Modelling maintenance of wakefulness in rats: comparing potential non-invasive sleep-restriction methods and their effects on sleep and attentional performance

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Keywords
cognition, survival analysis

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Accepted in revised form 30 August 2016; received 26 January 2016

DOI: 10.1111/jsr.12464

SUMMARY
While several methods have been used to restrict the sleep of experimental animals, it is often unclear whether these different forms of sleep restriction have comparable effects on sleep–wake architecture or functional capacity. The present study compared four models of sleep restriction, using enforced wakefulness by rotation of cylindrical home cages over 11 h in male Wistar rats. These included an electroencephalographic-driven ‘Biofeedback’ method and three non-invasive methods where rotation was triggered according to a ‘Constant’, ‘Decreasing’ or random protocol based upon the ‘Weibull’ distribution fit to an archival Biofeedback dataset. Sleep–wake architecture was determined using polysomnography, and functional capacity was assessed immediately post-restriction with a simple response latency task, as a potential homologue of the human psychomotor vigilance task. All sleep restriction protocols resulted in sleep loss, behavioural task disengagement and rebound sleep, although no model was as effective as real-time electroencephalographic-Biofeedback. Decreasing and Weibull protocols produced greater recovery sleep than the Constant protocol, mirrored by comparably poorer simple response latency task performance. Increases in urinary corticosterone levels following Constant and Decreasing protocols suggested that stress levels may differ between protocols. Overall, these results provide insight into the value of choosing a specific sleep restriction protocol, not only from the perspective of animal welfare and the use of less invasive procedures, but also translational validity. A more considered choice of the physiological and functional effects of sleep-restriction protocols in rodents may improve correspondence with specific types of excessive daytime sleepiness in humans.

INTRODUCTION
Excessive daytime sleepiness remains a substantial unmet medical need for a diverse range of patients (Balkin et al., 2011; Roth and Roehrs, 1996). In order to expedite mechanistic insights and discovery of novel therapies, accurate modelling of sleep restriction in animals remains a significant research area. As the physiological properties of sleep and consequences of its restriction seem well conserved across several species (Phillips et al., 2010), rodent sleep restriction experiments are considered a valuable model of impairment to screen putative pharmacotherapies (Loomis et al., 2015; McCoy and Strecker, 2011).

While several approaches to restrict sleep have been developed in rodents, there has been little within-experiment comparison to identify the most biologically valid means. An ideal methodology utilizes detection of sleep onset via electroencephalogram (EEG)–'Biofeedback', to trigger a
stimulus to wake the animal (Rechtschaffen and Bergmann, 1995; Wurts and Edgar, 2000). Such methodology applies the minimum necessary stimulus, which can be accurately and objectively quantified. However, downsides arise from the requirement of invasive surgical techniques.

Non-invasive methods of sleep restriction, such as the gentle handling procedure whereby rodents are kept awake by laboratory personnel, introduce the possibility of confounding variations in protocol, and such experiments are difficult to perform with high throughput (Franken et al., 1991; Meerlo et al., 2001; Winsky-Sommerer et al., 2008). Platform methods maintain wakefulness by requiring rodents to balance on a disc over water. Animals fall into the water due to loss of motor tone, particularly during rapid eye movement (REM) sleep (Mendelson et al., 1974). This method is associated with elevated plasma corticosterone and adrenocorticotropic hormone levels (Andersen et al., 2005; Suchecki et al., 1998), and impacts thermoregulation (Van Hulzen and Coenen, 1981; Youngblood et al., 1997), which may introduce confounding factors. In contrast, enforced activity methods use stimuli that induce waking by triggering a righting reflex or avoidance movements (Leenaars et al., 2011; Wurts and Edgar, 2000). Typically, a constant interval will be used to enforce activity, but there is often discrepancy between the rate at which waking stimuli are applied, which can range from 60 to 225 stimuli per hour (Baud et al., 2013; Stephenson et al., 2015). As waking stimuli are not aligned with sleep need, they can be excessive when sleep restriction commences and increasingly insufﬁcient as it progresses. Enforced activity protocols with a decreasing inter-stimulus interval can better match the increasing sleep pressure as restriction continues (Leenaars et al., 2011).

