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Impaired spatial working memory and altered choline acetyltransferase (CHAT) immunoreactivity and nicotinic receptor binding in rats exposed to intermittent hypoxia during sleep.

Barry W. Row^a, Leila Kheirandish^a, Yu Cheng^a, Peter P. Rowell^b, and David Gozal^{a,b}

^a Kosair Children's Hospital Research Institute, Department of Pediatrics, University of Louisville, Louisville, KY 40202

^b Department of Pharmacology & Toxicology, University of Louisville, Louisville, KY 40202

Abstract

Exposure to intermittent hypoxia (IH), such as occurs in sleep disordered breathing (SDB), is associated with cognitive impairment, neurodegeneration, oxidative stress, and inflammatory responses within rodent brain regions such as the basal forebrain. In this region, damage to cholinergic neurons correlates with working memory deficits in a number of neurodegenerative disorders, suggesting that degeneration of cholinergic systems may also contribute to the working memory impairments observed after IH exposures. We therefore examined basal forebrain choline acetyltransferase (CHAT) immunohistochemistry, nicotinic receptor binding in the prefrontal cortex (PFC), and working memory, in male rats tested on a delayed matching to place (DMP) task in the water-maze following exposure to either room air (RA) or intermittent hypoxia (IH; alternating 90 second epochs of 21% and 10% O₂ during sleep). IH-treated animals displayed impaired working memory with respect to controls, along with significant reductions in CHAT stained neurons in the medial septal nucleus, in both the vertical and horizontal limbs of the diagonal band, and the substantia innominata after 14 days of IH exposure. In addition, increases in nicotinic binding and receptor affinity in the PFC was observed after 14 days of IH exposure. Thus, a loss of cholinergic neuronal phenotype in the basal forebrain may contribute to the cognitive impairments associated with CIH exposure. However, compensatory mechanisms may also be activated in other brain regions, and may provide potential therapeutic targets for the cognitive impairments associated with SDB.

Introduction

Sleep Disordered Breathing (SDB) is an increasingly common condition that imposes substantial neurocognitive morbidity in both adult and pediatric populations [6,8,60]. Although the exact nature of the cognitive deficits associated with SDB is still a matter of debate, numerous studies have associated SDB with attention deficits as well as with dysfunction in higher-level cognitive skills that underlie the behavioral flexibility associated with problem-solving and reaching a goal, commonly referred to as "executive functioning" [2,9,27,50,52, 68]. These patterns of behavioral morbidity are thought to represent dysfunction of fronto-subcortical systems involved in attention and executive functions, particularly those that

Corresponding Author: Barry Row, Ph.D., Kosair Children's Hospital Research Institute, University of Louisville, 570 South Preston Street, Suite 321, Louisville, KY 40202, USA., Phone: 502-852-4073, Fax: 502-852-2215, Email: b0row001@louisville.edu.

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involve the dorsal prefrontal (PFC) cortex, as well as regions which share substantial reciprocal connections with the PFC, such as the hippocampus [4,8,18,19].

Recent developments in animal models mimicking the intermittent hypoxia (IH) that characterizes patients with sleep apnea supports a critical role for IH in the induction of substantial brain dysfunction. Indeed, learning and memory deficits, regionally-selective neurodegeneration and apoptosis, alterations in neurotransmitter systems, increased inflammation and gliosis, and increased levels of reactive oxygen and nitrogen species have all been reported [17,23,29,36,40,41,58,66,70,72]. Therefore, the collective evidence derived from both clinical and animal studies suggests that the intermittent hypoxia encountered by patients suffering from SDB contributes to functional disruption of hippocampal, prefrontal, and subcortical circuitry, and may play an important role in the neurocognitive deficits observed with these patients.

