

# Physostigmine Reverses Cognitive Dysfunction Caused by Moderate Hypoxia in Adult Mice

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**BACKGROUND:** Cognitive changes associated with moderate hypoxia in rodents may result from the diminished functioning of central cholinergic neurotransmission. We designed this study to examine whether treatment with physostigmine (PHY), an acetylcholinesterase inhibitor, could improve the impairment of working memory after hypoxic hypoxia.

**METHODS:** We randomized 90 Swiss Webster, 30–35 g mice (6–8 wks) to three hypoxia groups at fraction of inspired oxygen,  $FiO_2 = 0.10$  (1. no treatment; 2. PHY 0.1 mg/kg intraperitoneally administered immediately before; or 3. after hypoxia), or to two room air groups (given either no treatment or PHY after an insult). An object recognition test was used to assess short-term memory function. The object recognition test exploits the tendency of mice to prefer exploring novel objects in an environment when a familiar object is also present. During the 15 min training trial, two identical objects were placed in two defined sites of the box. During the test trial performed 1 h later, one of the objects was replaced by a new object with a different shape. The time spent exploring the two objects was automatically recorded by a video camera and associated software. The performance was analyzed with ANOVA, followed by *post hoc* comparisons using the Newman-Keuls test when appropriate. *P* values  $<0.05$  were considered significant.

**RESULTS:** Untreated mice subjected to hypoxia at  $FiO_2 = 0.1$  spent significantly less time exploring a novel object on testing day 1 than did untreated mice breathing room air. Performance of the mice subjected to hypoxia, who received physostigmine after, but not before, the insult did not differ from the control group.

**CONCLUSION:** Moderate hypoxia impairs rodents' performance in a working memory task. It appears that changes are transient, because the cognitive functioning of the mice returned to the baseline level 7 days after treatment. Postinsult administration of PHY prevented deterioration of cognitive function. An increased level of acetylcholine in the central nervous system may be responsible for the improved performance of the hypoxia-treated mice.

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**D**ecreased oxidative metabolism in the brain causes cognitive dysfunction, possibly due to impairment of central neurotransmission (1). In this view, any perioperative somatic disturbance (e.g., hypotension, hypoxemia, anemia, etc.) interfering with supply, use, or removal of substrates for metabolism would lead to a relatively stereotyped constellation of symptoms. A "final common neurotransmitter pathway" may underlie many of the etiologies of postoperative cognitive impairment (2).

Although abnormalities in several neurotransmitter systems were implicated in the development of delirium, reduced cholinergic function (and, possibly, increased dopaminergic activity) is the most likely

candidate (3,4). Clinical studies support the hypothesis that impairment of central cholinergic transmission contributes to delirium (5). The integrity of central cholinergic systems is disrupted in animal models of impaired learning and memory that are induced by scopolamine or atropine (6). Reduced levels of acetylcholine (ACh) and diminished cholinergic transmission have been found in response to hypoxia (7,8), which produces cognitive dysfunction (9,10).

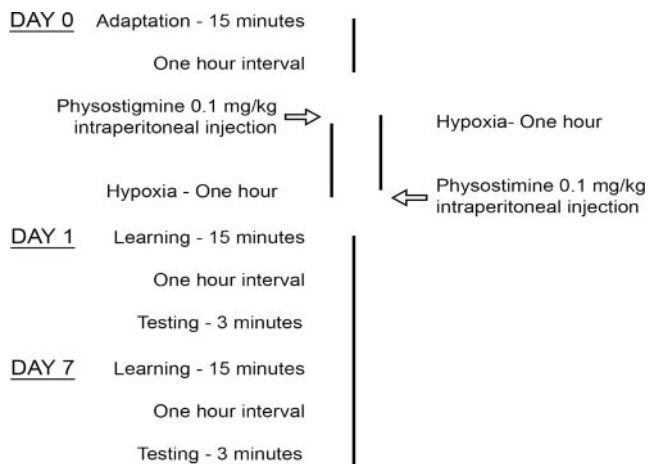
Facilitation of cholinergic neurotransmission may counteract functional deficits in mammals with impaired cholinergic function. Phenserine, a cholinesterase inhibitor, improved learning in a spatial memory task when cholinergic function had been impaired by scopolamine (11). Systemic application of physostigmine (PHY), a clinically relevant cholinesterase inhibitor, normalized learning-impaired behavior in transgenic mice expressing high catalytic activity of human acetylcholinesterase (12). Cholinomimetic drugs such as rivastigmine, tacrine, and donepezil are widely used to treat cognitive dementia related to reduced central cholinergic transmission (13). Hence, it is reasonable to assume that increasing concentrations of ACh in the central nervous system

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**Figure 1.** Experimental schedule.

