Behavioral Neuroscience Comprehensive Exam Question

Sleep behavior is heavily influenced by interactions between circadian and homeostatic mechanisms. These interactions, which have been modeled by Borbely and others, vary across individuals, between the sexes, and among species, and they change across the lifespan. In some species, like us, they typically produce a relatively long sleep bout at one time of day whereas in other species, like rats, they produce a pattern of many shorter bouts whose frequency and duration vary across the day-night cycle. A great deal is known about the neural substrates mediating sleep/arousal state, and researchers are just beginning to get a handle on how these systems are influenced by neural mechanisms mediating circadian and homeostatic influences on sleep. Very little is known about how these mechanisms differ in species with relatively long consolidated sleep bouts and ones with shorter more frequent bouts whose patterning changes from day to night. Most of the research on these mechanisms is done on the latter species (e.g. rats) although the general aim is to understand the former (e.g. humans).

For this part of your comps, your mission is to consider this issue and:

(1) Review the highlights of the literature germane to it, which includes (a) the basics of the Borbely model, (b) key components of neural pathways that modulate sleep/wake state, and (c) the neural/physiological basis of circadian and homeostatic regulation of sleep/arousal state. You are not expected to be exhaustive, but to identify key findings and principles relevant to the question of "how could species differences in these neural systems account for differences in the degree to which sleep occurs in long consolidated bouts at one phase of the day/night cycle, or in shorter bouts whose frequency/duration changes from day to night?"

(2A) Develop hypotheses, or alternative hypotheses, that might provide an answer to this question.

(2B) Design a research plan (like in an NRSA NIH application you are all exposed to) that would enable you to test these hypotheses. This will involve clearly spelling out the predictions that the hypothesis makes and how those predictions would be tested. We do not expect you to come up with a plan that could definitely establish whether your model is correct or not, but something that would represent a first step in its evaluation. To this end, we recommend that you propose 2-3 experiments that address different aspects of the problem. Assume that you have unlimited resources with which to conduct your research but that you will be required to comply to standard ethical guidelines.

(2C) Discuss potential outcomes of the proposed work and how you would interpret these outcomes, as well as potential pitfalls or limitations of the proposed experiments along with ideas on how one might address such limitations.

(2D) Wrap things up by indicating what the work would accomplish overall, why this would be important and what the next questions might be.

Sleep and sleep like states are ubiquitous behaviors observed in most every species from humans and non human primates to aplysia and drosophila (Tobler 1995). Sleep, like eating, drinking and mating are thought to be crucial to mammalian physiology as evidenced by the tight regulation of time and duration of sleep, as well as the rebounds following sleep deprivation (Beersma 1998). However, this timing and duration of sleep throughout the 24-hour light-dark cycle varies significantly between species. Extreme light-dark preferences are found in some animals, while others show a minimal degree of preference for either period. Under normal circumstances humans, as well as most apes, sleep in a single consolidated, or monophasic pattern, throughout the night. This monophasic pattern is in contrast to most other mammalian species who exhibit a polyphasic sleep pattern, showing fragmented sleep bouts interrupted by long active periods throughout the 24-hour day (Tobler 1995). Currently, the regulatory mechanisms that underlie the differences between monophasic and polyphasic sleepers are not understood. In order to elucidate possible mechanisms underlying this difference I will first present principles crucial to the understanding of 1) the Borbley, two-process model of sleep, 2) the key neural systems which modulate sleep and arousal and 3) the neural and physiological basis of circadian and homeostatic sleep regulation.

The Borbley Model

The Borbley, or Two Process Model, is a model of behavior used to describe the timing of alteration between sleep and wakefulness as the result of two processes; 1) the homeostatic need for sleep and 2) a circadian process keeping track of subjective time through environmental light cues (BorbÈly 1982; Beersma 1998). This model postulates that a homeostatic process (S) rises during the wakeful periods and declines during sleep and interacts with a circadian process (C) that is independent of sleep and waking (BorbÈly 1982; Borbely and Achermann 1999).

