Temporal trajectories of novel object recognition performance in mice exposed to intermittent hypoxia

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ABSTRACT   Intermittent hypoxia is one of the major perturbations of sleep-disordered breathing and has been causally implicated in neurocognitive deficits. However, the reversibility of such deficits is unclear.

Male C57BL/6J mice were exposed to either intermittent hypoxia or room air for 3–240 days, and then half were randomly selected and allowed to recover in normoxic conditions for the same duration of the previous exposure. A novel object recognition (NOR) test was performed.

NOR performance was stable over time in room air. Intermittent hypoxia induced significant reductions in recognition index that progressed over the first 45 days and stabilised thereafter. Normoxic recovery of recognition index was essentially complete and indistinguishable from room air in mice exposed to shorter intermittent hypoxia times (<90 days). However, significant residual deficits emerged after normoxic recovery following prolonged intermittent hypoxia exposures (p<0.01). In addition, gradual attenuation of the magnitude of recovery in recognition index occurred with increasingly longer intermittent hypoxia exposures (MANOVA p<0.0001).

Intermittent hypoxia during the resting period reduces NOR performance in a time-dependent fashion. Reversal of NOR performance deficits is unlikely after prolonged intermittent hypoxia duration. These findings suggest that early recognition of sleep apnoea and effective treatment are critical for restoration of the adverse cognitive effects of the disease.

Introduction

Obstructive sleep apnoea (OSA) is a highly prevalent clinical condition characterised by repeated episodes of upper airway obstruction during sleep, which imposes not only substantial cardiovascular and metabolic morbidity, but is also implicated in deleterious consequences on cognitive and behavioural functioning [1, 2]. Evidence pointing to structural brain changes in neural sites underlying cognitive functioning may ultimately account to the neuropsychological impairments [3–12]. However, the reversibility of such functional deficits has been highly variable, with studies showing major improvements while others were unable to detect any functional changes [13, 14].

An extensive body of work using rodent models has demonstrated that chronic exposures to intermittent hypoxia during the rest period, in the absence of significant sleep fragmentation, is accompanied by neurodegenerative changes, increased oxidative stress and inflammation, and impaired spatial learning in the Morris water maze [15–24], and that genetic or pharmacological manipulations of oxidative stress pathways attenuated intermittent hypoxia-induced deficits [25–27]. However, the recovery from such intermittent hypoxia-induced central nervous system structural and functional alterations has not been systematically investigated. We hypothesised that more chronic intermittent hypoxia exposures would be associated with a reduced capacity to reverse cognitive functioning alterations induced by intermittent hypoxia. Here we report on our findings using the novel object recognition (NOR) task as the functional reporter assay [28–31].

Methods

Mice

A total of 360 male C57BL/6J mice (20–22 g; 8 week-old) were purchased from Jackson Laboratories (Bar Harbour, ME, USA), housed in a 12-h light–dark cycle (lights on from 07:00 h to 19:00 h) at a constant temperature (26°C). Mice were housed in groups of four in standard clear polycarbonate cages, and were allowed access to food and water *ad libitum*. All behavioural experiments were performed during the light period (between 09:00 h and 12:30 h). Cages containing four mice were randomly assigned to either intermittent hypoxia or room air (room air) exposures. The experimental protocols were approved by the Institutional Animal Use and Care Committee at the University of Chicago (Chicago, IL, USA; ACUP Protocol # 72043) and are in close agreement with the National Institutes of Health Guide in the Care and Use of Animals. All efforts were made to minimise animal suffering and to reduce the number of animals used. Upon completion of the experimental paradigm mice were euthanised.

Intermittent hypoxia exposures

Animals were maintained in four identical commercially-designed chambers (300×200×200 mm; Oxycycler model A44XO, BioSpherix, Redfield, NY, USA) operated under a 12 h light–dark cycle (07:00 h–19:00 h) for 3, 7, 14, 30, 45, 60, 75, 90, 120, 180 or 240 days prior to behavioural testing. Oxygen concentration was continuously measured by an O_2 analyser, and was changed by a computerised system controlling gas outlets, as previously described [23, 25–27], such as to generate oxyhaemoglobin nadir values in the 65–72% range every 180 s. Ambient temperature was kept at 22–24°C. For controls (room air), normoxic gas was flushed periodically into the chambers at the same frequency as intermittent hypoxia. Upon completion of the designated intermittent hypoxia or room air exposures, two cages of mice were randomly assigned to normoxic recovery while the other two cages for each condition underwent novel object recognition testing as described below. As such there were four experimental conditions as follows: room air; intermittent hypoxia; room air with room air recovery; and intermittent hypoxia with room air recovery) for each of the 11 time points (3, 7, 14, 30, 45, 60, 75, 90, 120, 180, 240 days of exposure). In the recovery groups, after any of these exposures was completed, it was followed by an identical period to the exposure in normoxia (supplementary figure S1).