(CNS) after injury might improve cognitive function that is diminished as a result of that injury.

The present study was designed to determine whether administration of PHY before or immediately after the hypoxic episode prevents hypoxia-induced cognitive dysfunction. We examined memory and learning impairment in mice subjected to moderate hypoxia for 1 h. PHY, a centrally acting cholinesterase inhibitor, may improve mouse performance if ACh levels in the CNS are responsible for cognitive changes associated with the moderate hypoxia reported earlier (10). We used an object recognition test (ORT) to assess deficits in short-term memory on Days 1 and 7 after a hypoxic episode. ORT is widely used in rodents to test recognition memory allowing the assessment of acquisition, consolidation, and retrieval of information (14,15). It is based on the innate tendency of mice to explore an unknown object in preference to a familiar one.

## METHODS

The study was approved by the Institutional Animal Care and Use Committee of the NY University Medical Center. Swiss Webster male mice (30–35 g, 6–8 wk old) were used in all experiments. Mice were housed in groups of five per cage with a 12-h light/dark cycle and were tested in the normal day-light phase. Food and water were available *ad libitum*.

The ORT apparatus consists of a black Plexiglas open field square arena (48 cm × 48 cm) with 18-cm high walls. The light intensity in the arena is about 30 lux, and there are no areas of shadow within the arena. At the beginning of all sessions, mice were released in the center of the arena with their heads oriented away from either object. The objects to be discriminated were made of glass, plastic, or metal and were in duplicate. They were heavy enough so that they could not be displaced by mice. After each session, animals were returned to their home cages, while the arena and objects were cleaned with water to avoid any possible odorant cues.

Figure 1 shows the sequence of events. In an adaptation session 1 h before hypoxia, mice were

placed in the arena in the presence of objects (these objects were used only for habituation) for 15 min (see below). During the training session 1 day after adaptation and hypoxia treatment, mice were permitted to explore two new identical objects for 15 min. For the testing session performed 1 h later, animals were presented with a fresh version of the object seen during training (the familiar object) and a new object, which they had never seen (the novel object), for a 3-min testing period. Preliminary studies in our laboratory found that longer exposure lessened mice's interest toward novel objects. Care was taken to avoid object and place preference by changing the role of objects (familiar versus new object) and their position in the box during each individual testing session on Days 1 and 7.

The amount of time spent exploring the objects is automatically monitored by a tracking system (SMART, San Diego Instruments, San Diego CA), which records the time spent (in seconds) in the zones containing objects. Each zone represents 15% of the total surface area of the arena as defined by the SMART system. A Recognition Index (RI) quantifies the exploratory behavior by measuring the time spent exploring the novel object divided by the total time spent exploring both objects multiplied by 100. As was determined in our pilot studies, neurologically intact mice spend significantly more time exploring the novel object when tested 1 h after the learning session. In contrast, mice with short-term memory deficits do not exhibit significant differences in object differentiation, presumably due to impaired ability to discriminate between familiar and novel objects.

Experimental data were also used to determine an Attention Index, which is defined as the time spent exploring either the novel or familiar object divided by the total length of the testing period (3 min) × 100. The Attention Index was similar across all treatment groups of mice suggesting that all mice were equally motivated and able to explore objects.