Spatial working memory tasks have been used to assess executive function in rodents [61] and are largely dependent on an intact and fully functioning prefrontal cortex and hippocampus [24,28,39,55,69]. Modulation of prefrontal and hippocampal function by forebrain cholinergic systems plays a key role in these processes, as both the prefrontal cortex and hippocampus receive substantial innervation from cholinergic cell bodies located in the basal forebrain. Indeed, loss of such forebrain cholinergic neurons has been correlated with working memory deficits in a number of neurodegenerative disorders [12,49,54]. The increased oxidative damage and neurodegenerative changes within the basal forebrain of mice exposed to intermittent hypoxia suggest that degeneration of cholinergic systems may also contribute to the working memory impairments observed after IH exposures [66,71,72]. We therefore used our now well established rodent model of intermittent hypoxia that reproducibly mimics the recurring hypoxic events that occur in severe forms of SDB in humans [29,58], and examined the effects of IH on spatial working memory, basal forebrain choline acetyltransferase (CHAT) immunohistochemistry, and nicotinic receptor binding in the prefrontal cortex (PFC) of male rats.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (175–200 g) were obtained from a commercial supplier (Charles River, Portage, MI). Animals were housed in groups of four in clear polycarbonate cages (55 x 45 x 35 cm) with food and water available *ad libitum*. Rats were randomly assigned to be housed in IH (n=12), or room air (RA, n=12). Animals were kept on a 12-hour light/dark schedule (6:00 am–6:00 pm), and all behavioral testing was conducted during the light phase. The experimental protocols were approved by the Institutional Animal Use and Care Committee and are in close agreement with the National Institutes of Health guide for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Intermittent Hypoxic Exposures

Animals were housed in identical commercially designed chambers (30"x20"x20"; Oxycycler model A44XO, BioSpherix Instruments, Redfield, NY) operated under a 12 hour light-dark cycle (6:00am – 6:00pm). Oxygen concentration was continuously measured by an O₂ analyzer, and the desired gas profile was administered by a computerized system regulating gas outlets [29]. Intermittent hypoxic exposures designed to mimic the oxyhemoglobin desaturations occurring in patients with SDB, consisted of a cyclical pattern of 10% and 21% oxygen every 90 sec (IH; 26 cycles/hour) during the light phase throughout the duration of the experiment, while control rats were exposed to 21% oxygen (RA) throughout. The nadir

chamber oxygen concentration was 10%, and was reached within 30 sec for each cycle, such that the overall time spent at 10% oxygen during each cycle was 90 sec, with return to normoxia requiring approximately 15 sec. Ambient temperature was kept at 22–24°C.

Morris Water Maze

The Morris water maze can be configured to test performance that reflects types of learning and memory traditionally defined as spatial reference, spatial working, and non-spatial reference. The maze consisted of a white circular pool, 1.8 m in diameter and 0.6 m in height, filled to a level of 35 cm with water maintained at a temperature of 27°C. Pool water was made opaque by addition of 150 ml of non-toxic white tempera paint. A Plexiglas escape platform (10 cm in diameter) was positioned 2 cm below the water surface and could be placed at various locations throughout the pool. A circular white curtain surrounded the pool and extended from the ceiling to the floor. For spatial tasks that depend on allocentric strategies, such as the one used in the present study, extramaze cues were hung from the curtain at fixed locations. Maze performance was recorded by a video camera suspended above the maze and interfaced with a video tracking system (HVS Imaging, Hampton, UK). Albino rats were tattooed with a black mark to allow video tracking.

Behavioral Testing

Spatial working memory was assessed in a Morris water maze using a modified delayed matching-to-place (DMP) procedure [31,64]. During each daily session, rats received 4 trials consisting of an initial training trial (information trial), and 3 subsequent retention or choice trials. Time intervals between trials 1 and 2 was 10 min. The time interval between trials 2, 3, and 4 was 30 sec. For each subsequent 4-trial session, the hidden platform was relocated quasirandomly to different quadrants and locations of the water maze. Performance was assessed by two measures, namely mean escape latencies and swim path distances. To assess working memory performance during DMP training, mean escape latencies and swim distances were derived from trial one (acquisition trial) and trial 2 (retention trial) of each training session and the savings ($T1 - T2$) were analyzed by a one-way repeated measures analysis of variance (ANOVA), followed by Newman-Keuls *post-hoc* tests when appropriate. A *p* value <0.05 was considered statistically significant.