Current views of this model posit that S represents some substance that is accumulated during wakefulness in an exponentially saturating manner until an upper threshold is reached. Sleep onset is triggered when S, or the corresponding substance which it represents, approaches this "sleep threshold" wherein S will then decline at an exponential rate. S has been found to decline specifically in slow wave sleep (SWS) and most dramatically in the earlier sleep cycles of the night (Beersma 1998). Conversely, awakening is triggered when S reaches a lower or "wake threshold" (Daan, Beersma et al. 1984).

According to the two-process model, the circadian pacemaker controls these two thresholds. The upper and lower thresholds run in parallel together and vary with the time of day, together they are process C of the two-process model (BorbÈly 1982; Beersma 1998). Although the homeostatic drive for sleep is independent, it is nonetheless modulated by environmental cues. These thresholds modulate the sensitivity of the brain to accumulating S in accordance with C (Daan, Beersma et al. 1984).

Evidence for this model can be seen in sleep deprivation studies in which the upper threshold for S accumulation is suspended, allowing S to build to greater than normal levels. Following sleep deprivation, increased levels of SWS in the recovery night are observed (Webb and Agnew Jr 1971). In opposition, extended sleep or napping quells S accumulation, resulting in a reduction of SWS in subsequent nighttime sleep (Borbely and Achermann 1999).

Sleep and Arousal Systems

Arousal

In the 1940's two researchers, Mouruzzi and Magoun, observed that electrical stimulation of the mesopontine reticular formation promoted arousal and that damage to this location produced a coma like state (Moruzzi and Magoun 1949; Kandel, Schwartz et al. 2000) This seminal work was later extended to characterize what is now known as the ascending arousal system, the main pathway regulating wakefulness. This system, located in the rostral pontine caudal midrbrain border, can be subdivided into

two paths 1) the monoaminergic cell groups innervating the hypothalamus and cortex and 2) the cholinergic cell groups innervating the thalamus (Saper, Chou et al. 2001).

The monoaminergic group can be further subdivided into the noradrenergic neurons of the Locus Coeruleus (LC) and the serotonergic neurons of the Dorsal Raphe Nucleus (DR). Projections from these two nuclei ascend and run through the posterior lateral hypothalamus where they are joined by histaminergic fibers from the tuberomammilary nucleus (TMN). All of the above paths then project diffusely to the entire cerebral cortex (Saper, Chou et al. 2001). Activity in the above nuclei have a very characteristic state dependent pattern of activity; that is they fire rapidly during wake, slow down during SWS and are completely silent during rapid eye movement (REM) sleep (McGinty and Harper 1976; Aston-Jones, Chiang et al. 1991).

The cholinergic thalamocortical projections of the ascending arousal system consist of the Peduculopontine and the Lateral Dorsal Tegmental (PPT-LDT) nuclei. These neurons project in a topographic fashion to the thalamic relay nuclei, the intralaminar nucleus and the reticular nucleus of the thalamus, all of which then project to the cortex (Herkenham 1980; Saper, Chou et al. 2001). These cholinergic projections have the same characteristic firing pattern as the above monoaminergic nuclei with one exception; in addition to firing during wake they also show activity during REM sleep (Saper, Chou et al. 2001).

Sleep

Due to the state dependent nature in the firing pattern of the neurons within the ascending arousal system, it would seem that there must be something providing inhibitory input to silence these neurons during sleep. Indeed, researchers have identified a cluster of neurons within the ventral lateral preoptic area (VLPO) of the hypothalamus that send direct projections to the wake centers of the arousal system. The VLPO is organized into two parts; a dense central cell cluster which projects especially densely to

the TMN, and a more diffuse component which preferentially projects to the LC and DR (Lu, Greco et al. 2000; Lu, Bjorkum et al. 2002; Saper, Cano et al. 2005). Retrograde labeling studies have found that the VLPO contain inhibitory GABAergic and galanergic neurons that show high levels of activity, as evidenced by fos-immuoreactiviy (IR), during sleep (Saper, Chou et al. 2001; Gaus, Strecker et al. 2002). The VLPO provides inhibitory input to silence wake promoting neurons during sleep via this GABA and Galinergic neurotransmission. In addition, there are reciprocal inhibitory connections from all areas the VLPO innervates, that is the PPT-LDT, TMN, DR and LC (Saper, Scammell et al. 2005).