On Day 0 of the study, five animals at a time were randomly assigned to treatment groups as follows: 1) Fraction of inspired oxygen,  $FiO_2 = 0.21$ , 2)  $FiO_2 = 0.21 + \text{PHY } 0.1 \text{ mg/kg}$ , 3)  $FiO_2 = 0.10$ , 4)  $FiO_2 = 0.10 + \text{PHY } 0.1 \text{ mg/kg}$  before hypoxia, and 5)  $FiO_2 = 0.10 + \text{PHY } 0.1 \text{ mg/kg}$  after hypoxia. PHY was dissolved in 0.9% sodium chloride and administered intraperitoneally (IP), immediately before or after hypoxic insult. PHY was administered immediately after the experiment in the control group. Animals were used only once and killed after behavioral testing. Data from three experimental sets were combined for analysis ( $n = 15$ ). The experimental procedure was described elsewhere (10). Briefly, five animals at a time were placed in an air-tight chamber where they were exposed to the experimental gas mixture for 1 h. Oxygen concentrations were measured continuously using a capnometer (Datex-Ohmeda Capnomac Ultima, Helsinki, Finland) and confirmed with an oxygen analyzer (Bacharach

Oxor II, New Kensington, PA). Mice were allowed 24 h to recover from the experimental treatment and then were tested in the ORT apparatus on Days 1 and 7.

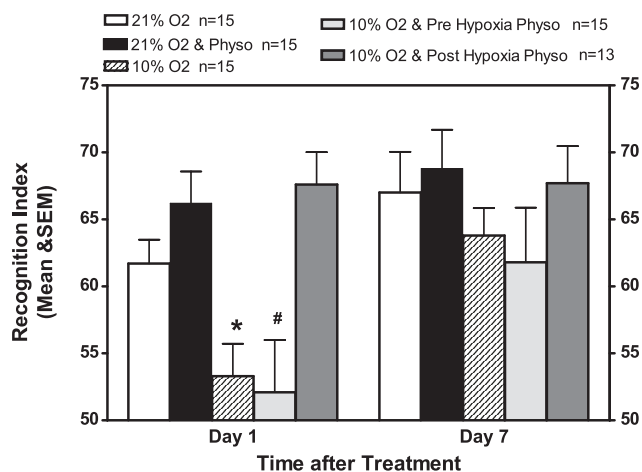
We assessed arterial oxygen saturation in a separate group of mice by using a Mouse-Ox pulse oximeter (STARR life Sciences, Allison Park, PA). These mice were anesthetized with ketamine (150 mg/kg)/xylazine (10 mg/kg) and then held loosely by the scruff of the neck while a pulse oximetry probe was placed on either thigh. Anesthesia was required, since awake mice continued to move after application of the rodent mask used to regulate oxygen concentration. Pulse oximetry data were recorded after a short period of adaptation.

The RI was the dependent variable. The experiment was designed as a  $2 \times 2 \times 2$  factorial with oxygen levels ( $F_{iO_2} = 0.21$  and  $F_{iO_2} = 0.10$ ) and (PHY) (treatment versus no treatment) as the between-group factors and test day (Days 1 and 7) as within group factor. Repeated measures ANOVA was used for comparisons of the session and treatment effects, followed by analysis of simple main effects by *post hoc* comparisons using Newman-Keuls test as appropriate. The results are presented as means  $\pm$  SEM. The confidence limit of  $P < 0.05$  was considered statistically significant.

## RESULTS

Hypoxia and PHY treatment were well tolerated by 88 of 90 randomly selected mice. One animal died during hypoxia treatment within approximately 40 min of exposure (PHY, hypoxia group). The second animal died within 20 min after PHY injection, possibly due to improper IP injection (PHY, hypoxia group). The oxygen saturation measured in separate groups of mice was (mean  $\pm$  SEM):  $F_{iO_2} = 0.21$  ( $n = 27$ ) =  $91.2\% \pm 0.3\%$  and  $F_{iO_2} = 0.10$  ( $n = 27$ ) =  $71.2\% \pm 1.5\%$ .

A  $2 \times 2 \times 2$  fixed effects analysis of variance revealed significant main effects of PHY treatment [ $F(1,54) = 10.1, P < 0.005$ ] and day of testing [ $F(1,54) = 8.21, P < 0.01$ ]. Oxygen level was not a significant source of variance. However, RI index appeared impaired on the first test day in the  $F_{iO_2} = 0.10$  group that did not receive PHY (Fig. 2). A one-way analysis of variance computed for the first day data revealed a significant difference among the four treatment groups [ $F(93, 54) = 8.17; P < 0.0005$ ]. *Post hoc* comparisons using Newman-Keuls tests showed that the  $F_{iO_2} = 0.10$  group exhibited a significantly ( $P < 0.05$ ) lower RI than all other groups. A one way analysis of variance computed for Day 7 did not show significant differences between groups, thereby, indicating that the memory impairment of the  $F_{iO_2} = 0.10$  group was transient and returned to control. This observation accords with a significant interaction determined between treatment with PHY and day of testing [ $F(1,54) = 4.25; P < 0.05$ ]. Mice exposed to hypoxia and treated immediately after the hypoxic



