep architecture was also affected by short-term sleep deprivation. Although time spent in SWS did not change during recovery, REM sleep showed a small, yet significant increase, primarily during the last two-thirds of the night. Tobler and Borbély (1988) also observed an increase in REM sleep following 24 hours of sleep

deprivation in pigeons (see also Newman et al. 2008), although, in contrast to our study, REM sleep increased primarily during the first half of the night. It is tempting to speculate that this difference in the timing of the increase in REM sleep during recovery is related to the presence of an increase in SWS-related SWA early in the night following 8 hours, but not 24 hours of sleep deprivation. As in mammals, increased SWS pressure early in the night may have delayed the increase in REM sleep following short-term sleep deprivation. In contrast, because SWS-related SWA (0.75 – 4.5 Hz) did not increase following 24 hours of sleep deprivation, the increase in REM sleep could occur unimpeded early in the night. A similar relationship between the duration of sleep deprivation and the characteristics of recovery sleep architecture has also been observed in rats, although the shift from an initial rebound in SWS-related SWA to one of REM sleep occurs somewhere between 24 and 96 hours in rats (Tobler and Borbély 1986, Rechtschaffen et al. 1999), in contrast to 8 and 24 hours in pigeons. As suggested for rats, the absence of an increase in SWS-related SWA following long-term sleep deprivation may indicate that the restorative function of REM sleep exceeds that of SWS, and therefore REM sleep takes precedence over SWS under these extreme conditions (Rechtschaffen et al. 1999, Rechtschaffen and Bergmann 1999a,b, Rechtschaffen and Bergmann 2002). Alternatively, the early REM sleep rebound following long-term sleep deprivation might reflect a homeostatic response to unintended selective REM sleep deprivation resulting from the failure to prevent short bouts of SWS (Benington and Heller 1999) and the occurrence of SWA in the waking EEG in chronically sleep deprived animals (Borbély et al. 1984, Tobler et al. 1990, Borbély 2001). The increase in REM sleep and decrease in SWA during SWS may also be a stress response related to long-term sleep deprivation (Feinberg 1999, Horne 2000). Along these lines, a recent gene expression study in rats showed that in comparison to short-term sleep deprivation (8 hours), long-term sleep deprivation (1 week) caused a more pronounced generalized inflammatory and stress response in the brain (Cirelli et al. 2006). Moreover, the expression of plasticity-related genes in the neocortex, such as BDNF (brain-derived neurotrophic factor), increased primarily following short-term sleep deprivation (Cirelli et al. 2006). This is interesting because the increase in SWA following short-term sleep deprivation may be mediated by BDNF in rats (Huber et al. 2007). Consequently, this difference in gene expression

following short- and long-term sleep deprivation may, in part, explain why SWS-related SWA only increases during recovery sleep following short periods of sleep loss.

Although time spent in REM sleep increased during recovery, the duration of REM sleep episodes actually decreased significantly during the recovery night when compared to the baseline night. A similar decrease in the duration of SWS episodes also occurred during recovery. Given that the duration of sleep (SWS and REM sleep combined) episodes was not affected by sleep deprivation, the reduction in the duration of SWS and REM sleep episodes apparently reflects more frequent switching between SWS and REM sleep during recovery. Interestingly, the decrease in the duration of SWS episodes suggests that the increase in SWA in pigeons was not mediated through an increase in SWS continuity, as observed in mammals where increased SWA is usually associated with decreased sleep fragmentation following sleep deprivation (Franken et al. 1991, Huber et al. 2000, Vyazovskiy et al. 2007).

Perspectives

Finally, the increase in SWA observed during sleep in the avian pallium following short-term sleep deprivation suggests that the mammalian neocortex is not necessary for the expression of a compensatory response in EEG activity. Perhaps this is not surprising given that neurons in the avian pallium exhibit the slow oscillations (Reiner et al. 2001) and connectivity (Rattenborg 2006) necessary to generate SWA in the first place. The ability to increase SWA in response to sleep deprivation in the avian pallium may involve cytoarchitecture and mechanisms similar to those involved in mammalian SWS homeostasis (Gvilia et al. 2000, Obál and Krueger 2003, Tononi and Cirelli 2006, Bourgin et al. 2007, Huber et al. 2007, Yasuda et al. 2007). Assuming that this is the case, it remains unclear whether these mechanisms and associated functions evolved through common descent from a stem amniote (the common ancestor to extant reptiles, birds, and mammals) that exhibited a similar precursor state, or independently in the respective ancestors of mammals and birds.

Although the apparent absence of SWA in the three-layered dorsal cortex of sleeping reptiles supports an independent origin for SWA in mammals and birds, some degree of homology may nonetheless exist between reptilian sleep and mammalian SWS. Specifically, sleeping reptiles show hippocampal spikes similar to those observed during SWS in mammals (Hartse 1994, Rattenborg 2006, 2007). Moreover, as in mammals, hippocampal spiking increases following sleep deprivation in reptiles (Hartse 1994). Thus at least one of the electrophysiological correlates of SWS and its homeostatic regulation are apparently present in reptiles. This suggests that the reptilian dorsal cortex may also exhibit neuronal activity (i.e., slow oscillations) similar to that which generates SWA in mammals (Steriade 2006). Even if neurons in the dorsal cortex are shown to exhibit slow oscillations (intracellular recordings are needed to resolve this issue), this activity may be largely asynchronous between neurons due to low corticocortical connectivity (Rattenborg 2006). In this regard, the synchronous neuronal activity that gives rise to SWA in mammals and birds may be an emergent property of their large, heavily interconnected pallia (i.e., the avian hyperpallium and the mammalian neocortex). Interestingly, this emergent property may perform emergent functions, not necessarily found in reptiles and other animals. Such functions may support their large, heavily interconnected brains and associated capacity to perform complex cognitive processes (Medina and Reiner 2000, Emery and Clayton 2004, Jarvis et al. 2005, Rattenborg 2006). Furthermore, functions associated with this emergent property may complement other sleep-related cellular processes that predate the evolution of mammals and birds (Cirelli et al. 2005, Cirelli 2006). Clearly, additional studies are needed to clarify the evolutionary history of the functions of sleep and the mechanisms underlying its regulation in vertebrates and invertebrates.

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CHAPTER 4

Local Sleep Homeostasis in the Avian Brain: Convergence of Sleep Function in Mammals and Birds?

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Abstract

The function of the brain activity that defines slow wave sleep (SWS) and rapid eye movement (REM) sleep in mammals is unknown. During SWS, the level of electroencephalogram slow wave activity (SWA or 0.5 – 4.5 Hz power density) increases and decreases as a function of prior time spent awake and asleep, respectively. Such dynamics occur in response to waking brain use, as SWA increases locally in brain regions used more extensively during prior wakefulness. Thus, SWA is thought to reflect homeostatically regulated processes potentially tied to maintaining optimal brain functioning. Interestingly, birds also engage in SWS and REM sleep, a similarity that arose via convergent evolution, as sleeping reptiles do not show similar brain activity. Although birds deprived of sleep show global increases in SWA during subsequent sleep, it is unclear whether avian sleep is likewise regulated locally. Here, we provide the first electrophysiological evidence for local sleep homeostasis in the avian brain. After staying awake watching David Attenborough's The Life of Birds with only one eye, SWA and the slope of slow waves (a purported marker of synaptic strength) increased asymmetrically in the hyperpallium – a primary visual processing region – increasing only in the hyperpallium neurologically connected to the stimulated eye. Asymmetries were specific to the hyperpallium, as the non-visual mesopallium showed a symmetric increase in SWA and wave slope. Thus, hypotheses for the function of mammalian SWS that rely on local sleep homeostasis may apply also to birds.

Introduction

The function of the brain activity that defines slow wave sleep (SWS) and rapid eye movement (REM) sleep in humans and other mammals is an unanswered question in neuroscience (Cirelli and Tononi 2008, Mignot 2008). At the neuronal level, SWS is seen as a slow (~ 1 Hz) oscillation of membrane potentials between a depolarized up-state with action potentials and a hyperpolarized down-state without (Steriade et al. 1993, Steriade 2006). The oscillation is synchronized among neurons by cortico-cortical connectivity (Amzica and Steriade 1995, Hill and Tononi 2005) and manifested in the electroencephalogram (EEG) as high-amplitude low-frequency waves, typically quantified as 0.5 – 4.5 Hz power density or slow wave activity (SWA;

Riedner et al. 2007). The level of SWA increases and decreases as a function of prior time spent awake and asleep, respectively (Borbély 2001, Dijk 2009). Additionally, SWA increases locally in the brain in response to local brain use during wakefulness (Kattler et al. 1994, Vyazovskiy et al. 2000, Huber et al. 2004, Yasuda et al. 2005a, Cajochen et al. 2008, Hanlon et al. 2009, Landsness et al. 2009, Määttä et al. 2010). Thus, SWA is thought to reflect homeostatically regulated processes potentially tied to maintaining optimal brain functioning (Krueger and Obál 1993, 2003, Benington 2000, Benington and Frank 2003, Tononi and Cirelli 2003, 2006, Rector et al. 2009, Diekelmann and Born 2010, Greene and Frank 2010, Rattenborg et al. in press).

Birds are the only animals, outside of mammals, known to engage in unequivocal SWS and REM sleep (Klein et al. 1964, Ookawa and Gotoh 1964, Low et al. 2008), a similarity that may have arisen via convergent evolution, as sleeping reptiles do not show similar brain activity (Rattenborg 2006, 2007). Although it has recently been shown that sleep deprived birds show a global increase in SWA during subsequent sleep (Martinez-Gonzalez et al. 2008, Rattenborg et al. 2009, Tobler 2011), it is unclear whether this effect reflects brain use *per se* or is mediated by central brain regions involved in the 'whole-brain' regulation of sleep (Komarova et al. 2008, see also Saper et al. 2005, Szymusiak and McGinty 2008). Below, we provide the first electrophysiological evidence for such local sleep homeostasis in the avian brain, which indicates that the level of SWA depends on the degree of brain use during prior wakefulness in birds.

Methods

Seven adult homing pigeons (*Columba livia*, 3 males, 4 females, genetically sexed) were housed individually in wooden enclosures (79 cm length x 60 cm width x 60 cm height; see Martinez-Gonzalez et al. 2008 for details). Each cage was equipped with four video cameras, one in each corner, and a ceiling-mounted infrared illuminator (940 nm) for night recordings. A flatscreen computer monitor (41 cm length x 34 cm width) was mounted on one side. Birds were maintained on a 12:12 light:dark photoperiod with lights on at 0800 hrs. Pigeons were returned to the breeding aviary at the end of the study. All methods were approved by the

Government of Upper Bavaria and adhere to the NIH standards regarding the care and use of animals in research.

Implanting EEG Electrodes

To record the EEG, pigeons were implanted with electrodes symmetrically placed over visual processing and non-visual processing regions of each hemisphere. The surgical procedures outlined below are similar to those used previously by our group (Martinez-Gonzalez et al. 2008). Briefly, a stereotax-mounted pigeon was anesthetized with isoflurane (1.5 – 2.0% vaporized in 1.0 LPM O2). Eight holes (0.5 mm diameter) were drilled through the cranium to the level of the dura; holes were arranged as two rows of four. The anterior row was positioned at AP +13.0 mm while the posterior row was positioned at AP +9.25 mm (Karten and Hodos 1967). Within a row, holes were drilled 2.0 and 6.0 mm lateral (L 2.0 and L 6.0) of the midline overlying each hemisphere. The medial electrodes (L 2.0) of both rows were positioned over the hyperpallium apicale while the electrodes placed more laterally (L 6.0) were seated over the mesopallium. The mesopallium is a non-visual region that has been implicated in higher cognitive processes, such as motor-learning and innovation (Timmermanns et al. 2000, Mehlhorn et al. 2010). Conversely, the hyperpallium is a primary visual processing region comparable to the primary visual (striate) cortex in mammals (Medina and Reiner 2000). In pigeons, each hyperpallium receives visual input primarily from the contralateral eye (Karten et al. 1973). As a result, visual stimuli presented to only one eye causes EEG activation in the contralateral hemisphere (Vyssotski et al. 2009). An additional hole was drilled over the cerebellum for the reference electrode, and another was drilled along the midline 2.0 mm anterior of the anterior row for the ground. All electrodes were gold-plated, round-tipped pins (0.5 mm diameter), glued in place using cyanoacrylic adhesive. Electrode wires terminated at a connector fixed on the head with Paladur® dental acrylic (Heraeus Kulzer, www.heraeus-kulzer.com). After a post-operative recovery period of at least 2 weeks, the feathers around the left and right eye were clipped and a Velcro® ring (2.5 cm diameter) was glued around the eyes using a non-toxic water-soluble skin glue. The bird's headplug was then connected to the recording cable, which in turn attached to a ceiling-mounted commutator (Plastics One®, Inc.,

www.plastics1.com). Baseline recordings commenced after at least 1 week of habituation to these recording conditions.