Novel object recognition test

The NOR test was initially described by ENNACEUR and colleagues [28–30] and others [31], and consists of a non-matching-to-sample task to evaluate preference for novelty, working and recognition memories. To avoid a learning effect from the repetition of behavioural tests, no baseline measures prior to the experimental protocol were performed and instead normoxic controls were employed. Mice were subjected to two consecutive days of habituation during the last two days of intermittent hypoxia for each of the exposures. During the habituation procedures, animals were placed in opaque rectangular cages (40×25×15 cm) covered with sawdust, without objects for 15 min·day^{-1}. In the third experimental day, two identical objects, O1 and O2 (9×8.5×8.5 cm), were placed in two corners of each of the cages, and 5 cm away from the walls. Animals were allowed to explore the objects and the environment for 5 min (training phase). Mice were then placed back to their home cages for 5 min while the objects O1 and O2 were replaced by one identical object (O3) and one different (T1) located in the same O1 and O2 positions. Mice were then

placed again in the testing cage and allowed to explore the familiar (O3) and the novel (T1) objects for 5 min. This period was denominated experimental phase 1 (EXP1). 24 h later, the animals were subjected to confirmation test 2 (EXP2), following the same procedure as EXP1. Of note, EXP2 is routinely used to confirm that the novel object has indeed become familiar during EXP1, and therefore provides EXP2 provides an additional level of confidence that the novel object was indeed assessed, explored and finally acquired by becoming familiar.

All experimental phases were conducted in a sound-isolated room. The objects and cages were cleaned with 70% ethanol solution to remove any odour cues. All experimental phases, were recorded from a vertical point of view with by a video camera suspended above the experimental area and interfaced with a video tracking system (HVS Imaging, Hampton, UK). Exploration was scored when the animals touched or directed their heads to at least 2 cm from the object for at least 5 s. Frequency and time spent exploring the novel (T1) and familiar objects (O3) were analysed and quantified by a blinded operator who was unaware of the mouse exposures. To avoid spatial and object bias, the position of objects was alternated between trials, and the choice of familiar *versus* novel object was changed from mouse to mouse. Mice have been shown to spend more time exploring the novel compared with the familiar object. However, the duration of each trial is important as a preference for the novel object only lasts for the first 5 min, after which time preference diminishes as both objects become familiar and are explored equally. The discrimination between novel and familiar objects was evaluated by the T1 and O3 exploration times. Only trials in which at least 10 s of exploration occurred were retained for analysis. To investigate the information retention by the animals, the recognition index (RI) was calculated as RI=T1/O3. The ability to recognise the novel object was considered to be present when the recognition index was superior to 1.0.

Data analysis

We used multivariate MANOVA model (SPSS 21, Chicago, IL, USA) to allow full assessment as to whether different exposure conditions and durations on NOR were present. The MANOVA model included: time exposures (11 time points) and two between factors: 1) room air and 2) intermittent hypoxia recovery. All F statistics are reported using Pillai's Trace. The interaction of three different factors, *i.e.*, time, condition and recovery were determined using a mixed model repeated measures MANOVA. To further elucidate the nature of identified interactions between groups, the data were analysed by one-way ANOVA. Firstly, overall statistical significance was determined for the exposure duration in the treatment groups (room air and intermittent hypoxia). In addition, statistical significance for recovery was assessed, followed by *post hoc* Holm–Sidak analyses, as needed. For all comparisons, a p-value <0.05 was considered to achieve statistical significance.

Results

Exposures to intermittent hypoxia were accompanied by progressive reductions in the usual body weight accrual trajectory which stabilised around 60 days exposures and thereafter (figure 1; n=7–8 mice per experimental group). Normoxic recoveries after intermittent hypoxia were accompanied by accelerated body weight gains, which exceeded the corresponding age-matched body weights of mice exposed to room air, particularly among the intermittent hypoxia-exposed mice subjected to more prolonged recovery periods (figure 1; p<0.002).

