Altitude acclimatization improves submaximal cognitive performance in mice and involves an imbalance of the cholinergic system

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Guerra-Narbona R, Delgado-García JM, López-Ramos JC. Altitude acclimatization improves submaximal cognitive performance in mice and involves an imbalance of the cholinergic system. J Appl Physiol 114: 1705–1716, 2013. First published April 18, 2013; doi:10.1152/japplphysiol.01298.2012.—The aim of this work was to reveal a hypothetical improvement of cognitive abilities in animals acclimatized to altitude and performing under ground level conditions, when looking at submaximal performance, once seen that it was not possible when looking at maximal scores. We modified contrasted cognitive tasks (object recognition, operant conditioning, eight-arm radial maze, and classical conditioning of the eyeblink reflex), increasing their complexity in an attempt to find performance differences in acclimatized animals vs. untrained controls. In addition, we studied, through immunohistochemical quantification, the expression of choline acetyltransferase and acetyl cholinesterase, enzymes involved in the synthesis and degradation of acetylcholine, in the septal area, piriform and visual cortices, and the hippocampal CA1 area of animals submitted to acute hypobaric hypoxia, or acclimatized to this simulated altitude, to find a relationship between the cholinergic system and a cognitive improvement due to altitude acclimatization. Results showed subtle improvements of the cognitive capabilities of acclimatized animals in all of the tasks when performed under ground-level conditions (although not before 24 h), in the three tasks used to test explicit memory (object recognition, operant conditioning in the Skinner box, and eight-arm radial maze) and (from the first conditioning session) in the classical conditioning task used to evaluate implicit memory. An imbalance of choline acetyltransferase/acetyl cholinesterase expression was found in acclimatized animals, mainly 24 h after the acclimatization period. In conclusion, altitude acclimatization improves cognitive capabilities, in a process parallel to an imbalance of the cholinergic system.

Hypobaric hypoxia is a type of oxygen deprivation characteristic of altitude, under natural conditions, although it can also be a consequence of certain aviation incidents. Hypobaric hypoxia alters many physiological processes in an elevation- and duration-dependent fashion (33). For instance, the exposure of an organism to transient hypoxic stress activates the respiratory and circulatory systems, as well as the adrenal glands, and affects both the release and effects of neurotransmitters in the central nervous system (41). However, these complex physiological acclimative responses cannot prevent early impairments in the cognitive functions of subjects exposed to hypobaric hypoxic conditions. For example, spatial and memory impairments have been reported in adult rats exposed to different simulated altitudes (18, 35). In contrast, it has been shown that transient exposure to hypoxia may not only prevent these disorders, but actually improve learning capabilities (41). Taken together, these data suggest that both the level and the duration of the hypobaric exposure are determinant factors that can induce either acclimatization or functional deficits.

In a previous study (17), our laboratory showed that acclimatization to high altitude prevented the impairment of classical eyeblink conditioning generated under hypobaric hypoxic conditions (∼5,000 m), but did not improve it when acclimatized animals performed the same task at ground level. Although still a matter of controversy, it is generally accepted that other motor and cognitive performances, such as the practice of certain sports, can be improved in people trained at altitude or in hypobaric chambers (2). Because of that, our first aim in this work was to look for an improvement of cognitive skills in animals acclimatized to altitude and tested under ground-level conditions. We modified selected cognitive tasks (object recognition, operant conditioning, eight-arm radial maze, and classical conditioning of the eyeblink reflex), increasing their complexity with the aim of obtaining submaximal performance, to help the finding of performance differences in acclimatized animals vs. untrained controls.