To better characterize the effects of enforced activity sleep-restriction protocols, the present study compared three non-invasive enforced activity protocols with an EEG-Biofeedback protocol. All rats were implanted for EEG recordings, so that all four protocols could be objectively compared during the sleep restriction and recovery period. Urinary corticosterone was measured as a marker of stress. Rats were also trained to perform a simple response latency task (SRLT) to serve as a functional behavioural index of the efficacy of each protocol.

MATERIALS AND METHODS

All experimental protocols were approved by the local Animal Welfare Ethical Review Body, and carried out in accordance with the UK Animals (Scientiﬁc Procedures) Act 1986.

Adult, male Wistar rats (n = 16, approximately 270–300 g at time of surgery; Charles River Laboratories, Margate, UK) were implanted with electrodes for long-term EEG/electromyogram (EMG) recordings (Data S1). In addition to the main study, a historical dataset, obtained from 42 rats that underwent EEG-driven Biofeedback sleep restriction and compared with 31 non-sleep-deprived rats, was used to devise the non-Biofeedback sleep restriction protocols.

These rats were treated in a manner similar to those used in the main study.

Sleep restriction protocols

Four different protocols were used to induce sleep restriction. Firstly, an EEG/EMG- signal-based sleep restriction method used real-time Biofeedback of ongoing sleep/wake states to prevent sleep. With the Biofeedback method, detection of non-REM (NREM) or REM sleep epochs by the SCOR-E2004® program activates a motor to roll the cylindrical chamber around its axis for 8 s (26° of rotation at 11.5 cm.s⁻¹), thereby initiating the righting reﬂex and waking the rat. Three other algorithms were devised for chamber turning based upon historical Biofeedback protocol data. The non-invasive, non-Biofeedback protocols were driven by predetermined sequences that activated the chamber for an equivalent number of times across the sleep restriction period as the Biofeedback protocol did. The three algorithms were: ‘Constant’, where the chamber was triggered at a constant rate of 1 turn per min; ‘Decreasing’, where the interval between chamber turns decreased linearly in proportion to time spent in sleep restriction (Y = −5X + 80, i.e. an initial interval of 80 s that declined each hour by 5 s); and ‘Weibull’, where the chamber turns were based on a Weibull distribution fit to the intervals between sleep attempts in the historical Biofeedback dataset (Data S1). In all sleep restriction protocols, the chamber turned in a pseudo-random direction to prevent habituation.

Seven days prior to study start; all subjects were sleep restricted for 5 h using the Biofeedback protocol to habituate animals to the procedure. Subsequently, a crossover study design was conducted where each animal randomly received all sleep restriction treatments as well as a control condition (no sleep restriction) over 5 weeks, with an interval of 1 week between each treatment.

Sleep–wake variables were recorded during a 24-h baseline period (ZT0–ZT24), the subsequent 11-h sleep-restriction period (ZT0–ZT11), performance of the Simple response latency test (SRLT) and also during the subsequent 36-h recovery period. Wake and sleep continuity were assessed by computing bout length (i.e. a continual episode of the state delimited by three or more consecutive 10-s epochs) and/or using survival analysis.

Simple Response Latency Test

Simple response latency task training was performed as described in Data S1 and based upon previous methodology (Loomis et al., 2015). Briefly, the task is structured as follows: a house light acts as a preparatory cue, followed by a variable interval (range 4–6 s), after which the magazine light is illuminated. A period of 10 s allows the rat to perform a nose poke to receive a reward. An interval of 30 s is permitted between trials. Upon successful completion of the training, where criterion performance was determined to
be >75% trial completion efficiency across five successive days, rats were subjected to 40 min of SRLT on three consecutive days (pre-, test- and post-sleep restriction sessions). On pre- and post-days, rats were tested between ZT2 and ZT4, while on the test day SRLT was performed immediately after the 11-h sleep-restriction period (i.e. ZT11). The number of completed trials and response omissions were used as performance measures.