NOR performance was stable over time in room air-exposed mice and no differences emerged with advancing exposure durations or during recovery the normoxic periods of room air-exposed mice (figure 2). Indeed, the recognition index fluctuated in a relatively narrow margin across all room air-exposed groups with median values of approximately 3.0 (figure 2a). Intermittent hypoxia was accompanied by significant reductions in the recognition index performance that rapidly progressed over the first 45 days and appeared to stabilise thereafter, such that no further significant and readily detectable changes in recognition index occurred in intermittent hypoxia after 45 days or longer exposures (figure 2a). Normoxic recovery in intermittent hypoxia-exposed mice led to normalisation of the recognition index (indistinguishable from corresponding normoxic controls in the recovery period), but only among mice exposed to the shorter intermittent hypoxia exposures (<90 days; intermittent hypoxia-recovery *versus* intermittent hypoxia, p<0.0001; intermittent hypoxia-recovery *versus* room air-recovery, p>0.05). However, when mice were exposed to intermittent hypoxia for periods longer than 90 days and allowed to recover the same amount of time in room air as the preceding intermittent hypoxia exposure duration, significant residual deficits in the recognition index emerged (intermittent hypoxia-recovery *versus* room air recovery, p<0.01; intermittent hypoxia-recovery *versus* intermittent hypoxia, p>0.05). Although ANOVA procedures identified a trend towards improvement after 90 days intermittent hypoxia exposures during normoxic recovery, there were no significant differences in the recognition index between intermittent hypoxia and intermittent hypoxia-recovery at the 120-, 180- and 240-day time points. In addition, assessment of changes in recovery index over time revealed gradual attenuation in the magnitude of recovery with
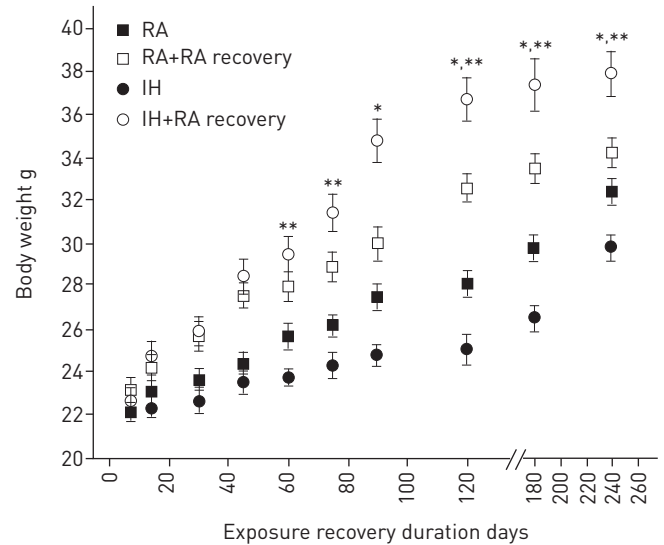
FIGURE 1 Changes in body weight in mice exposed to intermittent hypoxia (filled circles) or room air (filled squares) for 3–240 days and in mice undergoing normoxic recovery after such exposures for the same duration as corresponding initial exposure (open symbols). n=7–8 mice for each of the 22 experimental groups. Data are presented as mean±SD; *: room air–room air-recovery *versus* intermittent hypoxia–room air recovery p<0.001; **: room air *versus* intermittent hypoxia p<0.001.

increasingly longer intermittent hypoxia exposures despite corresponding lengthier durations of normoxia (MANOVA p<0.0001). Of note, the differences reported were not related to reduced duration of exploratory activity among intermittent hypoxia-exposed mice (figure 2b). However, reductions in exploratory activity emerged with age, and were therefore particularly apparent among the recovery groups undergoing longer duration exposures (p<0.01, ANOVA; figure 2b).

Discussion

This study shows that exposures to intermittent hypoxia mimicking moderate-to-severe OSA are accompanied by body weight reductions and cognitive deficits as illustrated by performance in the NOR task test. More importantly, our current study indicates that the longer the intermittent hypoxia exposures, the larger the effect on body weight will occur during application of treatment that is 100% effective and adherent (*i.e.*, normoxic recovery), which will then translate into increased rather than reduced body weight over time. Conversely, time-dependent reductions in NOR performance occur during the early timeframe of intermittent hypoxia exposures (initial 45 days or so) and appear to stabilise thereafter even if intermittent hypoxia exposures are continued. In contrast, when ideal therapy is provided (simulated here by returning mice to normoxia and as such mimicking ideally optimal continuous positive airway pressure therapy), the recovery of the recognition index to normal normoxic values is progressively attenuated such that for exposures lasting longer than 90 days, residual deficits in NOR performance remain significant after normoxic recovery periods of similar duration. If the present findings are applicable to patients suffering from OSA, then it becomes imperative that diagnosis and effective treatment occur as early as possible in the course of the disease, if the full benefits of therapy, as reflected by complete reversibility of the morbid phenotype, are sought.