We also addressed the study of the putative neurotransmitter system involved in an improvement due to the acclimatization process. In this regard, it has been reported that an important cause of memory deterioration at high altitude is the impairment of neurotransmission, and that cholinergic dysfunction is one of the mechanisms involved, restorable through the administration of acetylcholinesterase (AChE) inhibitors (23, 24, 25). Moreover, AChE inhibitors have been reported to have ameliorative effects on other cognitive impairments, such as dementia or Alzheimer’s disease (4), and to act as a therapeutic tool to accelerate angiogenesis, at least in the cardiovascular system (13). In turn, the cholinergic function has been reported to be modulated by free chelatable zinc during hypobaric hypoxia-induced neuronal damage (39). At the same time, the medial septum has been described as one of the limbic nuclei with a large population of cholinergic cells (8, 27), playing an important role in spatial memory (37). The loss of septal cholinergic cells has been related with memory and cognitive impairments in pathological aging (31). For all of these reasons, we decided to study, with the help of selected immunohistochemical techniques, changes in the septal cholinergic system of animals submitted to different hypobaric hypoxic states. Additionally, we studied changes in cholinergic projections to the hippocampus and the piriform cortex (two structures widely recognized as involved in cognitive processes) during the acclimatization process. As a control, we also studied changes in cholinergic expression in the visual cortex.

MATERIAL AND METHODS

Subjects. Studies were carried out in male, 2-mo-old C57BL/6 mice obtained from an official supplier (Animal House of the University of Seville, Spain). A total of 198 animals were kept under standard conditions of temperature with a 12:12-h light-darkness cycle and

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allowed ad libitum access to commercial mice chow and water. All electrophysiological and behavioral studies were carried out in accordance with European Union guidelines (2003/65/CE) and Spanish regulations (BOE 252/34367-91, 2005) for the use of laboratory animals in chronic experiments. The experimental protocols were also approved by the local Ethics Committee of the Pablo de Olavide University (Seville, Spain).

For object recognition, operant conditioning in the Skinner box, and eight-arm radial maze tasks, four groups of animals were established: 1) control mice (C); 2) animals not acclimatized to the simulated altitude and tested in hypobaric (≈5,000 m) conditions (H); 3) animals acclimatized to the simulated altitude (≈5,000 m) during 2 wk and tested under ground level conditions (AG); and 4) animals acclimatized during the same period and tested in hypobaric (≈5,000 m) conditions (AH). For control purposes, an additional pseudoconditioned group was used for classical eyelink conditioning (see Table 1).

Object recognition. For object recognition, a total of 66 mice not previously submitted to simulated altitude were first habituated individually to an open field (45 cm × 25 cm) inserted in a home-made cylindrical hypobaric chamber (85 cm high × 80 cm in diameter) provided at the top with a transparent methacrylate window to allow external videotape recording of the experiments, which was carried out with a video camera (BLV-9AI, ECV, Madrid, Spain) and Pinacle 11 system software (Avid Technology, Burlington, MA). The hypobaric chamber served as a sound attenuator, but it also provided the white noise (45 dB) produced by the air-renewal system. The recording room was dimly illuminated, avoiding projection of the dim light directly through the window of the hypobaric chamber. Since the desired simulated altitude was achieved in ≈7 min, the animals were placed during that time inside an opaque cylindrical restrictor located at the center of the chamber floor; this restrictor was subsequently removed to allow the mouse to explore freely.

Sessions lasted 10 min and were repeated for four sessions. During the training session, performed under ground-level conditions for all of the four groups, two identical unknown objects (blocks of a construction toy; A1 and A2) were placed into the open field, and the animals were allowed to explore them for 10 min. The time spent exploring each object and the total exploration time were quantified. During retention sessions, 60 min after the training session, animals were placed back into the same open field, and, with the aim of obtaining sub maximal performance, one of the familiar objects was replaced, not by a novel object, but by a new block (B1), attached to the first, while the other was substituted by an identical one (A3), to avoid odor recognition. Mice were allowed to explore the objects freely for 10 min. The time spent exploring each object and the total exploration time were again quantified. The preference index for the novel objects (estimated as the percentage of the total exploration) was used to measure recognition memory. Similar procedures were carried out during the test sessions performed 12 h (objects B2 and C1) and 36 h (objects C2 and D1) later, in which the complexity of the objects was increased by assembling new blocks. This object sequence is represented in Fig. 1. One-half of the mice were submitted to a simulated altitude of 5,000 m during the retention sessions to evaluate the effect of the hypobaric hypoxia on this task. One-way ANOVA, performed with the PASW Statistics 18.0 for Windows package (SPSS, Chicago, IL), was used to detect significant differences between groups.