**Locomotor activity, body temperature and corticosterone levels**

Locomotor activity and body temperature were recorded throughout the study period. Corticosterone levels were assessed by urinalysis during SRLT task performance at the end of each sleep-restriction period, as previously described (Loomis and Gilmour, 2010). Corticosterone (CORT) was measured using a commercially available ELISA kit (Immunodiagnostic Systems, Tyne and Wear, UK) and expressed relative to creatinine excretion to correct for individual urine production rates across days (ng CORT μmol creatinine⁻¹). Creatinine measurements were assayed using mass spectroscopy (Greendale Laboratories, Woking UK). Urine samples were collected during the operant task, i.e. for 40 min starting at ZT11 (at the end of the sleep restriction protocol), while rats were housed on gridded trays underneath. Urine spots were collected using Pasteur pipettes, and all urine voided during the operant task for each individual rat was pooled into Eppendorf tubes and frozen at -20°C for subsequent analysis.

**Statistics and survival analysis**

Statistical analyses were performed using SAS (version 9.2, SAS Institute, Cary, NC, USA) and JMP (version 8, SAS Institute, Cary, NC, USA) software packages. Variables were analysed using a repeated-measures mixed-effect model. Sleep restriction ‘treatments’ and period were fixed-effect variables, corresponding measures in the 24-h baseline were covariates, and subject a random effect. A compound symmetry structure was used in the model. Statistics are reported as model, $F\text{DFNUMERATOR, DFDENOMINATOR, }P\text{-value}$. For example: ANCOVA, $F_{4,38.1} = 45.61, P < 0.0001$; REM sleep (ANCOVA, $F_{4,39.1} = 37.28, P < 0.0001$); sleep (ANCOVA, $F_{4,39.7} = 103.53, P < 0.0001$); sleep bouts lengths ($F_{4,39.3} = 37.81, P < 0.0001$); and number ($F_{4,37.4} = 60.68, P < 0.0001$) was present between the sleep-deprivation conditions. Compared with the non-restricted control group, 125 ± 16, 152 ± 17 and 127 ± 16 min of NREM sleep were lost under the Constant, Decreasing and Weibull protocols, respectively (all $P > 0.001$, $T_{55} > 0.74$), which represented 38 ± 5, 47 ± 5 and 39 ± 5% of the time spent in NREM sleep during the control, undisturbed condition. REM sleep loss resulted in deficits of 39 ± 3, 42 ± 3 and 41 ± 3 min relative to controls under the Constant, Decreasing and Weibull protocols, respectively (all $P < 0.001$, $T_{55} > 15.23$). For all non-invasive sleep restriction protocols, average sleep bout lengths were significantly shorter than compared with the control condition (all

**RESULTS**

**Wake continuity during sleep restriction**

The Biofeedback protocol maintained wakefulness between 40 and 60 min per hour throughout the 11-h restriction period, where the number of sleep attempts progressively increased over time (Fig. 1a). With regard to the average wake bout length, the progressive decrease in wake maintenance followed a non-linear decay (Fig. 1b), and the survival of the population of wake bouts decreased in proportion to length of sleep restriction (Fig. 1c). Hourly hazard ratios, relative to the first hour of sleep restriction (Fig. 1d), show that the likelihood of falling asleep first increased significantly after 3 h of the Biofeedback protocol, compared with that measured at 1 h. By the 11th h, the hazard ratio had significantly increased, while the average wake bout length significantly decreased.