Experimental Design

Baseline EEG and video recordings were obtained for one 12 hr night starting at lights off (2000 hrs) following an undisturbed day. At 1200 hrs the next day, a Velcro®-ringed cardboard cap was attached to the eye-ring around the left eye; bandage tape around the margin further reduced the input of ambient light to the eye. This method of monocular occlusion is frequently used in studies of brain lateralization (Halpern et al. 2005). Given that the avian brain is functionally lateralized (Rogers and Andrew 2002), it is conceivable that the two hemispheres would respond differently to sleep loss (Kattler et al. 1994, Vyazovskiy et al. 2000, 2002). However, we chose to cap only the left eye (chosen randomly) in the present study, because SWS-related SWA increases symmetrically between the hemispheres in pigeons subjected to enforced wakefulness without unilateral visual stimulation (Martinez-Gonzalez et al. 2008). This approach also reduced the impact on the birds, as each pigeon was deprived of sleep only once. After the eye-cap was secured, the computer monitor began to show moving, non-repetitive video of wild birds (David Attenborough's The Life of Birds, BBC Video) continuously, without audio, for the next 8 hrs until lights off. Although the pigeons usually oriented their uncapped eye towards the monitor (Figure 1), occasionally they had to be re-positioned by the experimenter standing in front of the open cage door. During this period, the birds were also gently stimulated (by the experimenter) to stay awake whenever EEG-signs of sleep (i.e., slow waves) appeared in the hyperpallium or mesopallium of either hemisphere. Because pigeons housed in the laboratory spend around 50% of the last 8 hrs of the day asleep (Martinez-Gonzalez et al. 2008), the pigeons were deprived of 4 hrs of sleep. This sleep deprivation procedure has been shown to effectively reduce sleep to less than 10% of baseline values, and residual sleep is fragmented into short bouts of < 4 s (Martinez-Gonzalez et al. 2008). At lights off, the monitor was turned off, the eye-cap removed and the pigeons were allowed to sleep undisturbed for the next 12 hrs (i.e., 'recovery' sleep). Recordings concluded at lights on the following day.

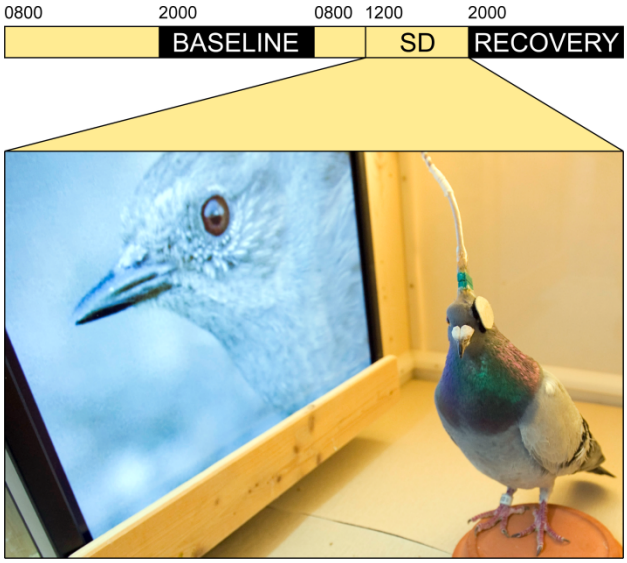

Figure 1. Experimental design: a 12 hr baseline night, 8 hr period of bihemispheric sleep deprivation with unilateral visual stimulation (SD) and a 12 hr recovery night. Photograph shows the experimental environment during the treatment (Copyright: Axel Griesch).

Processing EEG Signals

Eight unipolar EEG derivations were referenced to the cerebellum and digitally recorded at 200 Hz using commercially-available amplifiers (Embla® A10) and visualized in real-time with Somnologica Science v. 3.3.1 (Embla®, www.embla.com). The low-cut finite impulse response (FIR) filter was set at 0.5 Hz (-6 dB at 0.5 Hz and 0 dB at 0.8 Hz) and the high-cut anti-aliasing FIR filter was set at 100 Hz (-20 dB at 100 Hz and 0 dB at 80 Hz). The Embla® A10 system automatically applies the anti-aliasing filter after first sampling the data at 2000 Hz; the data is then down-sampled to 200 Hz. For sleep scoring and data analysis, we created four bipolar EEG derivations for the left and right hyperpallium (AP +13.0 – +9.25, L 2.0) and left and right mesopallium (AP +13.0 – +9.25, L 6.0).

Sleep Scoring

Twelve hr baseline and recovery night EEG recordings were scored for SWS and REM sleep using 4 s epochs with the aid of video recordings. An epoch was scored as SWS when the majority of the epoch showed slow (≤ 4 Hz) waves with amplitudes at least twice that of alert wakefulness. The appearance of slow waves was usually associated with behavioral signs of sleep onset (e.g., immobility, closure of at least one eye). All epochs were assessed for signs of SWS (i.e., 10,800 possible epochs of SWS per night). An epoch was scored as REM sleep when

at least 50% of the epoch showed high-frequency, low-amplitude EEG activity similar to that observed during alert wakefulness, but occurring with bilateral eye closure and behavioral signs of reduced muscle tone (e.g., drooping of the head). REM sleep was sampled at an interval of once per minute (i.e., 720 possible epochs of REM sleep per night), as simulations demonstrated that this sampling interval yielded REM sleep values similar to continuous scoring (see Supplementary Material for details).

Spectral Analyses

Fast Fourier transforms were performed on epochs of SWS and expressed as power density in 0.39 Hz bins from 0.78 – 25.00 Hz using Somnologica Science v. 3.3.1. Epochs containing artifacts and transitional epochs (i.e., > 2 < 4 s of SWS) were excluded from all spectral analyses. Spectral power density was calculated for each quarter of the baseline and recovery nights and expressed as a percentage of the 12 hr baseline night SWS mean per frequency bin.

Wave Slopes

The slope of slow waves during SWS has been used as a measure of synaptic strength (Esser et al. 2007, Riedner et al. 2007, Vyazovskiy et al. 2007, Bersagliere and Achermann 2010, Kurth et al. 2010, Leemburg et al. 2010), because steeper slopes are thought to reflect more synchronous alternations between up and down states of the slow oscillation resulting from increased synaptic strength (Esser et al. 2007, Vyazovskiy et al. 2009). Accordingly, slow waves are steeper (synapses are stronger) following extended periods of wakefulness in mammals (Vyazovskiy et al. 2007, Bersagliere and Achermann 2010, Kurth et al. 2010, Leemburg et al. 2010). To quantify potential changes in wave slope in response to unilateral visual stimulation during enforced wakefulness, we used the filter settings and definitions of Vyazovskiy and colleagues (2007). EEG signals were band-pass filtered using the MATLAB® (The Math Works, Inc., www.mathworks.com) function ‘bandpass’ from the Filter Design toolbox (function parameters: band-pass: 0.5 – 4.0 Hz, band-stop: < 0.1 Hz, > 10 Hz, rippel in band-pass 3 dB, attenuation in band-stop 20 dB). This filter exploits a Chebyshev Type II filter design (MATLAB®, function ‘design’ with parameter ‘Cheby2’). The filter was applied twice, left to right and right

to left (function 'filter' from the Filter Design toolbox), in order to maintain zero phase shift of the transform. The up slope (change in amplitude per duration from one negative peak to the next positive peak) and the down slope (slope from the positive peak to the next negative peak) were calculated from these band-pass filtered EEG signals. These calculations were repeated for each wave in an epoch and expressed as an epoch mean. Mean up and down slopes were calculated for the first quarter of the baseline and recovery nights, and expressed as a percentage of the all-night baseline mean.

Eye State

Pigeons can keep one eye open during SWS, a behavior associated with lower SWA in the contralateral hemisphere (Rattenborg et al. 2001). To determine if any asymmetries in the level of SWA on the recovery night were due to changes in unilateral eye opening, we examined instantaneous bilateral eye state at the start of each minute for epochs scored as SWS during the first quarter of the baseline and recovery nights, when the treatment effect is expected to be greatest.

Statistical Analyses

We conducted one, two or three-way repeated measures analysis of variance (rmANOVA) with factors variously as 'night' (baseline, recovery), 'brain region' (left, right hemisphere), 'quarter of night' and 'frequency'. Significant rmANOVAs were followed up by paired t-tests to determine the level(s) at which significance ($\alpha = 0.050$) was reached. N = 7 birds for all comparisons, except those involving the visually-deprived hyperpallium on the recovery night where N = 6 due to a technical problem. Wave slope and eye state analyses were conducted using paired t-tests. Statistical analyses were performed in SYSTAT 10 (©SPSS, Inc., www.systat.com).

Results

We present results on potential (i) changes in the amount of SWS and REM sleep across the baseline and recovery nights, (ii) changes in SWS-related spectral power density within the hyperpallium and mesopallium, (iii) changes in the slope of slow waves during SWS and (iv) changes in the occurrence of unilateral eye opening.

Amount of SWS and REM Sleep

The amount of SWS and REM sleep on the baseline and recovery nights was similar to those observed in a previous study on sleep regulation in pigeons (Martinez-Gonzalez et al. 2008). Specifically, the percentage of SWS decreased across the baseline ($F = 6.514$, $df = 3,18$, $P = 0.004$) and recovery ($F = 5.390$, $df = 3,18$, $P = 0.008$) nights, but did not differ between the two nights ($F = 1.732$, $df = 1,42$, $P = 0.195$; baseline night mean ± S.E. = 77.56 ± 1.69%, recovery night = 76.08 ± 1.95%). Conversely, the amount of REM sleep increased across the baseline ($F = 13.023$, $df = 3,18$, $P < 0.001$) and recovery ($F = 7.049$, $df = 3,18$, $P = 0.003$) nights, and there was significantly more REM sleep on the recovery night ($F = 6.789$, $df = 1,42$, $P = 0.013$; baseline night mean ± S.E. = 11.69 ± 1.33%, recovery night = 13.83 ± 0.49%), largely due to more REM sleep in the last quarter of the recovery night ($P = 0.012$), reflecting REM sleep homeostasis (Tobler and Borbély 1988, Rattenborg et al. 2009, Tobler 2011).

Spectral Power Density

When presenting results from spectral analyses, we begin with the hyperpallium followed by the mesopallium. Within a region of one hemisphere, we first compare data between the baseline and recovery nights followed by quantification of the degree of symmetry between the brain regions contralateral and ipsilateral to the uncapped eye.

Stimulated Hyperpallium: Spectral power density differed between the baseline and recovery nights in the stimulated hyperpallium ($F = 464.226$, $df = 1,3397$, $P < 0.001$). Specifically, 1.17 – 13.28 Hz power density was significantly higher during the first quarter of the recovery night (Figure 2). Power in the 0.78 – 4.69 Hz bandwidth (i.e., that which most closely approximates

SWA as typically defined in mammals) decreased across the recovery night ($F = 11.897$, $df = 3,261$, $P < 0.001$; Figure 2). Despite this decline, ≤ 2.73 Hz activity remained significantly higher than baseline throughout most of the recovery night (Figure 2). Circa 7.81 – 14.06 Hz power density was higher across the entire recovery night (Figure 2).

Visually-deprived Hyperpallium: Unlike the stimulated hyperpallium where the most predictive factor in the rmANOVA was 'night' (baseline or recovery), the strongest determinant of power density for the visually-deprived hyperpallium was 'quarter of night' ($F = 191.381$, $df = 3,3149$, $P < 0.001$). Accordingly, power density was not significantly different from baseline at most frequency bins across the recovery night, although in the second, third and fourth quarters 21.09 – 25.00 Hz activity was reduced relative to baseline (Figure 2).

Inter-hyperpallium: Across the baseline night, power density was not significantly asymmetric between the left and right hyperpallia ($F < 0.001$, $df = 1,3397$, $P = 0.996$; Figure 2). In contrast to this symmetry, the left and right hyperpallia showed a significant asymmetry during the recovery night ($F = 344.964$, $df = 1,3149$, $P < 0.001$). Specifically, there was a significant asymmetry in low-frequency (1.17 – 4.69 Hz) power density between the left and right hyperpallia, with the stimulated hyperpallium showing greater power (Figure 2). The magnitude of this low-frequency asymmetry attenuated across the recovery night (Figure 2). In the last half of the recovery night, power in some faster frequencies (> 6.25 Hz) was also significantly asymmetrical (Figure 2).

Mesopallium Contralateral to the Stimulated Eye: Spectral power density in the mesopallium contralateral to the stimulated eye differed between the baseline and recovery nights ($F = 380.673$, $df = 1,3397$, $P < 0.001$). Low-frequency (1.95 – 2.73 Hz) power density was significantly elevated during the first quarter of recovery sleep. Greater than circa 14.06 Hz activity was reduced across the recovery night (Figure 3).