Immunohistochemistry

Upon completion of behavioral testing, rats were anesthetized with pentobarbital (50 mg/kg i.p.) and perfused transcardially with 200 ml PBS at ambient temperature, and then with 2.5% paraformaldehyde in cold PBS containing 5% sucrose, pH 7.4. The brain was removed immediately from the skull after perfusion and placed overnight in a fixative containing 1% paraformaldehyde in PBS and 30% sucrose at 4°C. Coronal sections (40 microns) of the brain were initially incubated in 0.3% H₂O₂ for 30 minutes, washed several times in PBS, and blocked with a PBS/0.4% Triton X-100/0.5% TSA (Tyramide Signal Amplification, Perkin Elmer Life Sciences, Boston MA) blocking reagent/10% normal goat serum (Vector Laboratories, Burlingame CA) for 1 hour. Forebrain sections were serially incubated with primary antibody for choline acetyltransferase (CHAT, Chemicon, Temecula, CA; dilution - 1:1000). Sections were then incubated at room temperature for 1 hour in biotinylated anti-rabbit antibody (Vectastain Elite ABC kit, Burlingame CA; 1:600) in a PBS/0.5% TSA blocking reagent /10% goat serum solution. After 3 5-min washes, sections were incubated at room temperature with streptavidin-horseradish peroxidase diluted 1:100 in PBS/0.5% TSA blocking reagent. Subsequently, the sections were incubated with fluorescein tyramide reagent (green) diluted 1:50 in amplification diluent (Perkin Elmer Life Sciences, Boston, MA) for 2 minutes. Sections were then washed in PBS, and mounted onto glass slides. Negative controls were prepared by either omitting the primary or the secondary antibodies for either of the

primary antibodies. Sections were prepared from 4 animals from each of the treatment groups, and were visualized using a fluorescent microscope by an investigator who was blinded to the sample source.

Cell Counting

ChAT immunostained cells were readily identifiable as large cells with immunostained perikarya and radiating dendrites. As the ChAT immunostained cells were easy to detect due to their size and general lack of overlap in the tissue, it was possible to count all labeled cells apparent in a section and it was not therefore necessary to make an estimate of cell density. Isolating cells particular to the areas of interest was not difficult, as cells from each section tended to cluster within the respective regions.

Cell counts were performed with the aid of a video-based, computer-assisted image analysis system (Stereo Investigator software; Microbrightfield, Inc., Colchester, VT). Counts of ChAT-immunopositive cell profiles were performed at six rostro-caudal levels approximately 100–600 μm anterior to the decussation of the anterior commissure. To ensure that corresponding areas were analyzed in brains from different rats, cell counts were performed in predetermined regions of the Medial septum/vertical diagonal band (MS/vDB), horizontal diagonal band (hDB), and the basal portion of the substantia innominata (SIB), as defined by Paxinos and Watson (1998). Cell profiles were counted within the a particular region by superimposing a calibrated grid over the areas of interest. Corresponding fields were analyzed in each section by capturing the image viewed under a 10 $\times$ objective and counting the number of immunostained cell profiles. Sections were coded and analyzed blindly. The counts obtained from each of the six sections were added and normalized per total area sampled for each brain. Means obtained from four control and four IH-exposed animals were then compared and analyzed for statistical significance of differences. Since sections of preset, constant thickness were used and morphometric analysis of brain sections from control and IH-exposed animals did not reveal detectable differences in section dimensions we assumed that geometry of reference space in the sampled regions did not change as a result of intermittent hypoxic exposure. Furthermore, since for the purpose of this study we were interested in comparison of relative rather than actual cell number densities, no attempts were made to convert profile counts to actual cell numbers.

Nicotinic Acetylcholine Receptor (nAChR) Binding

In a separate set of experiments, nicotinic receptor density (B_{max}) in homogenized prefrontal cortex samples from rats exposed to 0 (room air or RA), 3, 7, and 14 days of IH was determined by a modification of the displacement assay outlined by Houghtling et al. (1995) using a saturating concentration of 5 nM [3H]-epibatidine ($K_d=0.4$ nM) [32]. The total number of specific [3H]-epibatidine binding sites was determined by the difference between total and non-specific binding (average of triplicates) and related to the tissue protein measured by the method of Bradford [10].

Results

Working Memory

Figure 1 illustrates that adult rats exposed to intermittent hypoxia were impaired with respect to control animals housed in room air on a delayed matching to place (DMP) procedure in the water maze. Peak impairment of working memory was observed after 5–7 days of IH exposure ($df(1,14)=18.808$, $p<0.001$ and $df(1,14)=5.914$, $p<0.029$ for pathlength and latency, respectively), with a partial recovery of function observed at 12–14 days, although the IH-treated animals were still impaired with respect to controls ($df(1,14)=10.453$, $p<0.006$ and $df(1,14)=4.785$, $p<0.046$ for pathlength and latency, respectively). No significant group

differences were observed after 1 day of exposure and no significant differences were found between groups in regards to swim speeds or on the latency and pathlength to locate a cued platform (data not shown), consistent with our previous findings that group differences were not due to sensorimotor impairments [29,57,58].