Operant conditioning in the Skinner box. Before training, mice were brought to 85% of their original weight through food deprivation from 7 days before and throughout the process. All of the experiments were carried out in a Skinner box measuring 15.9 cm × 14.0 cm × 12.7 cm (MED Associates, St. Albans, VT), located inside the hypobaric chamber, with the sound and light conditions detailed above. Conditioning programs, lever presses, and delivered reinforcements were monitored and recorded by a computer, using a MED-PC program (MED Associates).

In the first part of the experiment, 32 animals were rewarded with a pellet (Noyes formula P; 20 mg; Sandown Scientific, Hampton, UK) after pressing a lever, assuming a fixed ratio (1:1) paradigm (Fig. 2). The number of lever presses was quantified. Once the animals reached an asymptotic number of lever presses, and again with the aim of obtaining sub maximal performance, the complexity of the task was increased. To do that, animals were trained to obtain pellets by pressing the lever only when a small light bulb located above the lever was switched on. After 20 s, the light was turned off for 10 s and then turned on again. If the mouse pressed the lever during the dark time, the dispenser gave no food, and the time with the light off was extended for up to 10 additional seconds. In this second complex phase, the ratio of lever presses during the light-on/light-off periods was calculated and presented as the percentage of the ratio lever presses with light on/lever presses with light off obtained during the first session. A one-way ANOVA, performed with the PASW Statistics 18.0 for Windows package (SPSS), was used to detect significant differences between groups and between sessions.

Eight-arm radial maze task. For spatial memory evaluation, an eight-arm radial maze was designed, fitting the dimensions of the hypobaric chamber. Each arm (6 cm wide × 25 cm long × 15 cm high) was connected to an octagonal platform of 7.5 cm apothem. The distal end of each arm was equipped with a small cup to hold the food pellets (Noyes formula P; 20 mg; Sandown Scientific). A total of 40 animals were used. To increase the complexity, and with the aim of obtaining sub maximal scores, the end of only every other arm was marked to allow its identification, and pellets were placed in three of

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Table 1. Groups involved in the classical eyelink conditioning task

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Pseudo, pseudoconditioned; C, control; H, acute hypobaric; AG, acclimatized to hypobaria and tested under ground level conditions; AH, acclimatized to hypobaria and tested under hypobaric conditions; –, no; +, yes.
Figure 2. Operant conditioning in a Skinner box during a fixed-ratio (1:1) schedule (A) and a random complex program (B) aimed at detecting submaximal performance. In A, the number of lever presses from the 5th to the 10th sessions is represented, while in B the values are expressed as the ratio (lever presses during the light-on period to lever presses during the light-off period), with the time with the light off being increased when the animals pressed in that period and in which no food was dispensed. Values are means ± SE of the scores. A: one, two, and three symbols represent statistical differences between C (●), AH (▼), and AG (▲) groups with respect to the H (●) group: *P < 0.05, **P < 0.01, and ***P < 0.001, respectively. B: statistical differences between AG and H groups: *P < 0.05 and **P < 0.01. **Statistical differences between AG and C groups, P < 0.01. Bonferroni post hoc one-way ANOVA.

Surgical preparation for classical eyeblink conditioning. Under deep anesthesia (ketamine, 35 mg/kg and xylazine, 2 mg/kg ip), a group of 107 animals not previously submitted to simulated altitude were implanted with four electrodes in the upper eyelid of the left eye. Electrodes were made of Teflon-insulated, annealed stainless steel wire (50 μm in diameter, A-M Systems, Carlsborg, WA). One pair of electrodes was aimed toward the supraorbital branch of the trigeminal nerve and served for the application of electrical stimuli. The second pair of electrodes was implanted in the ipsilateral orbicularis oculi muscle and served for recording its electromyographic (EMG) activity. The four electrodes were connected to a four-pin socket (RS-Amidata, Madrid, Spain), which was affixed with dental cement to the cranial bone. The surgical procedure is schematized in Fig. 4A. After surgery, and before the beginning of the experiment, animals were kept for 5–7 days in independent cages, with free access to food and water, for a proper recovery. They were also maintained in individual cages for the rest of the experiment.

Classical conditioning procedures. For EMG recordings, animals were placed in individual (15 cm × 5 cm × 10 cm) methacrylate cages, located inside the hypobaric chamber, and the wires plugged into their implanted sockets were connected with the stimulating/recording system through a connection panel with which the chamber was equipped.