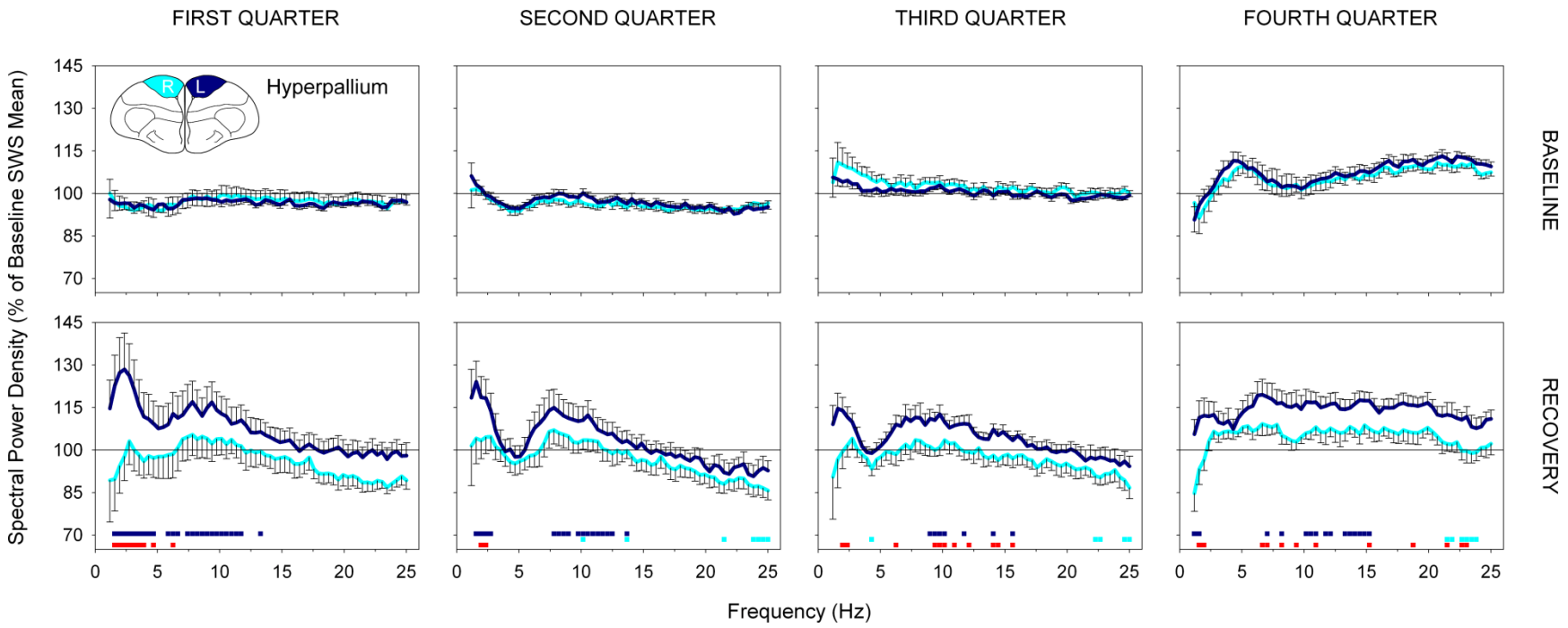

Figure 2. Spectral power density (0.78 – 25.00 Hz) across the four quarters of the baseline (top row) and recovery (bottom row) nights for the stimulated (dark blue) and visually-deprived (light blue) hyperpallia. Data are presented as mean ± S.E. Colored squares at the bottom of each recovery night plot reflect a significant pairwise comparison between the baseline and recovery night of the stimulated (dark blue) and visually-deprived (light blue) hyperpallia; red squares denote a significant asymmetry between the left (L) and right (R) hyperpallia during recovery sleep. Note the broad symmetry across the baseline night, and the decreasing low-frequency (< 5 Hz) asymmetry across the recovery night. Inset: frontal view of a transverse section through the cerebrum of a pigeon highlighting the hyperpallium.

Mesopallium Contralateral to the Deprived Eye: Spectral power density likewise differed between the baseline and recovery nights in the mesopallium contralateral to the deprived eye ($F = 13.661$, $df = 1,3397$, $P < 0.001$). The mean increase in low-frequency (1.17 – 3.13 Hz) activity during the first quarter of the recovery night did not reach statistical significance ($P < 0.100$), but was significant (≤ 1.56 Hz) during the second quarter (Figure 3). Power density of higher frequencies (circa 6.25 – 10.55 Hz) was also significantly elevated during recovery sleep (Figure 3).

Inter-mesopallium: Power density was not significantly different between the left and right mesopallia during baseline sleep ($F = 0.142$, $df = 1,3397$, $P = 0.707$; Figure 3). During the recovery night, however, despite a symmetric increase in < 3 Hz power density (Figure 3), the mesopallium contralateral to the stimulated eye responded differently to treatment than the mesopallium contralateral to the deprived eye ($F = 215.905$, $df = 1,3397$, $P < 0.001$). The mesopallium contralateral to the stimulated eye showed lower 6.25 – 17.58 Hz power density during the first quarter; this asymmetry was no longer detected in the fourth quarter (Figure 3).

Wave Slopes

Hyperpallium: The hyperpallium showed no significant asymmetry for the up ($P = 0.275$) or down ($P = 0.197$) slopes during the first quarter of the baseline night (Figure 4). During recovery sleep, however, the slope of slow waves in the stimulated hyperpallium was significantly steeper relative to baseline (up slope: $P = 0.027$, down slope: $P = 0.020$), but in the visually-deprived hyperpallium, the slope of slow waves was not significantly different from baseline (up slope: $P = 0.549$, down slope: $P = 0.271$; Figure 4). Consequently, there was a significant asymmetry in wave slope between the stimulated and visually-deprived hyperpallia in both the up ($P = 0.006$) and down ($P = 0.017$) slopes, with waves in the stimulated hyperpallium showing steeper slopes (Figure 4).

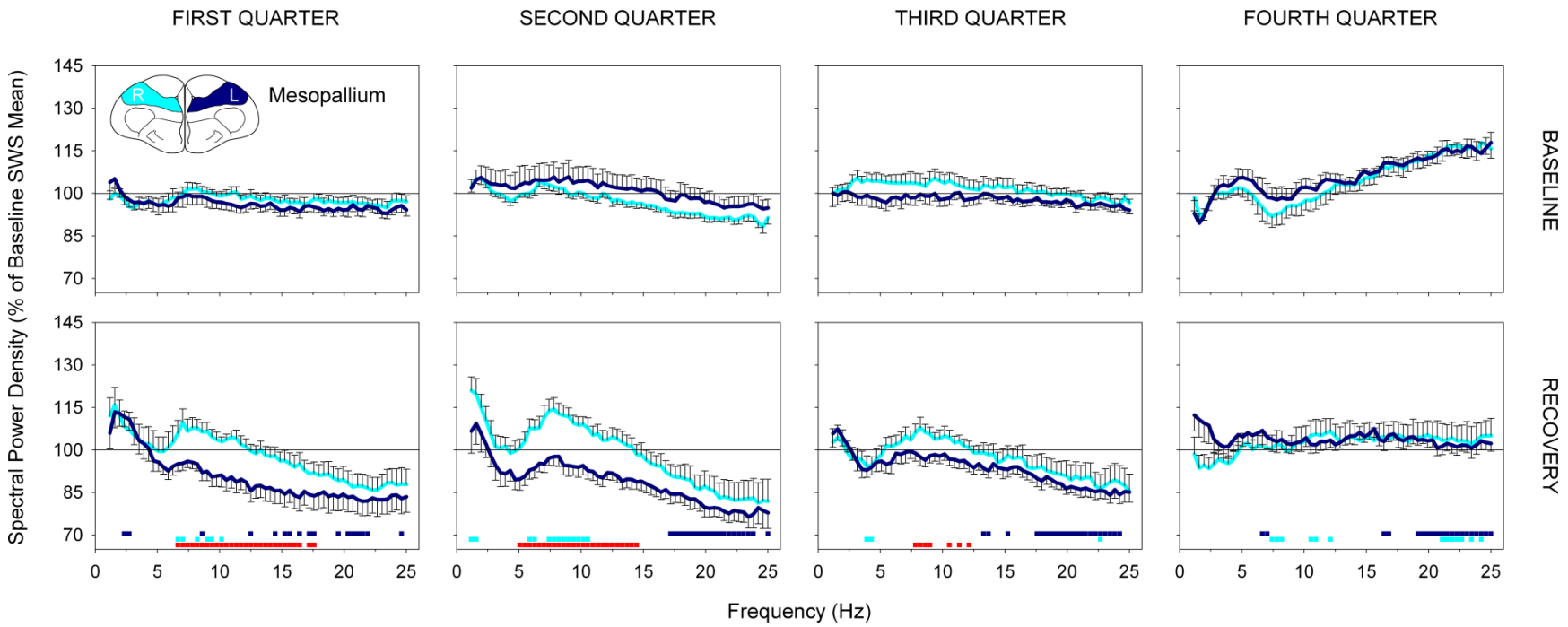

Figure 3. Spectral power density (0.78 – 25.00 Hz) across the four quarters of the baseline (top row) and recovery (bottom row) nights for the mesopallium contralateral to the stimulated eye (dark blue) and the deprived eye (light blue). Data are presented as mean ± S.E. Colored squares at the bottom of each recovery night plot reflect a significant pairwise comparison between the baseline and recovery night of the mesopallium contralateral to the stimulated eye (dark blue) and deprived eye (light blue); red squares denote a significant asymmetry between the left (L) and right (R) mesopallia during recovery sleep. Note the broad symmetry across the baseline night, and the < 5 Hz symmetry across the recovery night. Inset: frontal view of a transverse section through the cerebrum of a pigeon highlighting the mesopallium.

Mesopallium: Wave slopes were not significantly asymmetric between the left and right mesopallia during the first quarter of the baseline night (up slope: P = 0.884, down slope: P = 0.811; Figure 4). The up (P = 0.026) and down (P = 0.056) slopes increased during recovery sleep in the mesopallium contralateral to the stimulated eye, but the mean increase in slope in the mesopallium contralateral to the deprived eye was not significantly higher than baseline for the up (P = 0.143) or down (P = 0.136) slope (Figure 4). Nevertheless, wave slope was not significantly asymmetric between the left and right mesopallia during the first quarter of the recovery night (up slope: P = 0.845, down slope: P = 0.898; Figure 4).

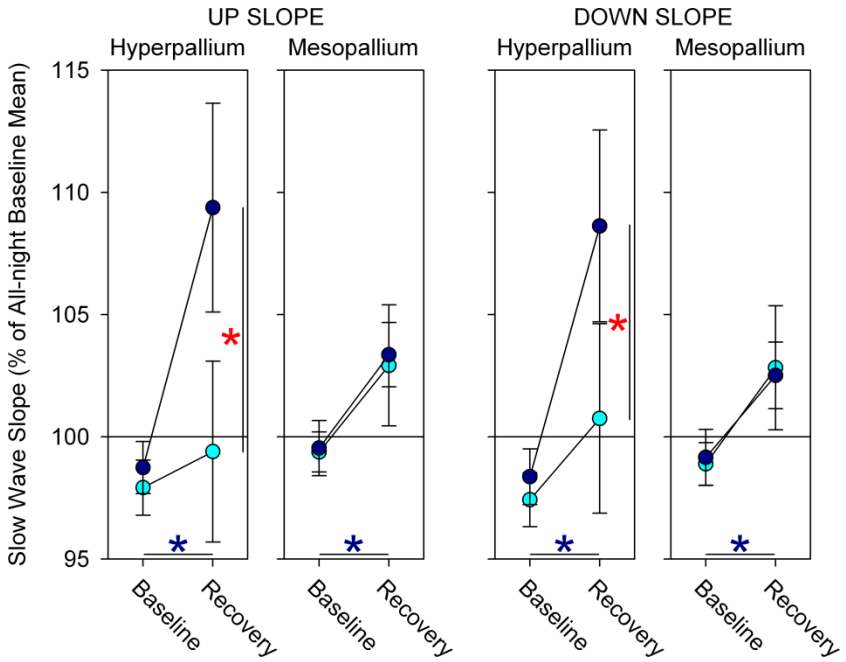

Figure 4. Up and down slopes of slow waves in the hyper- and mesopallium contralateral to the stimulated eye (dark blue) and deprived eye (light blue) during the first quarter of the baseline and recovery night. Data are presented as mean ± S.E. Significant changes in slope between the baseline and recovery nights are marked with an asterisk (contralateral to the stimulated eye in dark blue, contralateral to the deprived eye non-significant); significant asymmetries between the left and right hemisphere for a given region are denoted by a red asterisk. Note the asymmetry between the stimulated and visually-deprived hyperpallia during recovery sleep, with the stimulated hyperpallium showing steeper slopes, and the symmetric mean increase in the mesopallium.