The Sleep/wake Circuit

Thus, a clear picture emerges in which the transition between wake and sleep are brought about by the reciprocal inhibition of the ascending arousal system and the sleep promoting hypothalamic cell groups. When VLPO neurons fire rapidly during sleep they inhibit arousal cell groups and thus disinhibit themselves, reinforcing their activity. Conversely, when the ascending arousal system is firing during wakefulness, they inhibit the VLPO and reinforce their own firing. This reciprocal relationship creates a bistable circuit in which there are sharp transitions between discrete states of wake and sleep (Saper, Scammell et al. 2005).

This circuit is further stabilized by a group of neurons found in the lateral perifornical area of the hypothalamus containing the peptide Orexin. This group of neurons projects to all areas of the arousal system and reinforces wakefulness by increasing firing of the arousal nuclei, thereby inhibiting the somnogenic effects of the VLPO (Hagan, Leslie et al. 1999; Brown, Sergeeva et al. 2001; Eriksson, Sergeeva et al. 2001). This intricate balance between sleep and wake promoting pathways prevents flip flopping from small perturbations on one side or the other but will quickly shift states following large scale fluctuations such as those provided by circadian and homeostatic drives.

Circadian and Homeostatic Regulation of Sleep

The sleep-wake circuit comprises the machinery by which the body controls the behavioral state it is currently in, however, this machinery itself is under the control of circadian modulation as well as homeostatic regulation.

Circadian Modulation of Sleep and Arousal

All animals, whether they are mono- or polyphasic sleepers, have a circadian periodicity to their sleep wake cycle that approximates 24-hours (Kandel, Schwartz et al. 2000). These circadian patterns are endogenous rhythms whose activity is dependent upon a near 24-hour interaction of positive and negative transcriptional-translational feedback loop of "clock genes" with in the Suprachiasmatic Nucleus (SCN) of the anterior hypothalamus (Jin, Shearman et al. 1999). Though these rhythms are present in the absence of external cues (Hastings and Herzog 2004) under normal circumstances our circadian clocks are modulated or entrained by environmental timing cues, the most notable of these being light. Light entrains endogenous clock gene expression within these cells by means of the retinohypothalamic tract, a path that runs directly from specialized retinal ganglion cells to the SCN (Gooley, Lu et al. 2001). Light activates the SCN and promotes the active phase of the sleep-wake cycle in some animals (e.g. primates) while it promotes the inactive phase in others (e.g. rodents) (Edgar, Dement et al. 1993).

Although the SCN has some minimal outputs to the VLPO, the major route by which activity in the SCN modulates sleep and arousal is through the relay from the SCN to the ventral subparaventricular zone and finally to the Dorsal medial Nucleus (DMN) of the Hypothalamus (Chou, Bjorkum et al. 2002; Chou, Scammell et al. 2003). The DMN in turn has massive GABAergic input to the VLPO as well as Glutamatergic input to the orexin cell group (Chou, Scammell et al. 2003). Excitation of the DMN results in consolidation of wakefulness in a two-pronged effect by both inhibiting the VLPO as well as indirectly exciting the ascending arousal system via the orexin system.

Through this path environmental cues can modulate the arousal state within the appropriate circadian phase (Saper, Cano et al. 2005). Although the SCN modulates the propensity to sleep, it does not control sleep itself. It is the homeostatic need for sleep that is the ultimate regulator of sleep.

Homeostatic Regulation of Sleep

In his initial model Borbely proposed that the homeostatic process, S, increases in an exponentially saturating mechanism during wakefulness and decreases in an exponential fashion during sleep (BorbÈly 1982). Recent work has pointed to the molecule adenosine as being the substrate for the homeostatic drive, S.

In addition to acting as signaling molecule within the nervous system, adenosine is also the end product of adenosine triphosphate (ATP) break down, the primary fuel source of the cell. Adenosine is released from both neurons and glia through active and passive transport as a result of the intracellular-extracellular concentration gradient. Under conditions of compromised fuel stores intracellular concentrations of adenosine will rise until they exceed that of extracellular concentrations, at which point adenosine will be transported into the extracellular milieu. Adenosine can than either be degraded by the enzyme adenosine deaminase (ADA) or activate inhibitory A1 type adenosine receptors or excitatory A2 type adenosine receptors.