Both trace and delay conditioning paradigms were carried out. For this, animals were presented with a tone (6,000 Hz, 70 dB, 20 ms for trace, and 6,000 Hz, 70 dB, 250 ms for delay paradigm) as a conditioned stimulus (CS), followed 250 ms from its start by an electrical stimulation (100 μs, 2 × threshold) of the trigeminal nerve as an unconditioned stimulus (US). These parameters, milder than others used for similar experiments (17), were used with the aim of obtaining submaximal performance. CS-US presentations were separated at random by 30 ± 5 s. The CS and US parameters presented for

Figure 3. Results collected during the eight-arm radial maze task. The exploration-time ratio (the ratio between the time taken to find the three arms with pellets during each retention session to the time taken for that during the training session; A) and the visited-arms ratio (the ratio of visits to arms with pellets to visits to arms without pellets, normalized with respect to the same ratio obtained during the training session; B) are shown. Values are means ± SE of the ratios. Statistical differences with respect to the training level: *P < 0.05 and **P < 0.01. ***Statistical differences between sessions for the same group, P < 0.001. Bonferroni post hoc one-way ANOVA.
sessions of 50 trials each. Sessions analyzed to obtain learning curves, shown in Fig. 4. Results collected during classical eyeblink conditioning. A: schematic diagram of the experimental design. Stimulating electrodes were implanted in the supraorbital nerve for unconditioned stimulus (US), and recording electrodes were implanted in the ipsilateral orbicularis oculi (OO) muscle. B: superimposition of 5 EMG recordings from the OO muscle, illustrating the stimulus (Stim) and the R1 and R2 components of the reflex eyeblink response. C and D: schematic representation of the experimental design for trace (C) and delay (D) conditioning paradigms. From top to bottom are illustrated the tone used as conditioned stimulus (CS), the shock used as US, and a recording of EMG activity of the OO muscle collected during the fourth conditioning (C) session. E and F: eyelid conditioned responses collected from C and pseudoconditioned (PS), H, AG, and AH groups, during trace (E) and delay (F) conditioning paradigms. H1–H3 illustrate data collected from habituation (H) sessions; C1–C4 data are from C sessions, and E1–E3 data are from extinction (E) sessions. Exposure to the selected simulated altitude was carried out during C and E sessions. Values are means ± SE of percentage (% of conditioned responses (n ≥ 8). One, two, and three symbols represent statistical differences between C (●), AH (◇), and AG (▲) groups with respect to the H (○) group: P < 0.05, < 0.01, and < 0.001, respectively. *Statistical differences between AG and AH groups, P < 0.05. Bonferroni post hoc one-way ANOVA. G: schematic representation of H, C, and E sessions carried out during the performance of the classical eyelid conditioning tasks. Each of the 5 days of the experiment consisted of 200 trials, divided in four sessions of 50 trials each. Sessions analyzed to obtain learning curves, shown in E and F, are indicated by a number.