CHAT Immunohistochemistry

To assess whether IH was associated with changes in forebrain cholinergic neurons, immunohistochemistry for choline acetyltransferase (CHAT) was used as a cholinergic marker in forebrain sections from the medial septum (MS), the horizontal and diagonal band of Broca (vDB and hDB), and the substantia innominata (SI) of rats exposed to 14 days of IH and RA (Figure 2). Figure 3 illustrates that decreases in CHAT positive cells occurred following 14 days of IH exposure in the medial septum/ventral diagonal band complex (MS/vDB, $df(1,6) = 10.19$, $p < 0.019$), $p < 0.001$) and the hDB/SI ($df(1,6) = 8.53$, $p < 0.037$) in each of the brain regions examined.

Nicotinic Receptor Binding Assays

Figure 4 illustrates that exposure to IH resulted in a time-dependent increase in nicotinic acetylcholine receptor (nAChR) binding within the prefrontal cortex (panel A). Significant increases in Bmax were observed after 14 days of IH exposure ($df(3,12) = 5.25$, $p < 0.015$), in comparison to both RA controls and animals exposed to 3 days of IH. In addition, the overall nAChR Kd within the prefrontal cortex (panel B) was increased after 14 days of IH exposure ($df(3,12) = 11.933$, $p < 0.001$), indicating that exposure to IH increased receptor affinity within the in the prefrontal cortex as well as overall nAChR binding.

Discussion

Patients suffering from moderate to severe SDB suffer from a pattern of cognitive deficits that is consistent with disordered functioning of hippocampal-prefronto-cortical circuitry. Developmental models have established that exposure to the episodic hypoxia that characterizes SDB is associated with long-term alterations in working memory, even after cessation of the hypoxic challenge [17,35]. In the present study, we found that exposures to intermittent hypoxia also induces spatial working memory deficits in the adult rat, and is associated with decreased expression of CHAT positive neurons in the basal forebrain, as well as with increased nAChR binding and receptor affinity in the PFC of adult rats. These findings suggest that alterations of forebrain cholinergic systems, either directly, or through interactions with other neurotransmitter systems, may contribute to the cognitive deficits associated with IH exposures during sleep.

Patients with SDB display increases in systemic and circulating markers of oxidative stress, and recent evidence derived from rodent models clearly implicates oxidative stress as one of the most likely mechanisms underlying the adverse effects of IH on neuronal survival and function [14,38,56,58,67,70,72]. For example, severe hypoxic challenges are associated with decreases in forebrain and brainstem ChAT immunohistochemistry, suppression of hippocampal ChAT activity, as well as up-regulation of neuronal muscarinic receptor sites [21,51,65]. Cholinergic neurons in the basal forebrain are critically important for both proper hippocampal (HPC) and frontal cortical functioning, and are reportedly susceptible to the chronic excitotoxic processes and to the oxidative stress and inflammatory responses recruited by IH [11,21,46]. Indeed, the function of the forebrain cholinergic system is altered during normal aging, as well as in other pathological conditions associated with substantial oxidative stress, such as Alzheimer's disease [3,5]. The intermittent hypoxia paradigm used herein has also been reported to increase oxidative damage in sleep/wake centers of the basal forebrain, as well as being associated with long-term working memory deficits and alterations in

dopaminergic systems [17,66,72]. These reports are consistent with the view of neurodegenerative disorders as sharing similar pathogenetic mechanisms with the aging brain [42], and suggest that many of the neuronal changes occurring in IH may represent a form of accelerated brain aging through oxidative stressors.