**Figure 2.** The effect of hypoxia and physostigmine (PHY) administration on object recognition. The recognition index is defined as the percent of time that mice spent exploring a new object divided by the time that mice spent exploring either a familiar or a new object. *Post hoc* comparisons using Newman-Keuls tests showed that there is a significant difference between untreated mice with  $F_{iO_2} = 0.21$  and those with  $F_{iO_2} = 0.10$  (\*). The # indicates a significant difference between PHY-treated mice with  $F_{iO_2} = 0.21$  and mice with  $F_{iO_2} = 0.1$  treated with PHY before hypoxic exposure. There is no difference in performance of control versus PHY-pretreated mice subjected to  $F_{iO_2} = 0.1$  after hypoxic episode. Data are presented as mean  $\pm$  SEM \* $P < 0.01$ . # $P < 0.01$ .

event with PHY showed marked improvement in performance. Mice treated with PHY under room air conditions did not perform significantly better than control on either day. Likewise, a pretreatment with PHY did not improve the mice score on Day 1 (Fig. 2).

## DISCUSSION

Our results demonstrate that mice subjected to hypoxia at  $F_{iO_2} = 0.10$  ( $O_2$  saturation = 70%) for 1 h develop impairment in object recognition memory 1 day after the insult. When tested 7 days after hypoxia, the same mice exhibited normal performance. Hypoxic mice treated with PHY immediately after exposure did not show deterioration in ORT associated with a hypoxic episode. Pretreatment with PHY, however, did not improve mouse performance.

Mild to moderate hypoxemia in the perioperative period has been implicated as one of the factors contributing to postoperative (CNS) impairment, at least in the early postsurgery period. Clinical trials showed a correlation between oxygen saturation during the perioperative period and postoperative brain dysfunction, although a role of confounding factors (i.e., comorbidity, history of alcohol abuse, etc.) was not completely elucidated in these studies (16,17). Animal research confirmed that even mild hypoxemia might cause cognitive dysfunction (10,18,19).

Hypoxemia alters many physiological and psychological processes in a dose- and duration-dependent manner. The spectrum of derangement varies from

mild metabolic changes, including a change in neurotransmitter turnover, to cell membrane repolarization (i.e., cell death). In severe hypoxia, where the supply of oxygen is insufficient to maintain a normal level of energy substrates (i.e., adenosine triphosphate, creatine phosphates), homeostatic mechanisms of the brain fail. Of the numerous processes identified, excessive activation of glutamate receptors, accumulation of extracellular excitotoxic acids (i.e., glutamate) and intracellular calcium cations, excessive production of free radicals, and initiation of pathological apoptosis play a critical role in neuronal damage. By contrast, hypoxia too mild to decrease a supply of energy for the brain impairs functions of several neurotransmitter systems, including amino acid neurotransmitters, dopamine, and monoamines (9). Our goal was to study cognitive effects of mild to moderate hypoxia.  $F_{IO_2}$  of 6%–7.5% ( $P_{aO_2}$  of 35 mm Hg) did not change the cerebral metabolic rate for oxygen in volunteers (20). Rodents breathing  $F_{IO_2} = 0.08$  had normal cerebral metabolic rate of oxygen but decreased synthesis of ACh (8). Our choice of  $F_{IO_2} = 0.10$  is consistent with these results.

Changes in neurotransmitter turnover may result in the impairment of cognition occurring during and immediately after a hypoxic episode. Mouse performance was compromised 24 h after the insult but improved with time (on the seventh day) in our study. Return to baseline performance suggests that no permanent neuronal damage was produced by moderate hypoxia (as opposed to ischemia), at least as measured by the ORT. The observed recovery profile could not be explained by a simple restoration of ACh or other neurotransmitter levels in the brain after returning to normoxia. It is possible that changes in synaptic function (not just neurotransmitters turnover) lead to the observed behavior. A loss of synaptic plasticity has been proposed as a possible explanation of memory decline due to hypoxic episode (19). It is unclear how PHY administration immediately after a hypoxic episode improves mouse performance 24 h after treatment. One possibility is that hypoxia leads to hyperactivity and an upregulation of ACh receptors in the cerebral cortex in response to a decreased availability of ACh (21). An increased level of ACh, which is one of the effects of PHY, may preserve the functional integrity of a cholinergic synapse.