The EMG activity of the orbicularis oculi muscle was recorded using differential amplifiers with a bandwidth of 1 Hz to 10 kHz (Grass Technologies, West Warwick, RI). Data were stored directly on a computer through an analog-to-digital converter (CED 1401 Plus, Cambridge, UK), at a sampling frequency of 11–22 kHz and an amplitude resolution of 12 bits. Data were analyzed off-line for quantification of conditioned responses (CRs) with the help of the Signal Average Program (Cambridge Instruments, Cambridge, UK). As a criterion, we considered a CR those eyelid responses, recorded during the CS-US period, that presented the following characteristics: 1) the EMG activity lasted > 10 ms; 2) the EMG was not preceded by any spontaneous activity in the 200 ms preceding CS presentation; 3) the EMG activity was initiated > 50 ms after CS onset; and 4) the integrated EMG activity was at least 2.5 times larger than the activity recorded 200 ms before CS presentation (6). The EMG activity of the supraorbital nerve [for unconditioned stimulus (US)], and recording electrodes were implanted in the ipsilateral orbicularis oculi (OO) muscle. B: superimposition of 5 EMG recordings from the OO muscle, illustrating the stimulus (Stim) and the R1 and R2 components of the reflex eyeblink response. C and D: schematic representation of the experimental design for trace (C) and delay (D) conditioning paradigms. From top to bottom are illustrated the tone used as conditioned stimulus (CS), the shock used as US, and a recording of EMG activity of the OO muscle collected during the fourth conditioning (C) session. E and F: eyelid conditioned responses collected from C and pseudoconditioned (PS), H, AG, and AH groups, during trace (E) and delay (F) conditioning paradigms. H1–H3 illustrate data collected from habituation (H) sessions; C1–C4 data are from C sessions, and E1–E3 data are from extinction (E) sessions. Exposure to the selected simulated altitude was carried out during C and E sessions. Values are means ± SE of percentage (% of conditioned responses (n ≥ 8). One, two, and three symbols represent statistical differences between C (●), AH (◇), and AG (▲) groups with respect to the H (○) group: P < 0.05, < 0.01, and < 0.001, respectively. *Statistical differences between AG and AH groups, P < 0.05. Bonferroni post hoc one-way ANOVA. G: schematic representation of H, C, and E sessions carried out during the performance of the classical eyelid conditioning tasks. Each of the 5 days of the experiment consisted of 200 trials, divided in four sessions of 50 trials each. Sessions analyzed to obtain learning curves, shown in E and F, are indicated by a number.
**Immunohistochemistry.** For tissue processing, we followed experimental procedures described elsewhere (10, 12). Five groups of animals (C; acclimatized and perfused at 0 h and 24 h; and acute hypobaric and perfused at 0 h and 24 h) were deeply anesthetized with ketamine (35 mg/kg) and chloral hydrate (4%) and perfused transcardially with 5–20 ml of a phosphate-buffered saline (PBS) solution to remove the blood, and then with 100–150 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The brains were removed and postfixed for 4 h in the same fixative at room temperature. Afterwards, they were cryoprotected by overnight immersion in 30% sucrose in 0.1 M PB, at 4°C.

Tissue blocks were quickly frozen, and serial coronal sections (40 μm) were cut with a cryostat (Leica CM1900, Wetzlar, Germany). The left (L) hemisphere of each brain was marked to allow a later comparison with the right (R) one. Free-floating sections were processed by the avidin-biotin peroxidase complex procedure (11, 19, 30) to visualize immunoreactive (IR) sites. Briefly, sections were incubated for 30 min in PBS containing 3% normal bovine albumin serum (Sigma-Aldrich, Madrid, Spain) and 0.2% PBS/Triton X-100. Sections were incubated with specific polyclonal antisera against choline acetyltransferase (ChAT; Millipore, Bedford, MA) 1:100, and/or against AChE (Novus Biologicals, Littleton, CO) 1:400 in the above-mentioned solution for 48 h at 4°C. Then, and after several washes in PBS, sections were incubated in biotinylated rabbit anti-goat immunoglobulin (Invitrogen, Life Technologies, San Francisco, CA), and biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA), respectively, 1:250, followed by peroxidase-linked avidin-biotin peroxidase complex (Vector Laboratories). Peroxidase activity was demonstrated by the nickel-enhanced diaminobenzidine procedure (32). Additionally, and for immunofluorescence studies, donkey anti-goat Alexa Fluor 488 conjugated and donkey anti-rabbit Alexa Fluor 555 conjugated, respectively, 1:200 (Invitrogen Life Technologies, Paisley, UK) were used. Fluorescence photomicrographs (Fig. 5) were taken with an Axio Observer Z1 microscope equipped with an Apotome optical sectioning system (Carl Zeiss, Jena, Germany).