Recently it has been found that adenosine levels rise in the brain during wakefulness and fall during sleep (Porkka-Heiskanen, Strecker et al. 1997). More specifically adenosine levels have been shown to rise in areas surrounding the VLPO such as the basal forebrain(Porkka-Heiskanen, Strecker et al. 2000). It is thought that adenosine, acting via A2 type receptors within the VLPO and via A1 type receptors in surrounding areas, induce sleep. This hypothesis has been strengthened by several studies. Infusion of the adenosine agonist CGS21680 near the VLPO increases sleep and fos-IR in VLPO neurons (Scammell, Gerashchenko et al. 2001; Saper, Cano et al. 2005). Further, infusion of A1

receptor antisense oligonucleotides into the basal forebrain significantly reduced sleep (Thakkar, Winston et al. 2003). Thus, it could be argued that the physiological basis for the homeostatic drive of sleep is to replenish fuel stores. Adenosine, acting as regulatory negative feedback molecule, signals the current state of cellular energy availability, telling the body to sleep and refuel if adenosine concentrations are high.

Research Plan

Within this section I will first integrate the above principles into a hypothesis that could be used to explain the neural/physiological basis for the differences between mono- and polyphasic sleepers within a condensed NRSA format. I will then present a research plan that could serve as a springboard to test this hypothesis as well as potential pitfalls, alternatives and implications of the research.

Specific Aim and Background

Sleep is a behavior that is thought to be crucial to mammalian physiology and known to dominate our lives but which we still know so little about. There is no better illustration of this than in the differences between monophasic sleepers, who sleep in a single consolidated bout at on time of day, and polyphasic sleepers who exhibit fragmented sleep bouts which vary throughout the light dark cycle. According to the Two-Process model, sleep is induced when the homeostatic need for sleep (S) reaches an upper "sleep threshold", which is in turn modulated by external circadian cues (C).

As was introduced above, a recent a breadth of work has suggested that the ATP metabolite, adenosine, could be acting as a substrate for S. Adenosine has been found to accumulate in the brain during metabolically demanding periods of wakefulness and decrease during sleep. In addition, adenosine degradative enzymes are found to be more active during sleep (Chagoya, Hern·ndez et al. 1993). The time course of the variation in adenosine levels in the brain shows a nearly identical pattern to the proposed time course of S. Administration of adenosine agonists increases sleep expression in a

dose dependent manner and is selective to deep or SWS, which is known to be the homeostatic restorative component of sleep (Radulovacki, Virus et al. 1984). Adenosine has been shown to inhibit the arousal promoting noradrenergic neurons of the LC (Shefner and Chiu 1986) as well as mesopontine cholinergic neurons (Rainnie, Grunze et al. 1994). Finally, caffeine, a substance commonly used for its arousal properties, is a competitive antagonist of both A1 inhibitory and A2 excitatory adenosine receptors (Griffiths and Woodson 1988).

Despite the strong correlation between adenosine and the homeostatic drive for sleep, there is still almost no data linking changes in S to the difference between mono- and polyphasic sleep regulation. **Here I hypothesize that the difference between monophasic and polyphasic sleepers is in the sensitivity to accumulating levels of extracellular adenosine within brain regions know to be important in sleep and arousal**. More specifically, that polyphasic sleepers have an increased sensitivity to accumulating levels of extracellular adenosine than that of monophasic sleepers. This increased sensitivity to adenosine results in levels of adenosine accumulating to the "sleep threshold" within a relatively short duration of wakefulness. Due to the fact that the sleep threshold is set at a lower level in these animals, there are lower levels of adenosine to clear during restorative sleep, which results in shorter sleep bouts. The emerging pattern is that these animals will need to sleep more often but will also need to sleep for shorter durations. This is opposed to monophasic sleepers, which I am proposing have an increased threshold to adenosine buildup. Thus, they can allow extracellular adenosine to increase to a comparatively larger concentration throughout wakefulness and as a consequence must undergo longer bouts of restorative sleep to clear this buildup. The reason for light-dark variations in the length of sleep bouts, is due to the proposed modulatory role that C has on the sleep and wake thresholds (BorbÈly 1982). That is; circadian cues will either raise or lower the

sensitivity to adenosine's somnogenic effects depending on the phase of the day and whether the animal is nocturnal or diurnal.