Although the findings reported herein clearly indicate that changes in forebrain cholinergic systems occur following IH exposures, it is important to note that changes in other neurotransmitter systems are also likely to be involved. For example, dopaminergic mechanisms have been implicated in some of the behavioral effects of early post-natal IH exposure [17], including the long-term consequences associated with such exposures [35]. The basal forebrain cholinergic system has been clearly recognized as playing a major role in the modulation of fronto-cortical dopaminergic systems, as well as in working memory performance, suggesting that cholinergic systems may interact with dopaminergic circuits, and thereby may be involved in the behavioral phenotype associated with IH exposures [7,33,37, 47]. Additionally, cholinergic pathways act synergistically with glutamatergic transmission to regulate forms of synaptic plasticity such as long-term potentiation (LTP), the latter being adversely affected after IH exposures [30,34,53]. Finally, it is important to note that it is at present unclear whether these changes represent actual cell loss, or merely alterations in cellular phenotype, such as has been reported following exposure to hypoxia [22]. Given the importance of cholinergic basal forebrain neurons in cortical activation and behavioral states, it will be essential for future studies to identify the precise way in which such changes influence these processes [13,43].

In this study, we found that prolonged exposures to IH resulted in increased nAChR binding and receptor affinity in the PFC of the rat. This suggests that in addition to the adverse effects induced by IH, compensatory or homeostatic mechanisms may also be activated by such exposures. Nicotinic receptor regulation is a complex process, as receptor up-regulation has been reported following desensitization, inactivation of receptor function, and loss of cholinergic input [1,16,20,26,48,59,63]. The present study was confined to the examination of changes in nicotinic binding in the PFC, as central nicotinic receptors have been shown to play a key role in the attentional processes enabling working memory trace access during retrieval, as well as in the modulation of synaptic plasticity [25,45]. However, it was not our goal to explore how changes in nAChR distribution within neural networks would affect cognitive function, clearly a valuable and worthy aim that is well beyond the scope of the present study. Thus, future assessment is needed on changes in distribution, sub-type expression of specific nicotinic and muscarinic cholinergic receptor subtypes and their functional impact in the context of SDB [44].

Finally, we have proposed that the observed behavioral deficits associated with IH exposures are primarily mediated through impairments in prefrontal and/or hippocampal functioning [8,9]. The PFC, in conjunction with additional brain areas such as the hippocampus, amygdala, and caudate, has been argued to play a central role in a number of cognitive and executive processes, including working memory, decision-making, inhibitory response control, attention, and the temporal organization of goal-directed behavior [15,44]. Recent findings further suggest that these cognitive processes can be ascribed to functionally heterogeneous regions of the PFC. For example, the dorso-medial PFC has primarily been implicated in response selection and temporal processing of information, while ventral regions of the medial PFC have been implicated in more attention-related functions, such as stimulus detection, set-shifting, and behavioral flexibility [15]. Although our data clearly support that IH exposures are associated with significant learning and memory impairments, it is at present unclear whether the observed deficits are due to attention impairments, impaired formation of egocentric and/or allocentric spatial maps, or multiple prefrontal functional impairments, since our behavioral assessment protocols require the coordinated action of both frontal and temporal lobe structures

[62]. It will therefore be essential to extend current findings to other experimental paradigms of learning, and use more refined approaches that specifically examine particular aspects of cognitive and executive processes to determine the nature of PFC dysfunction occurring in our model of SDB.

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*, IH vs. RA, $p < .01$; #, IH vs. RA $p < .05$, ANOVA ($n=8$ per group)

Figure 1.

Mean pathlength savings (Acquisition trial, T1 – Retention trial, T2) in 16 rats exposed to RA ($n=8$) or IH ($n=8$) after 1, 5–7, and 14 days of IH (*, IH vs. RA, $p < .01$).

Figure 2.
Representative immunostaining for CHAT in medial septum (MS), diagonal band of Broca (DB), and the substantia innominata (SI) of rats exposed to RA or 14 days of IH.

(*, $p < .01$, ANOVA, #, $p < .05$, ANOVA; vs. RA)

Figure 3.

Mean number of CHAT positive cells in the MS/DB and SI of rats exposed to RA or 14 days of IH (*, IH vs. RA, $p < .01$; #, IH vs. RA, $p < .05$; $n=4$ per group).

(*, $p < .02$, ANOVA, #, $p < .001$, ANOVA; vs. RA)

Figure 4.

Mean nicotinic binding (Bmax, panel a) and affinity (Kd, panel B) in the PFC of rats exposed to RA (n=4) or IH (n=4) after 1, 5–7, and 14 days of IH (*, IH vs. RA, $p < .02$; #, IH vs. RA, $p < .001$; n=4 per group).