The non-invasive sleep restriction methods produced an equal total number of chamber turns to the Biofeedback protocol (Fig. 2a). For the Constant protocol, a total of 215 ± 15 min of sleep occurred, consisting almost entirely of NREM sleep. Only 3 ± 2 min of REM sleep was present, representing 1.4 ± 0.9% total sleep time (Fig. 2b). Average sleep bout lengths were 0.9 ± 0.1 min, and a total of 192 ± 9 sleep bouts were initiated (Fig. 2c and d). For the Decreasing protocol, a total of 179 ± 14 min of sleep was achieved, consisting predominantly of NREM sleep, with only 0.5 ± 0.1 min of REM sleep, accounting for 0.3 ± 1.2% total sleep time. Average sleep bout lengths were 0.9 ± 0.2 min long, and a total of 142 ± 11 bouts were achieved. For the Weibull protocol, 197 ± 13 min of sleep was achieved, with only 1.3 ± 1.9 min of REM sleep occurring (0.7 ± 1.0% total sleep time). Average sleep bout lengths were 1.0 ± 0.1 min long, and a total of 146 ± 10 bouts were achieved (Fig. 2c and d). A significant difference in total sleep (ANCOVA, $F_{4,38.1} = 45.61, P < 0.0001$); NREM sleep (ANCOVA, $F_{4,39.1} = 37.28, P < 0.0001$); REM sleep (ANCOVA, $F_{4,39.7} = 103.53, P < 0.0001$); sleep bouts lengths ($F_{4,39.3} = 37.81, P < 0.0001$); and number ($F_{4,37.4} = 60.68, P < 0.0001$) was present between the sleep-deprivation conditions. Compared with the non-restricted control group, 125 ± 16, 152 ± 17 and 127 ± 16 min of NREM sleep were lost under the Constant, Decreasing and Weibull protocols, respectively (all $P < 0.001$, $T_{55} > 0.74$), which represented 38 ± 5, 47 ± 5 and 39 ± 5% of the time spent in NREM sleep during the control, undisturbed condition. REM sleep loss resulted in deficits of 39 ± 3, 42 ± 3 and 41 ± 3 min relative to controls under the Constant, Decreasing and Weibull protocols, respectively (all $P < 0.001$, $T_{55} > 15.23$). For all non-invasive sleep restriction protocols, average sleep bout lengths were significantly shorter than compared with the control condition (all
Finally, average bout numbers were increased for all non-invasive sleep-restriction protocols relative to the control condition (all $P < 0.001$, $T_{55} > 2.92$). However, a significantly greater number of bouts was achieved during the Constant protocol compared with the Decreasing and Weibull protocols (all $P < 0.001$, $T_{55} > 3.47$).

Statistical comparison of the non-invasive sleep restriction protocols to the Biofeedback protocol showed that none of them was as effective at restricting NREM sleep. Relative to the Biofeedback protocol, the Constant, Decreasing and Weibull protocols allowed an additional 76/56, 49/37 and 74/69 min of NREM sleep, respectively (all $P < 0.001$, $T_{55} > 2.8$).

Furthermore, all three non-invasive protocols allowed significantly more and longer sleep bouts than the Biofeedback protocol (Constant: 193/93 bouts of 0.7/0.2 min; Decreasing: 135/11 of 0.7/0.2 min; Weibull: 139/10 of 0.8/0.2 min, all $P < 0.001$, $T_{55} > 2.8$).

Simple Response Latency Test

Relative to the control undisturbed condition, all sleep-restriction protocols reduced the number of completed trials (ANOVA, $F_{4,42.5} = 5.56$, $P < 0.001$) over the course of the task (Fig. 3a). The Decreasing and Weibull protocols showed similar declines in performance over time compared with the Biofeedback protocol. In contrast, trial completion rate following the Constant protocol decreased to a lesser extent during the last 20 min of the task (Fig. 3a). Omissions were significantly increased compared with the control condition for all sleep restriction protocols except the Constant interval protocol (Fig. 3b). Both Decreasing and Weibull protocols led to comparable increases in the omission rate compared with the Biofeedback protocol (40/53; 37/53 versus 4636, respectively). In contrast, the Constant protocol resulted in a significantly lower number of omissions compared with the Biofeedback protocol (22/53, $P < 0.001$, $T_{56} = -4.07$).

Locomotor activity, body temperature and urinary corticosterone

During the sleep restriction period, all four restriction protocols significantly increased locomotor activity to a similar extent (all $P < 0.001$, $T_{58} > 3.65$) relative to Control group (Fig. 4a). This was not accompanied by any significant changes in core body temperature (ANOVA, $F_{4,53} = 0.84$, $P < 0.5035$; Fig. 4b). Urinary corticosterone levels were significantly increased compared with the control group for both the Constant and Decreasing protocols ($P = 0.0003$ and $P = 0.027$, $T_{34} = 3.7$ and $T_{34} = 2.24$, respectively), whilst elevations following the Biofeedback and Weibull protocols were not significant ($P = 0.082$ and $P = 0.483$, $T_{34} = 1.75$ and $T_{34} = 0.69$, respectively; Fig. 4c).