Specific Aim: Determine if there is differential sensitivity to adenosine between monophasic and polyphasic sleepers

Sensitivity to adenosine will be probed between monophasic (squirrel monkey) and polyphasic (Rat) sleepers by first examining adenosine receptor distribution in specific areas known to be crucial for sleep and arousal, using both in *situ* hybridization as well as Immunohistochemistry (IHC). Following, quantitative levels of adenosine receptor mRNA and protein will be analyzed in these regions of interest using quantitative real time-polymerase chain reaction (qRT-PCR) and Western Blot Analyses. Finally, sensitivity to the adenosine agonist CGS21680 will be analyzed using intracerebral ventricle (ICV) infusion and fos-IHC. It is hypothesized that polyphasic sleepers will have an increased density (as compared to monophasic sleepers) of excitatory and inhibitory adenosine receptors in brain nuclei known to promote sleep and wake, respectively, which will result in an increased sensitivity to the sleep inducing effects of the adenosine agonist.

Approach and Experimental Design

Is there a differential distribution of adenosine receptors between monophasic and polyphasic sleepers*?*

Rationale: It is hypothesized that polyphasic sleepers are more sensitive to accumulating levels of extracellular adenosine than monophasic sleepers. It is further hypothesized that this increased sensitivity could be mediated by increased adenosine receptor density. Specifically, an increased density of A1 inhibitory adenosine receptors on neurons within arousal nuclei and increased density of A2 excitatory adenosine receptors within sleep nuclei, in polyphasic as compared to monophasic sleepers. In order to test this I will **1)** visually examine the location of A1 and A2 adenosine receptor mRNA and

protein and **2)** quantify this mRNA and protein in a time course spanning the 24 day. Comparisons will be made between the diurnal, monophasic sleeping squirrel monkey and the nocturnal, polyphasic Sprague Dawley Rat.

Experiment 1) Visualization: Animals (n=8/group, n=80/total) will be sacrificed by transcardial perfusion with 4% paraformaldehyde at zetigeber time (zt) 0, 6, 12, 18 and 24 hours. Half of the animals at each time will be used for *In situ* hybridization and the other half for IHC. Brains will be removed and post fixed overnight. Brains will then be sliced in the coronal plane at 20μm for *In Situ* and at 40μm sections for IHC. For *In Situ* hybridization, four adjacent sets of tissue sections will be mounted (one for sense and one for antisense probes of A1 and the same for A2), pre-hybridized, followed by overnight hybridization with sense and antisense probes of either A1 or A2 receptors. The slides will then be rinsed with RNAase and ethanol, dried and visualized on a phosphoimaging screen. For IHC, alternate free floating sections will be incubated with a primary rabbit anti -A1 or -A2 receptor antibody, followed by a biotin-conjugated, goat anti-rabbit secondary antibody. Bound peroxidase will be visualized with 0.05% 3-3'-diaminobenzidine tetrahydrochloride with 0.01% hydrogen peroxide using an ABC Elite kit. Slides from IHC or In Situ reacted tissue will be analyzed to determine the localization of A1 or A2 receptor mRNA and protein in the VLPO, LC, DR and PPT-LDT. Slides will also be counterstained with a cell body marker. Density of staining in each section will be determined in each individual nuclei and normalized to nuclei area (to account for inter-species variability in nuclei size).

	0h	12h	16h	18h	24h
Monkey	8	8	8	8	8
Rat	8	8	8	8	8

Experiment 2) Quantification: Animals (n=8/group, n=80/total) will be sacrificed by decapitation at

	0h	12h	16h	18h	24h
Monkey	8	8	8	8	8
Rat	8	8	8	8	8

zeitgeber time 0, 6, 12, 18 and 24 hours. Half of the animals at each time will be used for qRT-PCR and the other half Western Blot analyses. Brain will be removed and immediately frozen over dry ice. Following, brains will be sectioned at 600μm and the nuclei of interest will be microdissected out along the entire rostral-caudal axis of each region using the palkovits punch technique (Palkovits and Brownstein 1983). A1 and A2 adenosine receptor mRNA and protein will be measured in the LC, DR, PPT-LDT and VLPO. mRNA and Protein levels will be normalized to overall protein content sampled as determined by the Bradford method in order to account for interspecies differences. A t-test will then be performed to determine differences in A1/A2 distribution and concentration between species.