Before we discuss the potential implications of our findings, there are some methodological constraints that need to be delineated. First, we applied a "recovery treatment" duration that was identical to the duration of intermittent hypoxia exposures. In this context, we not only began all of the intermittent hypoxia exposures at the same age (8 weeks old), but we also undertook the precaution to pair each experimental group with its normoxic control to address and account for potential age-related decrements in NOR performance, thus enabling accurate estimates of complete recovery. We cannot therefore extrapolate whether longer periods of recovery particularly among the lengthier intermittent hypoxia exposures might have yielded improved NOR performances than those recorded in our experiments. However, we undertook this approach because the few studies examining recovery from intermittent hypoxia-induced morbidity have traditionally used the same duration for recovery as that for exposures, and similar to the present results, shorter exposures traditionally yielded reversal to baseline control measures, while longer exposures were fraught with residual alterations in the end-organ function of interest [32–34]. Of note, we reported less than complete recovery of water maze performance in rats after
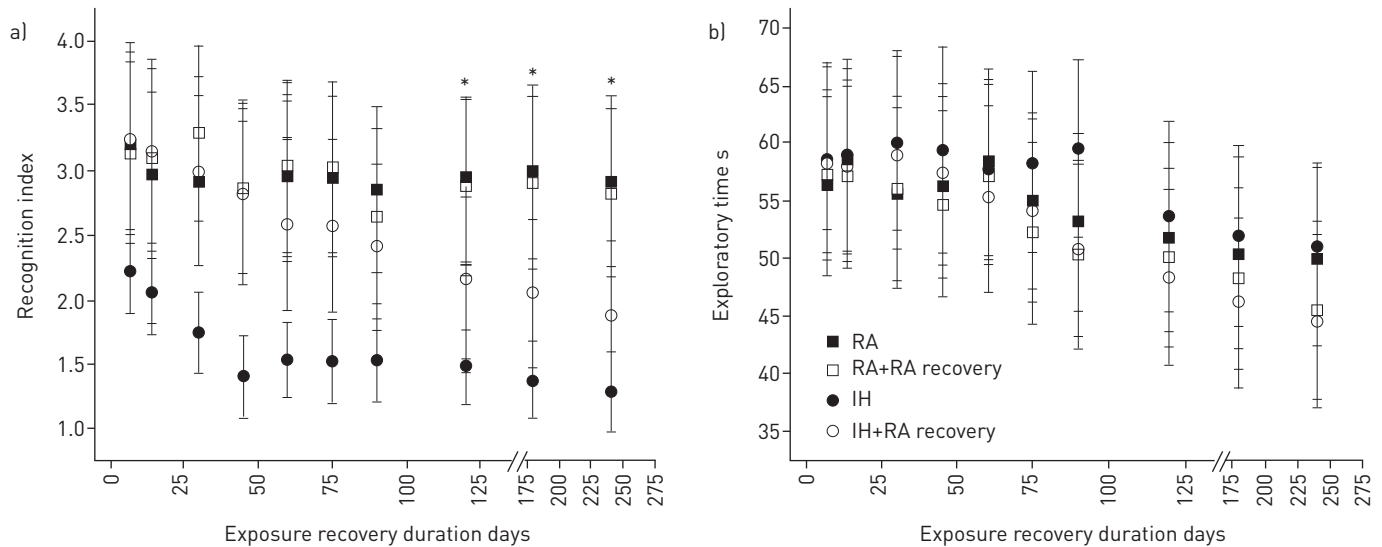
FIGURE 2 a) Changes in recognition index performance of novel object recognition task in mice exposed to intermittent hypoxia (filled circles) or room air (filled squares) for 3–240 days and in mice undergoing normoxic recovery after such exposures for the same duration as corresponding initial exposure (open symbols). Note that significant progressive declines in performance occurred in intermittent hypoxia till about 45 days exposure and did not continue to worsen thereafter. See text for more details. b) Duration of mouse exploratory activity during 5-min exposures to the novel object recognition arena in mice exposed to intermittent hypoxia (filled circles) or room air (filled squares) for 3–240 days and in mice undergoing normoxic recovery after such exposures for the same duration as corresponding initial exposure (open symbols). No differences emerged across experimental groups, but decline in the duration of exploratory activity emerged as a function of age in both intermittent hypoxia and room air-exposed mice (p<0.01, ANOVA). n=7–8 mice for each of the 22 experimental groups. Data are mean±SD. *: room air-room air-recovery *versus* intermittent hypoxia-room air recovery p<0.001.