Does this asymmetry in wave slope arise simply because of asymmetries in other wave parameters? That is, waves in the stimulated hyperpallium could be steeper because waves of greater SWA, higher amplitude or shorter period might be constrained to rise and fall more quickly (Riedner et al. 2007, Vyazovskiy et al. 2007, Bersagliere and Achermann 2010). To address this question, we matched slope and SWA data for the stimulated and visually-deprived hyperpallia. It is clear from Figure 2 that the stimulated hyperpallium exhibited high SWA values not found in the visually-deprived hyperpallium. Hence, we excluded the highest 20% of these SWA values, effectively removing the once-significant asymmetry in SWA between the left and right hyperpallia ($P = 0.666$). Despite now showing no significant inter-hyperpallial asymmetry in SWA, a significant asymmetry persisted in the up slope ($P = 0.022$) with steeper slopes still found in the stimulated hyperpallium, although this asymmetry was no longer significant for the down slope ($P = 0.112$). Next, the inter-hyperpallial asymmetry in wave slope was associated with a similar asymmetry in slow wave amplitude ($P = 0.004$) that arose because waves in the stimulated hyperpallium were of higher amplitude relative to baseline ($P = 0.049$) and waves in the visually-deprived hyperpallium were not ($P = 0.444$). By excluding the highest 20% of amplitude values in the stimulated hyperpallium, we removed this asymmetry ($P = 0.446$); however, the resulting asymmetry in wave slope was only marginally significant for the up slope ($P = 0.069$) and non-significant for the down slope ($P = 0.524$). Lastly, we calculated slow wave period in the stimulated and visually-deprived hyperpallia during recovery sleep to see if an asymmetry in period could explain the asymmetry in slope; however, no asymmetry in period was identified ($P = 0.858$).

Eye State

The proportion of SWS spent with unilateral eye opening did not differ significantly between the first quarter of the baseline and recovery nights (left eye closed / right eye open baseline mean ± S.E. = 17.99 ± 5.22%, recovery 26.32 ± 7.53%, $P = 0.238$; left eye open / right eye closed baseline 13.87 ± 6.23%, recovery 1.17 ± 0.62%, $P = 0.088$). The occurrence of bilateral eye closure during SWS likewise did not differ between the baseline and recovery nights (baseline 43.93 ± 11.02%, recovery 51.43 ± 9.83%, $P = 0.455$). Because only an increase in the proportion

of SWS spent with left eye open / right eye closed could influence the inter-hyperpallial asymmetry in SWA in the manner observed, this asymmetry in SWA was not due to changes in eye state. Moreover, this suggests that eye state alone may not be a good indicator of local sleep homeostasis, at least in pigeons under these conditions (Nelini et al. 2010).

Discussion

In this study, we provide the first electrophysiological evidence for local sleep homeostasis in the avian brain. Specifically, following unilateral visual stimulation during enforced wakefulness, SWA during recovery SWS was asymmetric in the hyperpallium – a primary visual processing region – with the greatest SWA observed in the hyperpallium contralateral to the stimulated eye. This inter-hyperpallial asymmetry appears to reflect a specific response to visual stimulation rather than a hemisphere-wide response (Lapierre et al. 2007), because the non-visual mesopallium showed a symmetric increase in SWA. This local effect is similar to those described in mammals (Kattler et al. 1994, Vyazovskiy et al. 2000, Huber et al. 2004, Yasuda et al. 2005a, Cajochen et al. 2008, Hanlon et al. 2009, Landsness et al. 2009, Määttä et al. 2010). Although many factors contribute to the local level of SWA (Krueger et al. 2008), recent studies suggest that increased SWA reflects synaptic potentiation (or strengthening) accrued during prior wakefulness in a use-dependent manner (Huber et al. 2007a). Accordingly, the SWA-related patterns identified here are mirrored by similar patterns in the slope of SWS-related slow waves, a potential marker of synaptic strength (Esser et al. 2007, Riedner et al. 2007, Vyazovskiy et al. 2007, Bersagliere and Achermann 2010, Kurth et al. 2010, Leemburg et al. 2010). The stimulated hyperpallium showed steeper slopes than those in the visually-deprived hyperpallium, independent of the level of SWA or wave period, while the mesopallium showed a symmetric increase in slope. However, the slope asymmetry in the hyperpallium was only marginally significant once the asymmetry in wave amplitude was taken into account, a finding that should be revisited with a larger sample size. In addition, other, more direct measures of synaptic potentiation (e.g., Liu et al. 2010) may be needed to confirm whether local increases in SWA truly reflect local potentiation in birds.

The lack of increased SWA or wave slope in the visually-deprived hyperpallium during recovery sleep may reflect the absence of a net change in synaptic strength. Indeed, in mammals, SWA can decrease locally in response to disuse alone, resulting from local synaptic depression (Huber et al. 2006). Along these lines, in an earlier study by our group using the same sleep deprivation protocol, but without unilateral visual stimulation, SWA increased symmetrically in both hemispheres (Martinez-Gonzalez et al. 2008), indicating that the reduction of visual input to the hyperpallium contralateral to the capped eye in the present study caused a reduction in SWA. SWA in mammals can also increase locally from baseline levels in response to time awake in the absence of sensory stimulation (Vyazovskiy et al. 2000). Specifically, rats subjected to enforced wakefulness and unilateral whisker removal, showed increased SWA during subsequent sleep in both the left and right barrel cortex (albeit to a lesser extent in the region contralateral to the cut whiskers; Vyazovskiy et al. 2000). Thus, the lack of change in SWA and wave slope in the visually-deprived hyperpallium could reflect the competing effects of decreased visual input and increased time awake, weakening and strengthening synapses, respectively. Overall, it appears that, as in mammals, SWA increases *and decreases* locally following use and disuse, respectively, during prior wakefulness in birds.

The response of frequencies faster than the SWA bandwidth was consistent with previous studies of sleep regulation depending on the brain region considered. For instance, in the stimulated hyperpallium on the recovery night, SWS power density increased out to (at least) 14 Hz, including a dip in activity around 5 – 6 Hz. Such patterns are not uncommon following sleep loss in mammals (Borbély et al. 1984, Tobler and Jaggi 1987, Tobler et al. 1990, Huber et al. 2000, Lesku et al. 2008) and birds (Jones et al. 2008a, Martinez-Gonzalez et al. 2008). However, the reason for the increase in higher frequencies is unknown. It has been proposed that such a response may reflect frequency-independent increases in neuronal synchrony due to a strengthening of synapses during prior wakefulness (Tononi and Cirelli 2006), but the activity in the mesopallium seemingly argues against this idea. While the low frequencies of the SWA bandwidth increased symmetrically in the mesopallium during recovery sleep, faster frequencies (6 – 18 Hz) were asymmetric between the left and right mesopallia,

with the mesopallium contralateral to the stimulated eye showing lower activity. Although the functional significance of this asymmetry remains unclear, three points are noteworthy. First, it is highly reproducible, being present in all birds. Second, the asymmetry is caused by two divergent patterns: an increase in 6 – 11 Hz activity in the mesopallium contralateral to the deprived eye and a decrease in 12 – 18 Hz activity in the mesopallium contralateral to the stimulated eye. Third, there is no clear relationship between the magnitude of the low-frequency asymmetry in the hyperpallium and the magnitude of the higher frequency asymmetry in the mesopallium, suggesting that the two phenomena are unrelated. Future studies employing methods for measuring pallial activity with higher spatial resolution (e.g., high density depth local field potentials or functional magnetic resonance imaging) may help elucidate the source and function of this interesting phenomenon in pigeons, as well as the increase in frequencies faster than SWA in mammals.

Several non-mutually exclusive factors could account for our finding of local sleep homeostasis in birds. Sleep regulatory substances, such as tumour-necrosis factor α and interleukin-1β, produced in response to waking neuronal activity, increase SWA during subsequent sleep in mammals (Yoshida et al. 2004, Yasuda et al. 2005b, Krueger et al. 2008) and might increase the level of SWA in birds as well. Local brain metabolism may also play a role in the use-dependent nature of SWA, as brain regions used more extensively during prior wakefulness can deplete their local stores of glycogen (Swanson et al. 1992), increasing extracellular adenosine, which in turn may increase SWA (Benington and Heller 1995, Scharf et al. 2008, Greene and Frank 2010). In addition, synaptic potentiation accrued during prior waking brain use may increase neuronal synchrony, and thereby SWA, locally during subsequent sleep (Tononi and Cirelli 2003, 2006, Huber et al. 2007a).

All of these factors may account for our results, but the latter ‘synaptic homeostasis hypothesis’ (Tononi and Cirelli 2003, 2006) is particularly appealing, because it provides an explicit mechanism for both the increase in SWA resulting from brain use, and the decrease in SWA with time asleep. Evidence for synaptic homeostasis in mammals and birds is as follows.

In response to brain stimulation during wakefulness, synapses strengthen in mammals, based on (1) molecular markers of potentiation (Cirelli et al. 2004, Vyazovskiy et al. 2008), (2) the frequency and amplitude of miniature excitatory postsynaptic potentials (Liu et al. 2010), (3) the slope and amplitude of cortical evoked responses (Vyazovskiy et al. 2008), (4) the slope of SWS-related slow waves (Esser et al. 2007, Riedner et al. 2007, Vyazovskiy et al. 2007, Bersagliere and Achermann 2010, Kurth et al. 2010, Leemburg et al. 2010), (5) the synchrony of transitions between up- and down-states among neurons (Vyazovskiy et al. 2009) and (6) a large-scale computer model of the thalamocortical system (Olcese et al. 2010). Synapses also appear to strengthen in a use-dependent manner in awake birds (this study), at least to the extent that slow wave slope reflects synaptic strength. Increased connectivity results in more synchronous slow oscillations during subsequent sleep (Vyazovskiy et al. 2009, see also Esser et al. 2007), as evidenced by a local increase in SWA in mammals (Kattler et al. 1994, Vyazovskiy et al. 2000, Huber et al. 2004, 2007a, Yasuda et al. 2005a, Cajochen et al. 2008, Hanlon et al. 2009, Landsness et al. 2009, Määttä et al. 2010) and birds (this study). In mammals, such plastic changes are facilitated (in part) by the potentiating and synaptogenic action of brain-derived neurotrophic factor (BDNF) expressed during wakefulness (Huang and Reichardt 2001), which increases the level of SWA during subsequent sleep (Huber et al. 2007b, Faraguna et al. 2008, Thompson et al. 2010). Although it is unknown whether a similar relationship exists between BDNF and SWA in birds, the expression of other genes involved in long-term potentiation is elevated during wakefulness when compared to sleep in the forebrain of white-crowned sparrows (*Zonotrichia leucophrys gambelii*, Jones et al. 2008b).

Under the synaptic homeostasis hypothesis, the synchrony of the slow oscillation is not only thought to reflect potentiation, but is also hypothesized to be the mechanism by which SWS reduces synaptic strength (Tononi and Cirelli 2006). Stimulation at a frequency similar to the slow oscillation induces long-term depression (Kemp and Bashir 2001, Collingridge et al. 2010) and so may the SWS-related burst firing of action potentials (Czarnecki et al. 2007, Olcese et al. 2010). The neuromodulatory milieu during SWS is also conducive to depression (Cirelli et al. 2005, Tononi and Cirelli 2006) and genes involved in this process are preferentially

expressed during sleep in mammals (Cirelli et al. 2004) and birds (Jones et al. 2008b). Regardless of the specific mechanism(s) by which SWS facilitates downscaling, several lines of evidence suggest that synapses weaken during SWS (Esser et al. 2007, Riedner et al. 2007, Vyazovskiy et al. 2007, 2008, 2009, Bersagliere and Achermann 2010, Kurth et al. 2010, Leemburg et al. 2010, Liu et al. 2010, Olcese et al. 2010). In addition to maintaining synaptic weights at an optimum level, synaptic homeostasis can also account for some of the enhancements in performance on various cognitive tasks observed in mammals post-sleep (Huber et al. 2004, Hanlon et al. 2009, Landsness et al. 2009, Määttä et al. 2010), perhaps by increasing the signal-to-noise ratio of relevant circuits (Hill et al. 2008, Olcese et al. 2010). Although recent evidence suggests that sleep plays a role in imprinting (Solodkin et al. 1985, Jackson et al. 2008), auditory discrimination (Brawn et al. 2010) and song learning (Dave and Margoliash 2000, Derégnaucourt et al. 2005, Crandall et al. 2007, Shank and Margoliash 2009, Gobes et al. 2010, Rauske et al. 2010) in birds, the role (if any) of synaptic downscaling in these processes is unknown.

If the interpretation above is correct, then slow oscillation-mediated synaptic downscaling may be a unique feature of mammalian and avian sleep (Rattenborg et al. 2009). Although downscaling also occurs in sleeping *Drosophila melanogaster* (Donlea et al. 2009, Gilestro et al. 2009), pointing to an 'ancient' origin for this sleep function, fruit flies appear to lack the mammal (or bird)-like slow oscillation during sleep (Nitz et al. 2002, van Swinderen 2006), suggesting that *Drosophila* have a downscaling mechanism unrelated to synchronous low-frequency neuronal activity. Understanding the reason for the different mechanism may provide insight into whether slow oscillation-mediated downscaling serves an additional function not found in flies, or a more efficient means for downscaling in relatively complex brains. Indeed, the highly interconnected brains of mammals and birds (Medina and Reiner 2000, Rattenborg 2006, 2007, Suárez et al. 2006, Medina and Abellán 2009), capable of performing complex cognition (Emery and Clayton 2005, Jarvis et al. 2005, Butler 2008), may depend on slow oscillation-mediated downscaling to maintain optimal functioning (Rattenborg

et al. 2009); however, further study is needed to resolve this important issue in the evolution of sleep and cognition.