Potential Outcome

It is expected that the Rat will show more of both visualized and quantified A1 receptor mRNA and protein within the LC, DR and PPT-LDT and more A2 receptor mRNA and protein in the VLPO than the Squirrel monkey. If the above increase is observed than this may be one of the mechanisms by which the polyphasic rat is more sensitive to adenosine. It will also be interesting to see if these levels of expression change with the subjective active or rest period within these animals; giving insight into a possible mechanism for the circadian modulation of sleep propensity. If no difference is observed than the mechanism regulating difference in sleep may not be adenosine sensitivity but rather a difference in buffering capacity. For example, the polyphasic sleeper could have lower levels of the adenosine degratory enzyme ADA. Similar alternative experiments could be undertaken to measure the ratio of adenosine to ADA in these brain regions. If there is a decreased buffering capacity you would expect to see a higher ratio in the polyphasic sleeper than in the monophasic sleeper.

Is there differential sensitivity to adenosine agonist in sleep-wake nuclei of monophasic and polyphasic sleepers?

Rationale: The hypothesis that difference in sensitivity to adenosine is what underlies polyphasic versus monophasic sleep patterns will be tested by investigating activity in sleep-wake nuclei following administration of an adenosine agonist. It is hypothesized that following adenosine agonist administration sleep nuclei will show a greater increase in activity while arousal nuclei will show a greater decrease in activity in polyphasic versus monophasic sleepers.

Experiment 3:

	Saline	CGS21680	CGS21680+ SCH58261	CGS21680+ DPCPX
Monkey	8	8	4	4
Rat	8	8	4	4

Animals (n=48 total, see table for groups) will be anesthetized and implanted with a cannula into the right lateral ventricle (brain slices will be examined for tracks to ensure correct placement). Following an 8 day recuperation period animals will receive ICV infusion of either the adenosine agonist CGS21680 (66.6pmol/kg bodyweight at) or 0.9% saline at 1.33µl/Kg bodyweight/min (Scammell, Gerashchenko et al. 2001). In addition, to determine which adenosine receptor type may be mediating any observed effects of CGS21680, two control groups will be pretreated with either the A1 receptor antagonist 8-Cyclopentyl-1,3-dipropylxanthine(DPCPX;1mg/kg; i.p.)(Anderson, Sheehan et al. 1994) or the A2 receptor antagonist SCH58261 (0.05 mg/kg; i.p.)(Cunha, Canas et al. 2008). Infusions will take place from either 5.00-7.00h zt or 15.00-17.00h zt (half of each group will be infused in either the light or dark phase to control for circadian variability). Two hours following initiation of drug infusion animals will be anesthetized, brains removed, sliced and IHC performed as described above using an anti-fos primary. Fos-IR neurons will be counted in all brain regions of interest and drug treated animals will be compared to controls to get a final value of change from baseline. A one-way ANOVA will be performed to detect any difference between groups.

Potential Outcome

It is expected that following infusion of the adenosine agonist there will be a greater increase (from baseline) in the number of fos-IR neurons in the VLPO in the poyphasic sleeper's brain as compared to the monophasic. It is also expected that there will be a greater decrease (from baseline) in the number of fos-IR neurons in the LC, DR, PPT-LDT in the polyphasic's brain as compared to the monophasic. Due to the fact that the same dose and flow rate (normalized to bodyweight) will be used, if these changes are observed, it would provide compelling evidence that the polyphasic sleeper's brain is indeed more sensitive to extracellular levels of adenosine. Further, it is expected that these changes in activity patterns will be mediated via A1 receptors in the arousal nuclei and A2 receptors in the sleep nuclei. Thus, we expect to see, solely, an increase in VLPO activity in the CGS21680+ DPCPX group and solely a decrease in arousal nuclei activity in the CGS21680+ SCH58261 group. Again the magnitude of these changes is expected to be greater in the polyphasic rat, reflecting an increased sensitivity to extracellular adenosine concentrations as compared to the monophasic squirrel monkey. If no difference is observed in the response of these nuclei to the adenosine agonist than it could be possible that the effects of accumulating adenosine are acting in other brain regions. To this end the investigation could be extended to look into other brain areas such as the thalamus, cortex and basal forebrain (Benington and Heller 1995). A potential problem could be the availability of adenosine to the brain areas of interest via ICV infusion. If this is the case than I propose to change the route of administration and use site-specific steerotaxic drug injections.