intermittent hypoxia exposures lasting only 14 days, while no residual deficits were apparent in NOR performance in mice exposed to a more severe nadir oxyhaemoglobin saturation for the same duration [15]. Secondly, we should emphasise that our major aim was to elucidate reversibility patterns of a cognitive function in the mouse as a function of intermittent hypoxia exposure duration rather than specifically identify which brain regions or neurobehavioral functions are more likely to favourably respond to cessation of intermittent hypoxia. Thus, we selected NOR, as this test was not only readily feasible and reproducible but was also not excessively laborious and time-demanding, particularly when considering the extensive number of mice that needed to be evaluated and the constraints imposed by the intermittent hypoxia exposures themselves. We should, however, remark that although widely employed, NOR task testing is still under debate as far as which brain regions underlie its performance characteristics [28–31, 35, 36]. As such, we propose to view current results using NOR as a general and non- specific brain region reporter assay of cognitive performance, rather than attempt to extrapolate specific reversibility constructs in particular brain regions. Accordingly, future studies should contemplate exploration of additional neurobehavioral tests that are more specifically designed to evaluate particular neural pathways and functionalities.

Shorter exposures to intermittent hypoxia resulted in body weight accrual decelerations that became less prominent with more prolonged intermittent hypoxia exposure duration. This temporal trajectory could be construed as reflecting an adaptive set of processes that ameliorate energy balance over time [34, 37, 38], in an environment that was set at temperatures slightly below thermoneutrality [39]. Conversely, accelerated weight gain occurred during the normoxic recovery period following intermittent hypoxia and more prolonged intermittent hypoxia exposures resulted in an overshoot of body weight relative to room air controls, a phenomenon that became statistically significant among mice exposed to intermittent hypoxia for 60 days or longer. Based on a recent study from our laboratory [34], we postulate that such accelerated weight gain upon termination of prolonged periods of intermittent hypoxia may reflect a process whereby only partial, rather than complete metabolic recovery occurs. We should point out that accelerated weight gain has been reported in patients with OSA receiving effective treatment [40, 41]. However, it will be important to perform a more in-depth exploration of the metabolic characteristics and adaptations that occur during both intermittent hypoxia and recovery at different critical time points, as identified in the current experiments.

Based on the different recovery trajectories of NOR task performance, several items emerge as particularly important, and could be potentially extrapolated to the clinical setting: first, if we were to mix all of the

intermittent hypoxia-exposed mice subjected to normoxic recovery as a single cohort (a common feature of clinical trials since the duration of OSA is generally if not universally unknown), we would have found a very large variance in the responses to therapy as far as cognitive function is concerned. This latter feature has emerged as a major issue in deciphering whether patients with OSA are cognitively affected and whether such deficits if present are reversible. It is therefore likely that the large variability in disease duration and severity along with the usually short periods of treatment before re-assessment of cognitive functioning may account for the discrepant findings across studies. Secondly, our study reveals that there is a progressive decline in NOR performance during the early phases of intermittent hypoxia exposures which then reach a relatively stable nadir at around 45 days of intermittent hypoxia. These findings are not only congruent with the only published study examining this issue [16], but are also reminiscent of the changes in water maze performance and neurogenesis reported in rats exposed to a similar model of episodic hypoxia [42], and suggest that the cognitively deleterious pathways activated by intermittent hypoxia are ultimately balanced by later recruitment of protective pathways and adaptive mechanisms that stabilise the overall neuronal and glial networks and maintain function. Conversely, the more prolonged intermittent hypoxia exposures were fraught with lesser ability to recover from the NOR task performance decrements. We should emphasise that although normoxic controls were incorporated into the study for all groups, we cannot exclude with certainty the possibility that ageing processes may hamper the functional recovery among the lengthier exposures. This possibility is further buttressed by the reduced duration of exploratory activity among the older mice, irrespective of whether they have previously been exposed to intermittent hypoxia or room air. However, the mechanisms underlying the reduced functional recovery capacity are potentially the most important elements to be identified in future studies to enable improved outcomes of OSA therapy through adjuvant interventions targeting such mechanisms.

In summary, we have shown that body weight and cognitive function exhibit temporally dependent trajectories in response to intermittent hypoxia exposures simulating OSA in mice. Furthermore, we also show that improved outcomes will emerge if the timing of reversal of intermittent hypoxia to room air occurs early in the duration of intermittent hypoxia, and that beyond a particular duration of intermittent hypoxia, complete recovery appears to be unlikely. If recapitulated in humans, these findings have far reaching implications for the detection and treatment of OSA.

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