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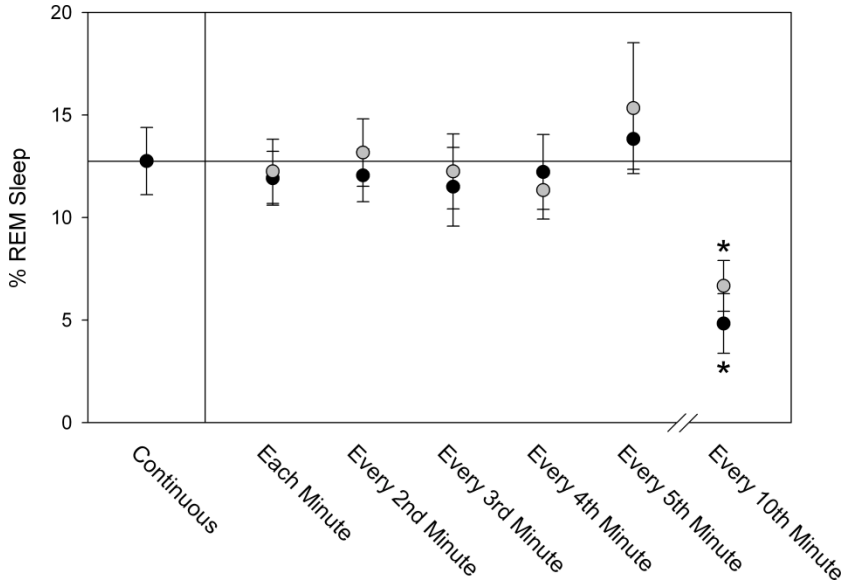
Supplementary Data

Avian REM sleep differs from mammalian REM sleep most conspicuously by its short duration (usually < 8 s) and large number of episodes per 24 hr day (hundreds). Each short episode of EEG activation must be confirmed as REM sleep (opposed to a brief period of alert wakefulness) using video recordings looking for behavioral signs of muscle atonia; the electromyogram is not a reliable indicator of REM sleep in birds. This procedure is a time-consuming process, and any sampling performed to reduce the number of possible REM sleep epochs would only reduce the time needed to accurately score avian REM sleep. Accordingly, some sleep studies in birds have sampled REM sleep by scoring only the first epoch of each minute (Rattenborg et al. 1999, 2004). Although this sampling regime was validated by those researchers (N. C. Rattenborg, *personal communication*), the verification was not published.

Here, we provide results from a simulation to determine the validity of various sampling intervals for REM sleep using the continuously-scored EEG recordings of Martinez-Gonzalez and colleagues (2008; N = 5 pigeons). REM sleep occupied 12.75 ± 1.64% (mean ± S.E.) of the 12 hr baseline night when scored continuously (Supplementary Figure 1). When scored at the top of each minute, the percentage of the baseline night devoted to REM sleep remained statistically unchanged relative to continuous scoring ($P > 0.24$; Supplementary Figure 1). There was likewise no significant difference when sampling at the top of every second, third, fourth or fifth minutes (all $P > 0.20$; Supplementary Figure 1). Sampling at the top of every tenth minute, however, resulted in a significantly inaccurate estimate of the amount of REM sleep ($P < 0.02$; Supplementary Figure 1). These basic results were repeatable using a +30 s offset to each sampling interval (Supplementary Figure 1). Scoring at the top of every fifth minute is as good as continuous scoring, but we nevertheless scored the top of each minute to maintain consistency with some previous sleep studies in birds (Rattenborg et al. 1999, 2004).



Supplementary Figure 1. Accuracy of estimating the percentage of a 12 hr night devoted to REM sleep (%REM sleep) when scored continuously (left-most black circle) by sampling the first epoch at the top of each minute, or every second, third, fourth, fifth or tenth minute (black circles). To determine the robustness of these estimations, further simulations were performed with a +30 s offset (grey circles). Only when sampling every tenth minute did the %REM sleep estimate differ significantly from continuous scoring (*$P < 0.02$). Data presented as mean ± S.E.

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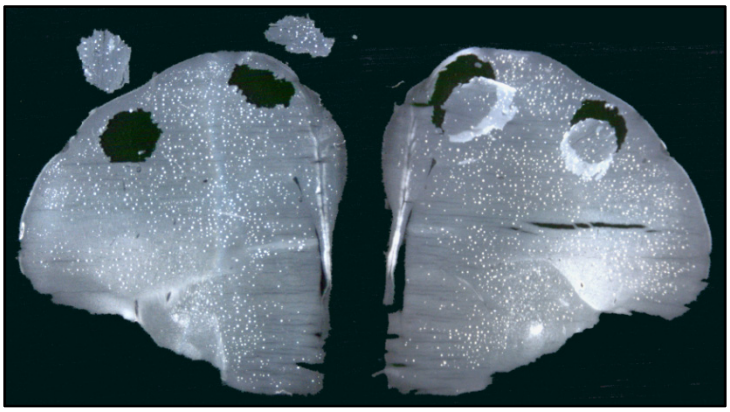
CHAPTER 5

Molecular Correlates of Local Sleep in the Pigeon (*Columba livia*)



Lesku JA, Dittrich F, Hertel M, Frankl C and Rattenborg NC. *In preparation*.

Abstract

Mammals and birds are the only animals known to engage in slow wave sleep (SWS) and rapid eye movement (REM) sleep. The level of SWS-related slow wave activity (SWA, 0.5 – 4.5 Hz power density) increases with prior waking brain use and decreases with time asleep. In mammals, brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (Arc) may mediate the increase in SWA. Although SWA in birds also increases following waking brain use, it is unclear whether BDNF and Arc contribute to this process, if at all. Using reverse transcription polymerase chain reaction, we measured the level of BDNF mRNA (*BDNF*) and Arc mRNA (*Arc*) in the hyperpallium and mesopallium of pigeons (*Columba livia*) in three conditions: at the end of the day, in the middle of the night and following unilateral visual stimulation during enforced wakefulness. *Arc* was higher at the end of the day relative to the middle of the night in both brain regions, and increased further following sleep loss; no asymmetry in *Arc* was observed in the hyper- or mesopallia following unilateral visual stimulation. These results suggest that *Arc* reflects the duration of preceding wakefulness, independent of waking activities. Conversely, *BDNF* was lowest at the end of the day and higher in the middle of the night in both brain regions. Moreover, *BDNF* increased asymmetrically in the hyperpallium, but counter to expectations derived from work on mammals, the increase was greater in the visually-deprived hyperpallium. These *BDNF* results suggest that, unlike in mammals, *BDNF* may not be related to waking brain use in pigeons, although additional studies are needed.

Introduction

Brain activity in sleeping mammals and birds alternates between two states, slow wave sleep (SWS) and rapid eye movement (REM) sleep. During SWS, neuronal membrane potentials alternate at ~ 1 Hz between a depolarized (up) state with action potentials and hyperpolarized (down) state without (Steriade et al. 1993, Reiner et al. 2001). This 'slow oscillation' is synchronized among neurons by cortico-cortical connectivity (Amzica and Steriade 1995, Vyazovskiy et al. 2009) and manifests in the electroencephalogram (EEG) as high amplitude slow (< 4 Hz) waves (Riedner et al. 2007), typically quantified as slow wave activity (SWA, i.e.,

0.5 – 4.5 Hz power density). The level of SWA is elevated following extended periods of wakefulness (reviewed in Borbély 2001, Tobler 2011), arising in part from synaptic potentiation (strengthening) during waking brain stimulation or use (Cirelli et al. 2004, Esser et al. 2007, Huber et al. 2007a, Riedner et al. 2007, Vyazovskiy et al. 2007, 2008, 2009, Hanlon et al. 2009, Bersagliere and Achermann 2010, Kurth et al. 2010, Leemburg et al. 2010, Liu et al. 2010, Olcese et al. 2010, Lesku et al. 2011). In mammals, several lines of evidence suggest that the increase in SWA is mediated by the action of proteins involved in activity-dependent neural plasticity, such as brain-derived neurotrophic factor (BDNF, Cirelli et al. 2004, Huber et al. 2007b, Faraguna et al. 2008, Thompson et al. 2010) and perhaps also activity-regulated cytoskeleton-associated protein (Arc, Huber et al. 2007b, Hanlon et al. 2009). Notably, Faraguna et al. (2008) microinjected BDNF and BDNF blockers unilaterally into the frontal cortex of awake rats, which caused a local increase and decrease, respectively, in SWA during subsequent SWS, suggesting that BDNF is causally involved in increasing SWA (see also Huber et al. 2007b). Although avian SWA also increases following waking brain use (Rattenborg et al. 2009, Lesku et al. 2011), whether this increase is mediated by molecular mechanisms similar to those in mammals is unknown.

We recently demonstrated that unilateral visual stimulation during enforced wakefulness increases SWA only in the hyperpallium stimulated during the deprivation procedure (Lesku et al. 2011). This inter-hyperpallial asymmetry in SWA was a specific response to the visual stimulation during prior wakefulness, as an asymmetry in the non-visual mesopallium was not observed. Here, we investigate the possible contributions of BDNF mRNA (*BDNF*) and Arc mRNA (*Arc*) to this local effect on SWA in the pigeon (*Columba livia*).

Methods

Pigeons were housed individually in wooden boxes (79 cm length x 60 cm width x 60 cm height) in a room maintained on a 12:12 light:dark photoperiod. Food and water were available *ad libitum*. A flatscreen computer monitor (41 cm length x 34 cm width) was mounted on one side of the cage. All methods were approved by the Government of Upper Bavaria.

Groups

Birds were randomly assigned to one of three groups. Pigeons experienced an undisturbed day and were euthanized either just before the lights turned off (Group 1, 'awake' baseline) or 6 hrs after lights-off (Group 2, 'sleep' baseline). The sleep-wake history of birds in Groups 1 and 2 differed, as pigeons housed under virtually-identical conditions are awake for 54% of the last 6 hrs of the day and only 17% during the first 6 hrs of the night (Martinez-Gonzalez et al. 2008). However, circadian time was also different between these groups. To distinguish between sleep and circadian effects on the level of *BDNF* and *Arc*, pigeons of Group 3 were sleep deprived and euthanized at the same time as Group 1. Specifically, each pigeon experienced an undisturbed 24 hr day, and 4 hrs into the following day, each bird was stimulated to stay awake whenever they showed behavioral signs of sleep (e.g., lack of movement, a slow eye blink, etc.). We have extensive experience sleep depriving pigeons with concurrent EEG recordings (Martinez-Gonzalez et al. 2008, Lesku et al. 2011) and hence, in the present study, were readily able to identify sleep onset by behavior alone. Just prior to the video presentation, the left eye of each bird was capped with a Velcro® ring (see Lesku et al. 2011 for details). During the presentation, the uncapped eye was oriented towards the monitor, which displayed moving, non-repetitive video of wild birds (David Attenborough's The Life of Birds, BBC Video). Group 3 pigeons were euthanized after 8 hrs of sleep deprivation with unilateral visual stimulation (i.e., just before lights-off). Thus, birds in Groups 1 and 3 were euthanized at the same circadian time, but birds in Group 3 were deprived of approximately 4 hrs of sleep (Martinez-Gonzalez et al. 2008) and the stimulated hyperpallium received heightened visual input and the visually-deprived hyperpallium received greatly reduced input (see Lesku et al. 2011).

Brain Processing and Reverse Transcription Polymerase Chain Reaction

At the appropriate time, a bird was removed from its enclosure, deeply anesthetized (5% isoflurane in 1 LPM O2 for < 1 min) and rapidly decapitated. The brain was removed quickly and snap frozen over liquid nitrogen. Transverse sections (40 µm) were made on a cryostat and tissues from the hyperpallium and mesopallium were micro-dissected manually under a stereomicroscope. Slices from which tissue was taken correspond to the location of EEG

electrodes in our previous study on sleep regulation in pigeons (Martinez-Gonzalez et al. 2008, Lesku et al. 2011). Total RNA was purified using the RNeasy Micro Kit and digested on a column with RNase-Free DNAse (Qiagen). Quality and yield of total RNA was determined on the Agilent 2100 bioanalyzer using the nanochip. Complementary DNA synthesis was carried out with the SuperScript III First-Strand Synthesis System for reverse transcription (RT) polymerase chain reaction (PCR) (Invitrogen), following the standard protocol using random hexamers and 60 ng of total RNA for each reaction. In each PCR run, the RT reactions were performed in duplicate and one non-RT control was included for each sample. For the determination of the relative amount of *BDNF* in each sample, equal volumes of RT and non-RT reactions were analyzed by real-time PCR using the Power SYBR Green PCR Master Mix (Applied Biosystems), with zebra finch specific primers designed against the prepro-sequence of *BDNF* (forward primer: 5'-CCC AAT GAA AGA AGC CAG TC-3'; reverse primer: 5'-TTC AAA AGT GTC TGC CAA CG-3') and β-actin (forward primer: 5'-AAC CGG ACT GTT TCC AAC AC-3'; reverse primer: 5'-CAC CTT CAC CGT TCC AGT TT-3') at a final concentration of 100 nM. The PCR reactions were run in duplicate for each RT and non-RT reaction in a final volume of 25 µl on a Mx3005P qPCR System (Stratagene) using the following thermal cycling protocol: 95 °C for 10 min, 45 cycles 95 °C for 30 sec, 60 °C for 1 min and 72 °C for 30 sec. A melting curve was generated at the end of each run to ensure product uniformity. For each sample the cycle threshold (Ct) was determined with RT and non-RT reactions and the ΔCt value was calculated (ΔCt = mean Ct for RT reaction – mean Ct for non-RT reaction). Based on the results of three PCR runs, an average ΔCt was calculated for each sample and normalized for the β-actin-mRNA level. Differences in the mean normalized expression level (Muller et al. 2002, Simon 2003) were tested for statistical significance ($\alpha = 0.050$) with paired t-tests. Data were log-transformed (when necessary) to meet the assumption of normality of residuals. N = 6 pigeons for all Groups except for the mesopallium data in Group 2 where N = 7. Statistical analyses were performed in Microsoft® Excel® 2007 (office.microsoft.com).