Effects during the recovery period

For all protocols, recovery sleep was significantly different between the sleep-deprivation conditions (ANOVA,
4,40.1 = 38.14, \( P < 0.0001 \), and occurred only during the first 12-h dark phase following sleep restriction (Fig. 5a, c and e). Relative to the control condition where rats slept ad libitum, total sleep time significantly increased in the first 12-h dark phase by 96 ± 13, 124 ± 14 and 110 ± 13 min after Constant, Decreasing and Weibull protocols, while it increased by 146 ± 13 min with the Biofeedback protocol (Fig. 5b). Relative to the Biofeedback protocol, the Constant

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and Weibull protocols resulted in less recovery sleep ($P < 0.001$, $T_{55} = -4.6$, and $P < 0.01$, $T_{55} = -2.73$, respectively), whereas the Decreasing protocol did not significantly differ from the Biofeedback protocol ($P > 0.1$, $T_{55} = -1.58$).

After sleep restriction, sleep showed greater continuity and depth, as inferred from the average sleep bout length and EEG delta power in NREM sleep. Average sleep bout lengths increased $2.0 \pm 0.1$, $1.7 \pm 0.1$, $1.7 \pm 0.1$ and $1.9 \pm 0.1$-fold after Biofeedback, Constant, Decreasing and Weibull protocols, respectively, compared with the control condition (Fig. 5c and d). EEG delta power in NREM sleep was also increased during the recovery period with $126 \pm 2$, $119 \pm 2$, $124 \pm 3$ and $121 \pm 2\%$ relative to the control condition after Biofeedback, Constant, Decreasing and Weibull protocols, respectively (Fig. 5e and f). The Constant protocol induced a significantly smaller increase in NREM sleep EEG delta power relative to the Biofeedback protocol ($P < 0.01$, $T_{55} = 2.81$).

**DISCUSSION**

This study shows that sleep restriction using enforced activity methods in rats can achieve similar functional deficits to that of an EEG-driven Biofeedback protocol, thereby offering non-invasive alternatives to this approach. Importantly, not all non-invasive protocols perform equivalently, and the ‘Constant’ protocol differed from the other protocols for several parameters of sleep–wake continuity and functional capacity.
Both Decreasing and Weibull protocols were superior to the Constant protocol at producing functional deficits and created a greater need for recovery sleep. The Weibull protocol may be the most broadly applicable protocol, particularly if extended periods of restriction involving saturation of homoeostatic sleep pressure are of interest.

The EEG-based Biofeedback protocol progressively reduced the capacity of rats to maintain bouts of wakefulness, an effect comparable to that measured in humans using the multiple sleep latency test (Bonnet and Arand, 2003). This protocol also produced deficits in performance for a SRLT, indexed by progressive task disengagement, as previously described (Loomis et al., 2015). These findings potentially show translational correspondence when compared with the effects of sleep deprivation in humans on psychomotor vigilance test performance (Lo et al., 2012; Van Dongen et al., 2003).

Survival analyses have previously been used to model naturally occurring wake bouts across a range of species (Lo et al., 2004). Furthermore, in humans with sleep apnea, survival analysis was used to quantify the relative risk of falling asleep during the multiple sleep latency test increased 2.11-fold in humans, comparable to the 2.02-fold increase observed following 11 h of sleep restriction in rats in the current study. Thus, the hazard ratio following a sleep restriction protocol in rodents may be useful for translation of sleep disturbance models to human conditions of excessive daytime sleepiness.

The effectiveness of a Weibull sleep restriction protocol was compared with that of a Biofeedback protocol and two other non-invasive methods (i.e. Constant and Decreasing protocols). All sleep restrictions reduced total sleep time to a similar extent, fragmented sleep and abolished REM sleep. However, for all parameters (except for REM sleep), non-invasive protocols were not as impactful as the Biofeedback protocol. The reduced NREM sleep loss and increased sleep bouts segregated the non-invasive protocol from the Biofeedback protocol. The Constant protocol allowed more and longer sleep bouts than either the Weibull or Decreasing protocols at the start of the sleep restriction, reflecting the relative inability of the Constant protocol to model the increase in homoeostatic sleep pressure that occurs over time.