Possible Pitfalls

Some of the potential problems as they relate to specific experiments have been discussed in the experimental design section as appropriate. The most obvious pitfall concerning the overall methodology is the trouble in comparing findings between species. The rat and monkey brain have clear anatomical and morphological differences. Though measures have been taken to circumvent these

issues (such as comparing sensitivity in terms of change from baseline, normalizing mRNA and protein findings to total amount sampled, normalizing drug dose and volume to bodyweight and normalizing visualization density to area of the respective nuclei) it is quite possible that this issue may confound results. If this is the case then other avenues may be explored. Such as an attempt to create a monophasic sleeping rat using viral mediated gene manipulation to knockdown or increase adenosine receptor expression within the respective sleep-wake nuclei of interest. This would again show that the neural basis of polyphasic and monophasic sleep is a differential sensitivity to adenosine. Beyond being an additional avenue to this research I believe manipulation of receptor expression would also be the next step in this line of logic.

Together the proposed experiments could serve as a starting point to determine if differential sensitivity to accumulating adenosine levels does in fact underlie differences between mono- and polyphasic sleepers. In addition to giving insight crucial to our understanding of sleep, of which we know so little about, this could also illuminate differences in brain metabolism and fuel storage capacity between species. Further, if this hypothesis is valid, this information could be applied in the development of drugs or therapeutics for sleep disorders (such as insomnia), more efficient and healthier wake promoting drugs and possibly even an attempt to make humans more efficient sleepers.

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Comments and feedback

Grade = Pass

The committee unanimously recognized this paper as exemplary. Below we list several reasons. 1) The writing is clear and concise, with excellent use of summary statements throughout that tie information and concepts together and which serve to remind readers of the most critical and salient ideas and facts. 2) The paper is also very well organized and extremely easy to read with new information coming at a comfortable pace so that a coherent picture emerges for the reader with minimal effort (exactly what one strives for when writing a grant!). 3) Information provided in the introduction was not only easy to follow but was also strategic, with obvious relevance to the proposed Research Plan that followed. 4) The student also did a very good job stating clear hypotheses and their predictions and discussing potential outcomes and pitfalls, with appropriate attention to alternative strategies and controls in the Research Plan. 5) Finally, this student did a beautiful job of considering how circadian mechanisms might influence a homeostatic mechanism underlying specie differences in sleep/wake patterns (pg 12, whether expression levels of adenosine receptors also change with Zetigeber). In sum, this was an exceptional paper that reflects creativity, solid deductive reasoning and excellent scientific writing skills —all of which are critical for development of a successful research plan. The committee wishes to congratulate this student. We believe that should she/he write a real NIH NRSA chances of actually obtaining funding would be excellent!

We have only minimal feedback to offer that might benefit the student when designing experiments and writing grants in the future.

- Possibly other statistical tests should be considered. The first experiment compares between two species and across time; a T test is proposed, presumably running multiple T tests that compares the two species at each time point. However, a 2 X 4 ANOVA would allow direct comparisons between groups along these two dimensions (species and time) and also allow one to assess how possible differences in adenosine receptor expression might depend on Zetigeber.

- Halving the number of animals in the two control groups in Expt #3 is unwise and raises concern about whether the student understands how reducing sample size affects statistical power; in short, it reduces one's ability to detect genuine differences (between control and experimental groups). Sample size matters, for all groups in the study. Given that the two so-called control groups (in which treatments are in fact given to test the involvement of different adenosine receptors) are further divided into day and night treatment groups (resulting in final Ns = 2 for the adenosine receptor antagonist groups), it would be highly unlikely that any effects of these treatments would be detected. Moreover, to call these groups "control groups" is a misnomer. If saline is the vehicle for the various proposed treatments, then the saline group is the only control group in this study.

- The kind of anesthesia was not specified nor did the student indicate any plans for management of post-operative pain.

- Age and sex of the animals was not specified.