Results

Group 1 and 2: Diel Rhythm and Interhemispheric Symmetry

The level of *BDNF* was significantly higher in the middle of the dark phase of the photoperiod relative to the end of the light phase in the hyperpallium and mesopallium; *Arc* showed the opposite pattern (Figure 1). *BDNF* and *Arc* were symmetric in both brain regions in both baseline groups (Figure 1).

Group 3: Interhemispheric Asymmetry?

Unilateral visual stimulation during enforced wakefulness was effective at inducing an asymmetry in *BDNF* in the hyper- and mesopallium. *BDNF* in the stimulated hyperpallium was lower relative to the visually-deprived hyperpallium (Figure 1). This inter-hyperpallial asymmetry was present in all birds. An asymmetry was likewise identified in the mesopallium with the mesopallium contralateral to the stimulated eye showing greater *BDNF* than the mesopallium contralateral to the visually-deprived eye (Figure 1). Unlike the increase in *BDNF* in the left and right hyperpallia following unilateral visual stimulation (relative to Group 1), the mesopallium contralateral the visually-deprived eye showed less *BDNF* relative to baseline (Figure 1). *Arc* increased in both brain regions relative to that observed at the end of an undisturbed day; however, these increases were not significantly asymmetric (hyperpallium $P = 0.293$, mesopallium $P = 0.173$, Figure 1).

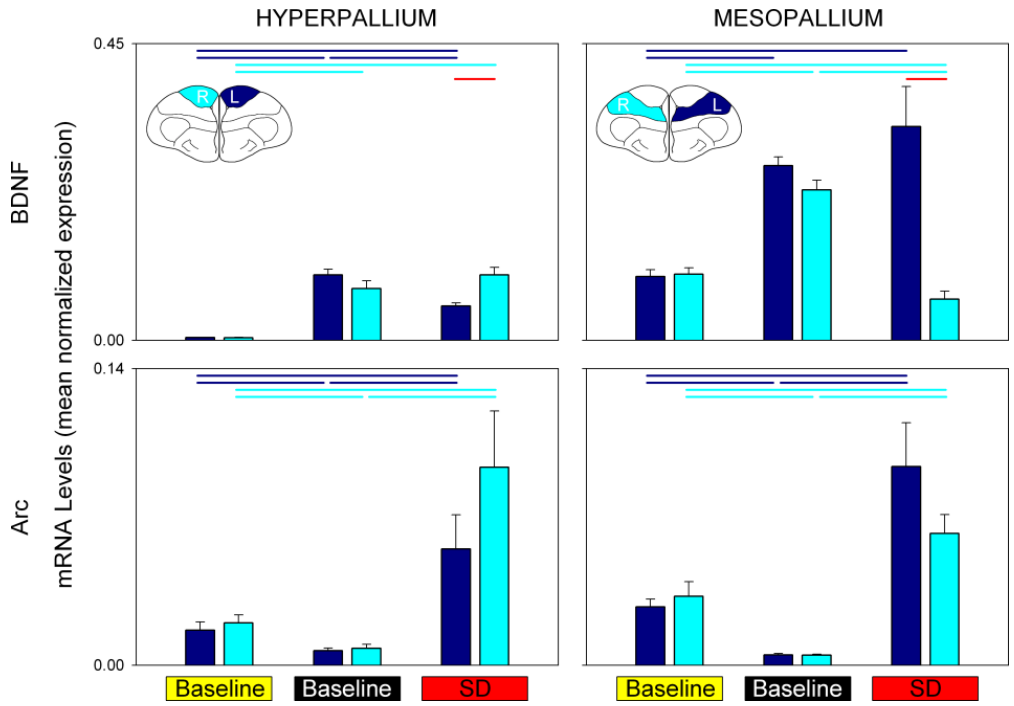


Figure 1. mRNA levels for *BDNF* and *Arc* in the left (dark blue) and right (light blue) hyperpallia and mesopallia under three conditions: at the end of an undisturbed day (yellow baseline), in the middle of the night (black baseline) and immediately following 8 hrs of daytime sleep deprivation (SD) with unilateral visual stimulation (red). In the latter group, the left (dark blue) and right (light blue) hemisphere is contralateral and ipsilateral, respectively, to the stimulated eye. Horizontal lines at the top of each plot reflect significant differences (a red line denotes a significance asymmetry within that brain region). Inset: frontal view of a transverse section through the cerebrum of a pigeon highlighting the hyperpallium (left panel) and mesopallium (right panel).

Discussion

Most of the patterns in the level of *BDNF* and *Arc* outlined above were unexpected. We address each in turn below.

BDNF mRNA was higher in the middle of the night than at the end of the day

BDNF is higher in awake rats, relative to asleep animals (Cirelli et al. 2004). Hence, we expected *BDNF* to be lower during the night, when pigeons sleep the most. The reason for this rat – pigeon difference is unclear, but may indicate that *BDNF* is regulated differently in mammals and birds. Unfortunately, we are unable to compare our results with the only other study of sleep-wake changes in gene expression in birds, because changes in *BDNF* were not reported in that study (Jones et al. 2008). Alternatively, it is possible that *BDNF* was up-regulated during REM sleep at night in (Group 2) pigeons (Martinez-Gonzalez et al. 2008). Indeed, another plasticity-related gene, *zif-268* (or *ZENK*), is up-regulated during REM sleep in rats (Ribeiro et al. 1999), although *BDNF* was not examined in that study. If *BDNF* is up-regulated during REM sleep in birds, then quantifying *BDNF* earlier in the night, when REM sleep is rare in pigeons (Martinez-Gonzalez et al. 2008, Lesku et al. 2011), might yield a mammal-like pattern of expression.

BDNF mRNA was higher in the visually-deprived hyperpallium relative to the stimulated hyperpallium following unilateral visual stimulation during enforced wakefulness

BDNF increases and decreases in a use- and disuse-dependent manner, respectively, in the visual cortex of rats (Castrén et al. 1992). Moreover, BDNF (protein) appears to be causally involved in increasing SWA in rats (Faraguna et al. 2008). In our previous study on sleep regulation in pigeons (Lesku et al. 2011), SWA increased only in the stimulated hyperpallium. For this reason, *BDNF* was expected to be higher in the stimulated hyperpallium – opposite to the pattern observed. Therefore, *BDNF* may be unrelated to waking brain use in pigeons; however, if true, it would still be unclear why *BDNF* was higher following enforced wakefulness than at the end of an undisturbed day.

BDNF mRNA was asymmetric in the (non-visual) mesopallium, with the higher level observed contralateral to the stimulated eye

As stated above, BDNF can increase SWA in mammals (Faraguna et al. 2008), but in our previous EEG study (Lesku et al. 2011), SWA increased symmetrically in the left and right mesopallia following unilateral visual stimulation during enforced wakefulness. The asymmetry in *BDNF* is unlikely to have been caused by the visual stimulation during the deprivation procedure *per se*, because visual input is transmitted principally by two ascending pathways neither of which project directly to the mesopallium in pigeons. The thalamofugal pathway projects from the retina to the contralateral nucleus opticus principalis thalami to the interstitialis hyperpallii apicale and hyperpallium densocellulare, which in turn project to the hyperpallium apicale; the tectofugal pathway projects from the retina to the contralateral optic tectum to the nucleus rotundus of the thalamus and then to the entopallium (Güntürkün et al. 1993). *BDNF* in the mesopallium contralateral the stimulated eye might be higher due to lateralized processing of the content of the film, because the mesopallium has been implicated in learning and memory (Timmermanns et al. 2000, Mehlhorn et al. 2010), some which is lateralized (e.g., Patel and Stewart 1988, Patel et al. 1988). However, currently the reason for this *BDNF* asymmetry in the mesopallium is unclear.

Unlike *BDNF*, patterns of *Arc* were more consistent with expectations based on studies on mammals. *Arc* was lower in the middle of the night (when pigeons are typically sleeping) than at the end of the day (when they have spent more time awake). This decrease at night is probably due to sleep rather than circadian time *per se*, because *Arc* increased above baseline levels (i.e., Group 1) following daytime sleep deprivation. These results are in accordance with the finding that *Arc* is higher during wakefulness, irrespective of the time of day, in the neocortex of rats (Cirelli et al. 2004, Huber et al. 2007b) and also in the forebrain of sparrows (Jones et al. 2008). Nevertheless, why the stimulated hyperpallium did not show more *Arc* than the visually-deprived hyperpallium is unclear (see Hanlon et al. 2009), unless the level of *Arc* in birds reflects time awake independent of waking activities.

The largely unexpected results reported above necessitate verification. For instance, another technique for quantifying levels of mRNA, such as *in situ* hybridization, could be employed, which provides greater spatial resolution per tissue sample (Gahr and Metzdorf 1997, Dittrich et al. 1999). In addition to increasing spatial resolution, adding more time points to visualize the time course of mRNA expression might reveal an (mammal-like) increase in mRNA early in the wake period and a decline during early sleep (see Huber et al. 2007b). A more direct approach, however, would be to locally apply BDNF protein to the hyperpallium and quantify the effect (if any) of subsequent SWA (as per Faraguna et al. 2008), given that BDNF protein, not mRNA, is the effector molecule in neurogenesis (Huang and Reichardt 2001, Numakawa et al. 2010). Such a direct assessment of the effect of BDNF on SWA would be particularly useful, because *BDNF* (mRNA) and BDNF (protein) levels have been shown to dissociate under certain conditions (Pollock et al. 2001, but see Karpova et al. 2010). Alternatively, proteins other than BDNF or Arc that increase SWA in mammals, such as tumor necrosis factor α and interleukin-1β (Krueger et al. 2008), might mediate the increase in SWA in birds. Lastly, we must also entertain the possibility that use-dependent changes in the level of SWA in birds do not reflect underlying changes in the strength of synapses, as they appear to in mammals (Cirelli et al. 2004, Esser et al. 2007, Huber et al. 2007a, Riedner et al. 2007, Vyazovskiy et al. 2007, 2008, 2009, Hanlon et al. 2009, Bersagliere and Achermann 2010, Kurth et al. 2010, Leemburg et al. 2010, Liu et al. 2010, Olcese et al. 2010). However, we found previously that the slope of SWS-related slow waves increased – a possible correlate of increased synaptic strength (Esser et al. 2007, Riedner et al. 2007, Vyazovskiy et al. 2007, Bersagliere and Achermann 2010, Kurth et al. 2010, Leemburg et al. 2010) – only in the stimulated hyperpallium, with a symmetric increase in wave slopes in the mesopallium, both of which argue against this idea (Lesku et al. 2011). Overall, this *BDNF* / *Arc* study provides several unexpected findings and more work is needed to fully understand them.

Acknowledgements

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General Discussion

At the outset of this dissertation, we set out to answer some fundamental questions about sleep in birds. How did avian sleep evolve? (Chapters 1 and 2) Is avian sleep regulated similar to mammals? (Chapters 3 – 5) What might birds tell us about shared sleep functions between mammals and birds? (Chapters 3 – 4) As a result of the studies presented herein, we have some answers to these questions. Of course, these answers create only new questions.