**Image analysis of immunostained cells.** For the quantification of ChAT- and AChE-diaminobenzidine-immunostained cells, one out of each eight coronal sections of five brains per group were processed, and the selected areas were photographed with a Leica DC 500 digital camera adapted to a Leica DMRE microscope in transmitted light mode, sup-

![Fig. 5. Distribution patterns of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in septum (A–D), piriform (E–H), and visual (I–L) cortices, and the hippocampal CA1 area (M–P). In the septum, there are two different populations of cells expressing the two enzymes, graphically separated by dashed lines in B, with the AChE-expressing ones being more medial, and the ChAT ones located laterally to them. In the piriform cortex, the two enzymes are uniformly distributed, principally between the dashed lines in F, the same as their colocalizations. A homogeneous distribution was found in the visual cortex, with the same bipolar ChAT-positive cells distributed in the upper layers (K and L). In CA1, the distribution is similar to that in the piriform cortex in the somatic line of pyramidal cells (between the dashed lines in N), although, in the surrounding interneurons, ChAT and AChE appear to be colocalized (O and P). Arrows, cells expressing AChE; arrowheads, cells expressing ChAT. Colocalizations are shown in yellow in D, H, and L. Scale bars, 50 μm. DAPI, 4',6-diamidino-2-phenylindole.](image-url)
ported with Leica IM 500 software (Leica Microsystems). Piriform cortex was photographed with a ×10 objective, while septum and the CA1 hippocampal area were photographed with ×5 and ×40 objectives, respectively. Special care was taken to maintain the intersection between the dorsoventral and the rostrocaudal axis at the same point of each captured image, to allow an equal analysis. Following the coordinates of Paxinos and Franklin (28), piriform cortex photomicrographs were taken from bregma 1.94 to −2.30 (524 analyzed slices), visual cortex photomicrographs from bregma −2.18 to −4.48 (161 analyzed slices), septum pictures were taken from bregma 1.10 to 0.62 (260 analyzed slices), and hippocampal areas were selected between bregma −1.34 and bregma −2.30 (146 analyzed slices). In each section, the desired structures were delimited following the schemes of Paxinos and Franklin (28) and are detailed in Figs. 6B, 7B, and 8B.

The immunoreaction against ChAT and AChE was compared between the C group, two groups of acute 394 mmHg = 5,000 m hypobaric hypoxic animals perfused 0 h (H 0h) and 24 h (H 24h) after 2 h of hypoxic exposure, and another two groups of acclimatized animals perfused 0 h (A 0h) and 24 h (A 24h) after 2 wk of acclimatization at 394 mmHg = 5,000 m. In all cases, except for the septum, the L and R hemispheres were analyzed separately.

ChAT and AChE immunostained structures were identified, and the relative IR area (the ratio IR area/area of interest) was quantified with the help of the Image J free image-analysis software, maintaining the threshold parameters throughout the analysis of each area. Statistical differences between groups and between hemispheres were compared using the one-way ANOVA test, with Bonferroni post hoc tests used to compare all the groups. The analysis was performed with the PASW Statistics 18.0 for Windows package (SPSS).

RESULTS

Object recognition. As is shown in Fig. 1, the object recognition task evidenced a progressive increase of the Exploration Index, a reference of memory performance, in the four experimental groups. Thus, from initial (0 h) indexes of 106.7 ± 6.2, 116.6 ± 7.0, 101.2 ± 3.8, and 109.6 ± 7.6 for the C, H, AG, and AH groups, respectively, animals reached indexes of 125.5 ± 7.5, 123.7 ± 11.3, 137.0 ± 6.1, and 123.0 ± 4.7 for the same groups in the session carried out 36 h later (mean ± SE, n = 16). No significant differences between groups were observed for any of the experimental sessions. Significant \( F_{(3,75)} = 5.16; P < 0.01 \) differences between performances for the 0-h and 36-h sessions were observed only for the AG group (Dunnett post hoc analysis of ANOVA; Fig. 1). Interestingly, a statistical difference \( F_{(3,66)} = 4.0; P < 0.05 \) was also observed for the AH group, for performances carried out at 12 h and 36 h (Bonferroni post hoc test).

Operant conditioning. The operant conditioning was carried out in two successive phases, the second aimed at finding putative differences between the three groups that obtained similar scores during the first phase.