Compromised cholinergic neurotransmission is implicated in a wide variety of cognitive disorders, including learning and memory impairment, dysfunction of the cortical arousal system, and attention deficit (22). Our goal was to study transient change in short-term memory, which is one of the characteristics of postoperative cognitive dysfunction. We used the Y-Maze test in our previous investigation (10). This test is based on the spontaneous preference of rodents for novel stimuli. The baseline mouse performance, however, deteriorated with repetitive testing, possibly due to familiarity with the maze and subsequent

disinterest. We chose the ORT for this series of experiments, as it does not require rule learning that may lead to diminished motivation. Instead, ORT requires only judgment as to whether the objects presented in the choice phase are novel or familiar to the rodent (15). Object recognition tasks are widely used in humans to test aspects of working memory (23). The cholinergic system is especially prominent in mediating recognition memory (22,24). Thus, object discrimination and novelty detection could be impaired by derangement of the cholinergic system induced by anticholinergic drugs or hypoxia.

PHY 0.1 mg/kg administered IP produced locomotor inhibitory effects in mice subjected to various behavioral tests 30 min after injection (25). This effect could have potentially interfered with animal ORT performance, since the test requires free movement of the animal between objects. We tested animals on posttreatment day 1, rather than immediately after PHY injection. The enzymatic activity of PHY recovered to 81% at 2 h and 100% within 24 h after IM injections and to 85% within an hour after IV injection (26,27). Thus, it is highly unlikely that our results are affected by the residual concentrations of PHY. In addition, we compared the Attention Index that indirectly evaluates motivational as well as motor components of mice behavior. Attention Indices did not differ among the groups.

Although posttreatment administration with PHY preserved recognition memory in hypoxic mice, administering the drug before insult produced no effect. The result is probably related to the pharmacokinetics of the drug. The half-life of PHY in the rat brain is 11 min and 16 min after IV and IM injections respectively. The brain to plasma ratio ( $B/r = 1.69$ ) peaks at 15 min after IV injection (26) and at 22 min after IM administration ( $B/r = 1.61$ ) (27). Although there are no data on the pharmacokinetics of PHY after IP injection, it is likely that the half-life of the drug in a mouse's brain is between 15 and 22 min. Hence, it is unlikely that pretreatment with PHY will produce a sufficient increase in the brain ACh level at the end of hypoxic treatment (60 min) to affect synaptic changes.

Our study has several important limitations. First, our conclusion that hypoxia-induced cognitive changes might be caused by impaired cholinergic transmission is an inference from the reversal of short-term memory dysfunction by PHY. It is possible that a PHY-induced increase in cerebral bloodflow (CBF) was responsible for the observed working memory improvement. This hypothesis was proposed to explain PHY-induced cerebral protection in the anoxic mouse (28). We did not measure either ACh level in cerebrospinal fluid or CBF. Although the mechanisms of injury induced by anoxia and moderate hypoxia are different, this possibility of short-term memory improvement secondary to the increased CBF cannot be excluded. Second, we used ORT and Y-Maze tests (in our previous study) to measure cognitive changes caused by mild hypoxia.

Both tasks assess short-term working memory. This investigation did not examine the effect of mild hypoxia on the retention of already acquired information (measured by radial maze test, water maze, or passive avoidance tests), which is an important component of postoperative cognitive dysfunction. Moreover, the effect of hypoxia and PHY administration on locomotion could have affected our results. Third, we did not measure physiological variables (except O<sub>2</sub> saturation). Therefore we cannot comment on the effect of hypo- or hypercarbia, arterial blood pressure, acid-base status, or the release of stress hormones on cognitive impairment.

We conclude that adult mice exposed to hypoxia (F<sub>IO<sub>2</sub></sub> = 0.1) develop a transient short-term memory deficit as measured by the ORT. We further conclude that the administration of PHY immediately after hypoxic insult preserves cognitive function. The results may have implications for understanding and treating cognitive changes in the immediate postoperative period.

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