Evolution of REM Sleep

We conducted the first electrophysiological sleep study on ostriches – members of the most basal group of living birds. Surprisingly, ostrich sleep electrophysiology is in some respects unlike that observed in any (known) mammal or bird. Although the hyperpallium of sleeping ostriches exhibits unequivocal slow wave sleep (SWS); during rapid eye movement (REM) sleep, the hyperpallium alternates between (1) the 'typical' activated electroencephalogram characteristic of REM sleep in Neognathae birds and (2) atypical SWS-like slow waves. These slow waves can be said to occur during REM sleep because they occur with classical brainstem-generated signs of REM sleep, such as reduced muscle tone, head and eye movements, and can be temporally adjacent to typical REM sleep hyperpallial activation. This is interesting, because basal mammals do something similar: the egg-laying monotremes show only slow waves during sleep (Allison et al. 1972, Manger et al. 2002), but with eutherian-like REM sleep brainstem-generated phenomena (platypus, Siegel et al. 1999) or brainstem unit activity characteristic of REM sleep in eutherians (echidna, Siegel et al. 1996). Moreover, if one calculates the amount of REM sleep as periods of reduced muscle tone with head and eye movements, then ostriches have more REM sleep than any other bird, just as the platypus has more REM sleep than other mammals using similar criteria (Siegel et al. 1999, Siegel 2005). Thus, the pattern of REM sleep evolution in birds appears to be remarkably similar to REM sleep evolution in mammals (Figure 1). Importantly, sleeping reptiles do not show mammalian / avian-like forebrain activation during sleep (Siegel et al. 1998), and sleeping turtles do not show eutherian-like brainstem activity (Eiland et al. 2001), suggesting that the similarities in REM sleep between basal mammals and basal birds is due to evolutionary convergence (Rattenborg et al. 2009).

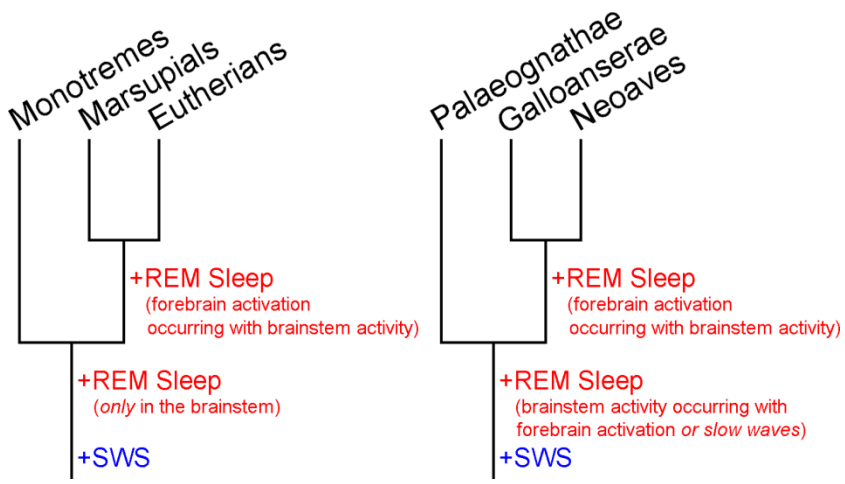


Figure 1. Cladogram for the three main groups of mammals (left) and birds (right) showing the appearance of slow wave sleep (SWS) and the different stages of rapid eye movement (REM) sleep evolution. Unequivocal SWS has been described in all mammalian and avian groups, suggesting that SWS was present in the most recent common ancestor to each group. 'Classical' REM sleep, characterized by forebrain activation with concomitant brainstem-generated phenomena (e.g., reduced muscle tone, eye movements) has been observed in marsupials and eutherians, Galloanserae and Neoaves, suggesting that it evolved only after the appearance of these more derived lineages. In the monotremes, REM sleep is a heterogeneous state with signs of REM sleep restricted to the brainstem occurring concurrently with electroencephalogram activity indicative of SWS. REM sleep in ostriches (Palaeognathae) alternates between this monotreme-like REM sleep state with that of 'classical' REM sleep, intimating a similar trajectory of REM sleep evolution in mammals and birds.

The presence of such a unique REM sleep state in ostriches raises several important questions about the evolution of sleep. What is the function of this state? Why was it inadequate for Neognathae birds? How does the brain alternate between the different types of REM sleep? The functional significance of REM sleep at the level of the brainstem in basal homeotherms is unclear, but may serve to warm the brainstem preparing it for wakefulness (Siegel 2011). Certainly identifying the function of this heterogeneous REM sleep state is important to our greater understanding of REM sleep, but it may be necessary to address more mechanistic questions first. For instance, the burst-pause pattern of brainstem activity during

REM sleep in eutherians is specific to REM sleep (Siegel et al. 1996, Siegel 2011). How brainstem and forebrain activity correlate in ostriches might provide insight how this state is generated and maintained, and, in turn, provide clues to its function. It would also be useful to determine whether the unique REM sleep state observed in ostriches is specific to ostriches, or to ratites, or to Palaeognathae birds in general. However, given the close-relatedness and gross similarities between ostriches and other large flightless ratites (Corfield et al. 2008), it is difficult to imagine why ostriches would be so different in their sleep neurophysiology.

Our work on ostriches – conducted on free-moving animals in a large naturalistic reserve – continues the push for electrophysiologically-based sleep research to move into more wild environments (Rattenborg et al. 2008). The study of sleep in the environmental and ecological context in which it evolved may provide more meaningful results than those obtained through a strict laboratory-based approach, at least in some animals (Calisi and Bentley 2009, Lesku et al. 2009). The eminent, early comparative animal sleep researcher, Truett Allison, said at a 1971 Congress in Belgium:

> "This symposium brought together for the first time many of the active [sleep researchers] in the field. […] Where are the zoologists, ethologists and other students of animal behavior? […] Animal behaviorists have been concerned almost entirely with waking behavior, partly because sleep may not appear a very interesting "behavior". […] It is not hard to predict that this state of affairs will end. A merging of the laboratory and field traditions into a comprehensive study of the physiology and behavior of animals sleeping in their natural habitat will occur in the next decade."

Unfortunately, Dr. Allison's four decade old vision has yet to be realized. Comparative animal sleep research, *using the electrophysiological tools of the discipline*, has never really caught on in the wild (Rattenborg et al. 2008). This is a pity as a complete understanding of sleep will only arise through the integration of animal behavior, ecology, neurophysiology and evolutionary biology. Perhaps Chapters 1 and 2 contribute in small part, towards such an integration.

Whole-brain Regulation of Avian Sleep

In Chapter 3, we provided the first experimental, electrophysiological evidence for mammal-like SWS homeostasis in birds. SWA at night was greater after a day when pigeons were kept awake (Martinez-Gonzalez et al. 2008). This is important because most prominent hypotheses for the function of sleep rely on such an increase in SWA following sleep loss (e.g., Tononi and Cirelli 2006, Scharf et al. 2008). For example, the increase in SWA could reflect increased brain metabolism during prior wakefulness, as waking brain use depletes brain stores of glycogen, increasing extracellular adenosine, which in turn may increase SWA (Scharf et al. 2008). Moreover, brain use increases the strength and number of synapses (or potentiation) in a use-dependent manner, which increases the neuronal synchrony during subsequent SWS and the level of SWA (Tononi and Cirelli 2006). However, because the increase in SWA in pigeons was symmetric between the left and right hemispheres, it could reflect the influence of central brain regions involved in the onset and maintenance of sleep following prolonged wakefulness (Komarova et al. 2008, see also Saper et al. 2005 and Szymusiak and McGinty 2008), instead of use-dependent increases in brain metabolism or synaptic strength.

Local Regulation of Avian Sleep

In Chapter 4, we distinguished between these alternatives by investigating whether avian SWS is regulated locally in response to prior brain use during wakefulness, as it is in mammals. If avian SWS is regulated only via central mechanisms, then local brain use should have no effect on the level of subsequent SWA. If avian SWS is regulated locally, then local brain use should cause a local increase in SWA. Using unilateral visual stimulation, with an experimental design otherwise identical to that used in Chapter 3, we demonstrated, for the first time, that avian SWS is homeostatically regulated locally in response to prior waking brain use (Lesku et al. 2011). That is, the hyperpallium connected to the stimulated eye showed increased SWA while the visually-deprived hyperpallium showed no change from baseline, likely reflecting the competing effects of time awake and reduced input. This inter-hyperpallial asymmetry was specific to the hyperpallium as the non-visual mesopallium showed a symmetric increase in SWA, reflecting increased time awake. In addition, we provided evidence that this local effect

may arise from a local increase in synaptic strength (measured as the slope of slow waves during SWS, Esser et al. 2007, Riedner et al. 2007, Vyazovskiy et al. 2007), in the hyperpallium connected to the stimulated eye. The mesopallium showed a symmetric increase in wave slope. Consequently, mammalian and avian sleep are both regulated locally (see also Rattenborg et al. 2009 and Tobler 2011).

Molecular Mechanisms underlying Local Sleep Homeostasis

Is the increase in SWA mediated by the same molecular mechanisms as in mammals? Neural plasticity genes, such as brain-derived neurotrophic factor (BDNF), increase SWA in mammals (Faraguna et al. 2008). Because SWA reflects synaptic strength, the synaptogenic action of BDNF likely more effectively synchronizes the slow oscillation underlying SWA via increased connectivity (Huang and Reichardt 2001, Vyazovskiy et al. 2009). In Chapter 5, we sought to determine if BDNF plays a similar role in birds as well, in addition to another plasticity-related gene, activity-regulated cytoskeleton-associated protein (Arc, see Hanlon et al. 2009). Surprisingly, neither BDNF or Arc mRNA were expressed asymmetrically in the hyperpallium in the manner predicted if they reflect waking brain use, as they do in mammals. Also unlike mammals, levels of BDNF mRNA were higher at night, when pigeons in the laboratory spend more time asleep (Martinez-Gonzalez et al. 2008, Lesku et al. 2011). This further suggests that BDNF might be unrelated to waking neuronal activity in birds. The local regulation of SWS in mammals and birds may be mediated by different molecular mechanisms, but further study is clearly needed.

Implications for our Understanding of the Function of Avian SWS

What does this study contribute to our understanding of the function of SWS in birds as well as the existence of a *core* or fundmental sleep function shared by mammals and birds? As outlined above, the demonstration of local sleep homeostasis in pigeons could reflect increased brain metabolism and / or increased potentiation in regions used more during prior wakefulness, which could subsequently increase the level of SWA. The latter 'synaptic homeostasis hypothesis' (see Figure 3 in the General Introduction) posits that the strength of

synapses increases during wakefulness in a use-dependent manner (Tononi and Cirelli 2006). Increased synaptic strength results in a more synchronous slow oscillation of neuronal membrane potentials underlying SWS, which increases the level of SWA (Vyazovskiy et al. 2009). The slow oscillation is thought to promote synaptic downscaling, because stimulation at a low frequency similar to the slow oscillation induces long-term depression (Collingridge et al. 2010). Consequently, the brain is prepared for further plastic changes during subsequent wakefulness.

Although each step of this hypothesis is well-supported by several lines of evidence in mammals, what evidence exists for synaptic homeostasis in birds? In response to brain stimulation during wakefulness, synapses appear to strengthen in birds, at least to the extent that slow wave slope reflects synaptic strength (Lesku et al. 2011) and the expression of genes involved in long-term potentiation is elevated during wakefulness in the forebrain of white-crowned sparrows (*Zonotrichia leucophrys gambelii*, Jones et al. 2008a). Results from our molecular study (Chapter 5) were inconsistent with expectations derived from work on mammals and must be revisited to determine if BDNF and Arc mediate the local increase in SWA following brain use in birds. Nevertheless, the available evidence suggests that synapses may strengthen during wakefulness. Like mammalian SWS, avian SWS is seen at the cellular level as the slow oscillation of neuronal membrane potentials (Figure 2). Increased connectivity accrued during wakefulness may result in a more synchronous slow oscillation during subsequent SWS, as evidenced by a local increase in SWA (Martinez-Gonzalez et al. 2008, Lesku et al. 2011). Synapses may weaken during avian SWS, because SWA decreases with time asleep (Jones et al. 2008b, Martinez-Gonzalez et al. 2008, Lesku et al. 2011, see also Rattenborg et al. 2009 and Tobler 2011) and genes involved in this process are preferentially expressed during sleep in birds (Jones et al. 2008a). In mammals, synaptic homeostasis can account for some of the enhancements in performance on various cognitive tasks observed after sleep. Whether slow-oscillation mediated synaptic downscaling promotes learning also in birds in unknown.