With regard to recovery sleep, compared with the Biofeedback protocol, the Decreasing protocol had a statistically
similar effect on all measured parameters (i.e. total sleep time, sleep bout length and EEG delta power), while the Weibull protocol resulted in significantly less total sleep time during recovery, and similar effects on sleep bout length and delta power. In contrast, the Constant protocol resulted in both less total sleep time during recovery and less EEG delta power than the Biofeedback protocol. These differences suggest that the manner in which sleep disruption occurs is important for determining subsequent recovery.

While none of the sleep restriction protocols achieved a complete deprivation of sleep, the well-characterized consequences of sleep restriction were evident in the post-sleep restriction period, i.e. inducing recovery sleep, elevating EEG delta power and enhancing sleep continuity. These results are in accordance with previous reports using sleep restriction in rodents. A constant sleep restriction protocol in mice resulted in a similar magnitude of sleep pressure (Baud et al., 2013), with decreases in REM sleep and fragmented sleep patterns across the course of sleep restriction. Leenaars and colleagues also showed similar outcomes of sleep restriction when using a form of linear increasing protocol in rats (Leenaars et al., 2011).

One of the hallmarks of sleep restriction both in rodents and humans is the increasing failure to respond in a timely fashion to an imperative cue, as measured by a SRLT such as the psychomotor vigilance test (Christie et al., 2008; Lim and Dinges, 2008; Oonk et al., 2015). Accordingly, all treatment groups in the present study displayed control-level performance for the first 20 min of the test, before performance of sleep-restricted animals began to deteriorate. While the performance deficit induced by the Decreasing and Weibull protocols was of similar magnitude compared with the Biofeedback protocol, the Constant protocol led to a significantly smaller deficit. This may be the consequence of a relatively weaker sleep burden, allowing the animals subjected to this protocol to maintain task engagement for longer. The poor SRLT performance following sleep restriction may result from a lack of motivation to perform the task, or an inability to maintain attention to the imperative cues, or a combination of both effects. Further work would be required to provide additional insights.

In our study, locomotor activity and temperature measures were not different between sleep restriction protocols, with locomotor activity increased to a similar degree by all protocols and body temperature showing no differences from the control condition. In contrast for urinary corticosterone levels, Biofeedback and Weibull protocols were the only protocols not to induce a significant increase in urinary corticosterone. Interestingly, the Constant protocol had the largest increase in urinary corticosterone levels despite producing the least sleep and functional deficits. Corticosterone levels elicited by this protocol were similar to a sub-maximal dose (<0.5 mg kg⁻¹) of nicotine or a 10-min forced swim test (Loomis and Gilmour, 2010). It may be speculated that the Constant protocol was more stressful as a result of anticipation of such regularly applied stimuli, although in other contexts predictable versus unpredictable stressful stimuli have not been shown to elicit significant differences in the stress response (Sudha and Pradhan, 1993).

Ultimately, the aim of this work was to expand the array of experimental rodent models available to study sleep deficits of potential relevance to human disease. Thus, speculation regarding translational validity is warranted. Each of the protocols tested may have utility in different translational contexts, depending on the clinical presentation of the sleep deficit. For instance, shift workers are likely to be exposed to constant external environmental stimuli during their shift, whilst patients with sleep apnea may display more sleep fragmentation towards the end of the night when muscle atonia during REM sleep enhances the likelihood of upper airway collapse (Mokhlesi and Punjabi, 2012). Well-characterized sleep restriction protocols in rodents are needed to develop treatment interventions for different forms of sleep deficit. Further work detailing mechanistic differences between human conditions of sleep deficit and how they relate to animal models of sleep restriction may therefore be of great value.

**AUTHOR CONTRIBUTION**

A. M., S. L., G. G. designed the study; S. L. ran the studies; A. M., S. L. and B. E. performed data analysis; A. M., S. L., K. W., R. W. S. and G. G. were involved in data interpretation and manuscript writing.

**CONFLICT OF INTERESTS**

At the time of completion of these studies, A. M., S. L., B. E., K. A. W. and G. G. were employees of Eli Lilly & Co. Ltd.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Data S1. Supplementary methods.

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