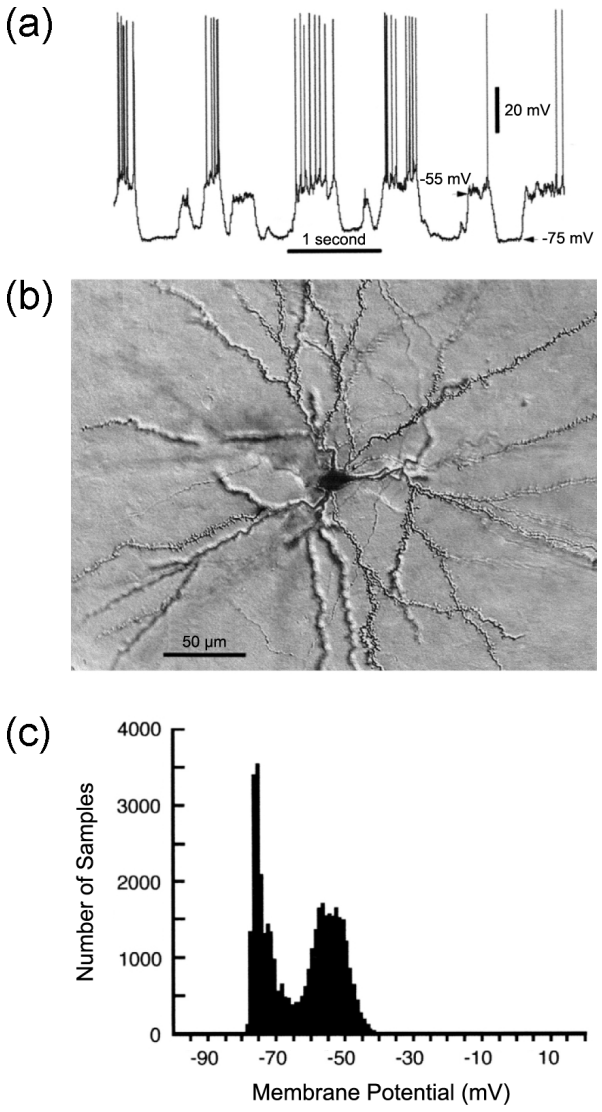


Figure 2. (a) Spontaneous slow oscillations of the membrane potential in a pallial neuron of an anesthetized pigeon between depolarized "up-states" (-55 mV) with frequent action potentials and hyperpolarized "down-states" (-75 mV) with no action potentials. (b) The slow oscillation was recorded from a neuron in the pallium. (c) Frequency histogram of membrane potentials showing the tendency of a neuron in the nidopallium caudolateral to be either in the down-state or up-state. Modified from Reiner et al. (2001) Physiology and morphology of intratelencephalically projecting corticostriatal-type neurons in pigeons as revealed by intracellular recording and cell filling. *Brain Behav Evol* 58:101-114.

Because of the work presented in this dissertation, we know that mammals and birds share a remarkably similar pattern of REM sleep evolution. We know that the homeostatic regulation of avian sleep occurs at global and local levels, similar to that observed in mammals, and this similarity may not be reflected at the molecular level. Now, new questions arise. Is the regulation of avian sleep, as demonstrated here in pigeons, common to birds, including the most basal Palaeognathae? In mammals, SWS regulation has been demonstrated experimentally in numerous eutherians (Tobler 2011), and monotremes show a decline in SWA during sleep (Siegel et al. 1996), suggestive of eutherian-like sleep homeostasis. Thus, SWS may be regulated similarly across mammals (Tobler 1995, 2011). But in birds, the phylogenetic

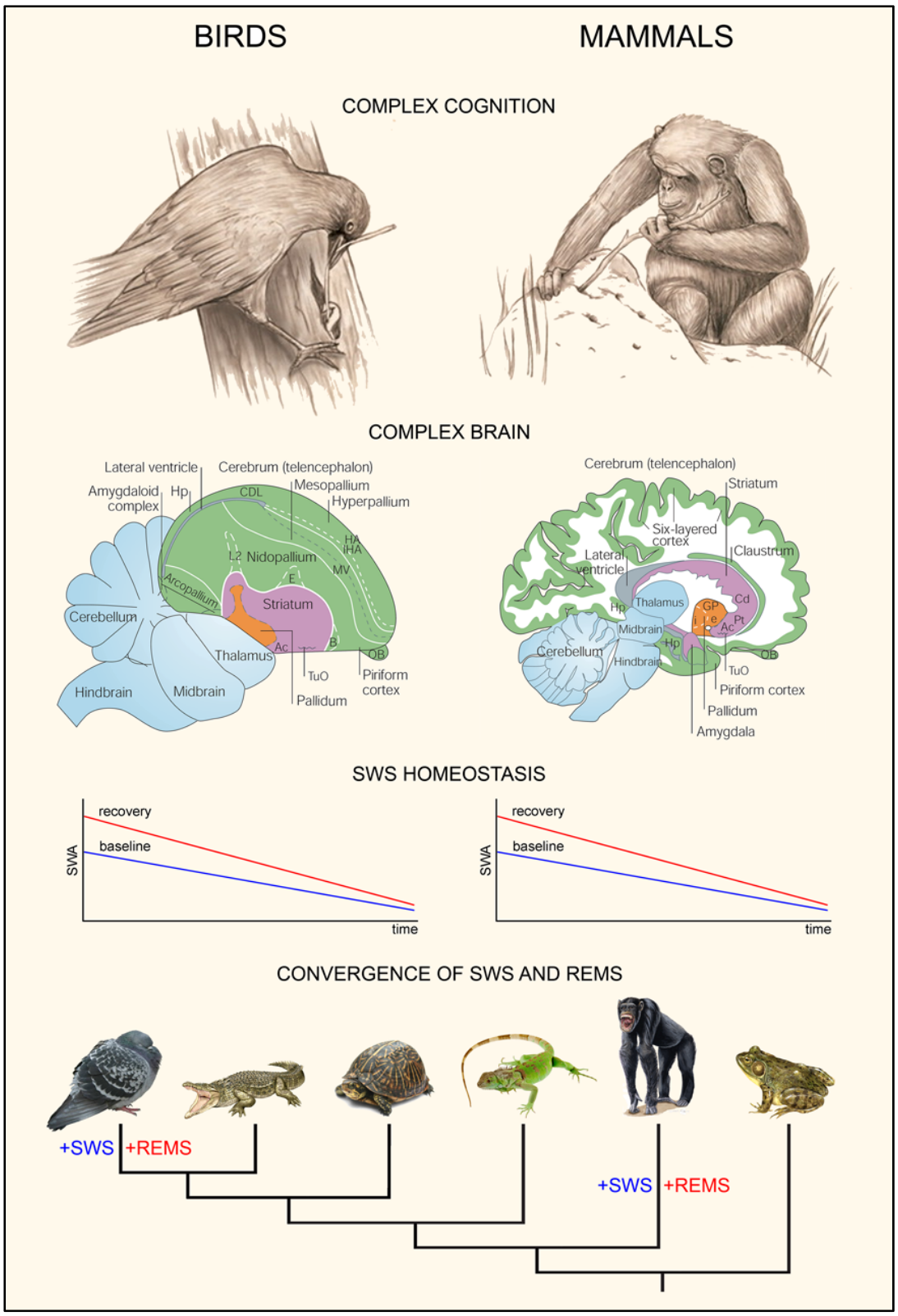
breadth of mammal-like SWS homeostasis is unknown. Shortly after our first description of sleep homeostasis in the pigeon, other researchers demonstrated a similar response in a songbird (Jones et al. 2008b). However, passerines and pigeons are both members of the Neognaethae (Hackett et al. 2008). Determining how sleep is regulated in the Palaeognathae might provide insight into how sleep is regulated in birds in general.

How might local sleep homeostasis be involved in facilitating waking cognition, as it is in mammals? Most studies on sleep-dependent learning in birds focus on the role of sleep in song development (e.g., Dave and Margoliash 2000, Derégnaucourt et al. 2005, Crandall et al. 2007, Shank and Margoliash 2009, Gobes et al. 2010, Rauske et al. 2010). Under models of memory consolidation in mammals, performance should improve with a period of sleep (Tononi and Cirelli 2006, Diekelmann and Born 2010). Whether the improvement reflects (1) the renormalization of synaptic strength, increasing the signal-to-noise ratio of relevant neural circuits (Tononi and Cirelli 2006), or (2) the transfer of new (volatile) memories from the hippocampus to the neocortex for more permanent storage (Diekelmann and Born 2010) is a topic of active debate, but the idea that sleep improves waking cognition is (generally) not. Perhaps in contrast to the general hypothesis for sleep-dependent memory consolidation, the quality of the song in juvenile zebra finches *decreases* across a night of sleep (Derégnaucourt et al. 2005, Crandall et al. 2007, Shank and Margoliash 2009). However, the degree to which the song deteriorates predicts the ultimate song quality, suggesting a positive role for sleep in song learning. How such a process operates under existing mammal-based hypotheses for memory consolidation (if at all) is unclear (Rattenborg et al. 2011).

Why would the brain activity of sleeping birds (and associated sleep homeostasis) more closely resemble that of mammals than their more closely-related crocodilian 'sisters'? While sleeping mammals and birds show unequivocal SWS and REM sleep in the EEG, studies on sleeping caiman revealed only wake-like, low voltage activity with intermittent high voltage sharp spikes in the EEG (Flanigan et al. 1973, reviewed in Rattenborg 2006, 2007). A similar EEG has been described in squamate lizards (Tauber et al. 1966, Flanigan 1973, Huntley 1987), and

turtles and tortoises (Flanigan 1974, Flanigan et al. 1974) engaged in sleep defined by behavioral criteria (e.g., quiescence, increased arousal threshold, stereotypic posture, etc.). In addition to independently evolving SWS and REM sleep, mammals and birds also independently evolved other traits thought to be relevant for either the genesis or function of these states (Figure 3).

Notably, the slow oscillation underlying SWS is synchronized by brain connectivity (Amzica and Steriade 1995, Hill and Tononi 2005, Esser et al. 2007, Huber et al. 2007, Vyazovskiy et al. 2009). The importance of connectivity for the genesis of high amplitude slow waves during sleep is also supported by comparative data (Rattenborg 2006, 2007), in that while the mammalian cortex and avian hyperpallium exhibit extensive connectivity and show such slow waves during sleep, the reptilian dorsal cortex has limited connectivity and does not (Medina and Reiner 2000, Rattenborg et al. 2009). Additionally, mammals and birds have uniquely large relative brain sizes (or encephalization); encephalization has been interpreted by some as an interspecific measure of cognitive abilities (Jerison 1985). Consistent with such an interpretation, some birds and mammals show forms of cognition that may be unique to these animals, such as tool manufacture and use (e.g., Emery and Clayton 2005). Thus, mammals and birds independently evolved SWS and REM sleep, similar sleep regulatory mechanisms, and relatively large, highly interconnected brains capable of performing advanced cognition. It is intriguing to speculate that this assemblage of traits, not found in other animals, may be functionally interrelated (Rattenborg et al. 2009). If true, then the study of avian sleep has much to tell about the function of sleep in mammals and birds alike.



(Caption on next page)

Figure 3. Not only did mammals and birds independently evolve slow wave sleep (SWS) and rapid eye movement sleep (REMS) as well as similar sleep regulatory mechanisms (bottom panels), but they also appear to have independently evolved complex cognition not found in other animals, such as tool manufacture and use, and complex, heavily interconnected brains (top panels). The drawing of tool use in a New Caledonian crow and a chimpanzee was modified from Emery and Clayton (2004) The mentality of crows: convergent evolution of intelligence in corvids and apes. *Science* 306:1903-1907. Schematic of a sagittal section through an avian (zebra finch) and mammalian (human) brain; Ac, accumbens; B, basorostralis; Cd, caudate nucleus; CDL, dorsal lateral corticoid area; E, entopallium; GP, globus pallidus (i, internal segment; e, external segment); HA, hyperpallium apicale; Hp, hippocampus; MV, mesopallium ventrale; IHA, interstitial hyperpallium apicale; L2, field L2; LPO, lobus parolfactorius; OB, olfactory bulb; Pt, putamen; TuO, olfactory tubercle; the large, white regions are axon pathways (i.e., white matter) in the cerebrum; lamina (cell-sparse zones separating brain subdivisions) are marked as solid white lines, primary sensory neuron populations are distinguished from neighboring regions by dashed white lines, and regions differing in cell density or size are demarcated by dashed grey lines; reprinted from Jarvis et al. (2005) Avian brains and a new understanding of vertebrate brain evolution. *Nature Rev Neurosci* 6:151-159. The graphs of SWS homeostasis do not reflect collected data, but rather patterns of SWA that are conceptually similar to data obtained in studies of sleep homeostasis (i.e., increased SWA during 'recovery' sleep following an extended period of wakefulness or increased brain use relative to 'baseline' sleep, and a decrease in SWA with time asleep). Overall figure modified from Rattenborg et al. (2009) Avian sleep homeostasis: convergent evolution of complex brains, cognition and sleep functions in mammals and birds. *Neurosci Biobehav Rev* 33:253-270.

General Conclusions

With this dissertation, we have expanded our understanding on the evolution and regulation of sleep in birds. We found that, in addition to independently evolving slow wave sleep (SWS) and rapid eye movement (REM) sleep, mammals and birds may share the same evolutionary trajectory in REM sleep evolution. We also found that the view that avian SWS is regulated differently from mammals is incorrect. Indeed, like mammalian SWS, avian SWS is regulated globally and locally following brain use during prior wakefulness; a response that may depend on underlying increases in the strength of synapses. Further study is needed to determine whether the molecular mechanisms responsible for increasing the intensity of SWS following waking brain use are also shared between mammals and birds. Overall, our results suggest that hypotheses for the function of SWS in mammals may also apply to birds, and thus represent a *core* or fundamental function of SWS. More broadly, our results expand upon studies showing remarkable similarities in waking cognition between mammals and birds, by showing that the avian brain functions in a mammal-like manner during sleep as well. Given that similar forms of cognition and sleep appear to have evolved independently in mammals and birds, the aspects of sleep unique to these groups (e.g., local sleep homeostasis) may be intimately involved in supporting the complex forms of cognition exhibited by mammals and birds. In conclusion, we hope that this dissertation makes a small contribution to our understanding of the evolution of sleep and sleep functions.

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