The first phase consisted of a fixed-ratio (1:1) schedule. During the first four sessions of this phase, a progressive increase in the number of lever presses was observed (data not illustrated). By the fifth session, the number of lever presses was 8.0 ± 2.6, 0.0 ± 0.0, 5.3 ± 2.0, and 7.2 ± 2.4 for the C, H, AG, and AH groups, respectively (Fig. 2A). Animals reached asymptotic values of 24.8 ± 4.5 (C), 0.5 ± 0.2 (H), 35.0 ± 4.9 (AG), and 32.3 ± 6.2 (AH) lever presses by the ninth session (mean ± SE, n = 7; Fig. 2A). The ANOVA of the collected data showed statistical differences between the H group and the other groups (C, AG, and AH) for the 6th \( F_{(3,30)} = 3.1; P < 0.05 \), 7th \( F_{(3,30)} = 3.8; P < 0.05 \), 8th \( F_{(3,29)} = 6.39; P < 0.01 \), 9th \( F_{(3,29)} = 9.7; P < 0.001 \), and 10th \( F_{(3,19)} = 6.2; P < 0.01 \) sessions.

During the second phase, the complexity of the task was increased: animals received the reward when pressing the lever during the light-on period (see MATERIALS AND METHODS). By the 12th training session, the percentage of the initial ratio of lever
presses with light on to lever presses with light off reached values of 86.5 ± 18.12, 103.3 ± 10.3, 478.7 ± 136.3, and 220.8 ± 23.1 for the C, H, AG, and AH groups, respectively (Fig. 2B). These results indicate that, compared with the other three groups (C, H, and AH), the AG group presented the best...
performance in this second phase of the operant conditioning task. Differences between the AG and the other groups were significant for the 7th \(F_{(3,28)} = 4.6; P < 0.05\), 9th \(F_{(3,28)} = 6.8; P < 0.01\), 11th \(F_{(3,28)} = 4.7; P < 0.01\), and 12th \(F_{(3,28)} = 6.7; P < 0.01\) training sessions. The scores achieved by the AH group were better, although nonsignificantly, than those of the C and H groups during the 11th and 12th training sessions.

Eight-arm radial maze task. The graphical representation of the two indexes (exploration-time and visited-arms ratios) obtained during the eight-arm radial maze task, compared with the training level, obtained during the first session at 0 h, are shown in Fig. 3. ANOVA of the collected data rendered significant differences for the C \(F_{(2,45)} = 5.1; P < 0.05\) and AG \(F_{(2,43)} = 9.4; P < 0.001\) groups. Surprisingly, as shown in Fig. 3A, both C and AG groups, tested under ground-level conditions, took significantly more time to find the arms with food in the 1-h session than in the 0-h one. Only the AG group improved its exploration-time ratio in the 24-h session compared with the 1-h one (1.64 ± 0.2 vs. 0.63 ± 0.08). With regard to the visited-arms ratio (Fig. 3B), only the AG group presented significant differences \(F_{(2,45)} = 3.6; P < 0.05\) with respect to the training level, in the 24-h session, reaching values of 1.43 ± 0.14 (mean ± SE, \(n = 8\)).

Classical conditioning with a trace paradigm. While, as expected, during the habituation sessions the percentage of putative CRs diminished slowly but steadily, during the conditioning sessions all of the groups, with the exception of the H group and the pseudoconditioned one (not represented), increased their percentage of CRs, reaching asymptotic values by the third conditioning session. Acclimatization at 394 mmHg \((≈5,000\ m)\) for 7 days restored learning and memory capabilities in mice, which, after this period, were conditioned under these same acute hypobaric hypoxic conditions (AH group). The percentage of CRs reached by the AH group was 27.4% for 7 days and conditioned at 35 m (AG group), the percentage of CRs was significantly higher than those of the H and AH groups from the first conditioning session [reaching values of 66.7 ± 4.9 (mean ± SE, \(n = 8\)) in the fourth one], while the AH group only reached significant differences with the H group until the fourth conditioning session. One-way ANOVA applied to the conditioning data indicated that there was a significant difference between groups \(F_{(3,27)} = 7.9; P < 0.01\). Additionally, there is a significant effect of session \(F_{(3,8)} = 18.6; P < 0.001\), but not for group-by-session interactions \(F_{(9,8)} = 0.9; P = 0.4\).

During extinction, only the AG group showed a percentage of CRs significantly higher than that reached by the nonacclimatized one conditioned at 5,000 m (H group). The same analysis applied to the extinction data showed significant differences between the groups \(F_{(3,27)} = 5.0; P < 0.01\), with a significant overall effect of session \(F_{(2,54)} = 15.4; P < 0.001\), but no significant group-by-session interaction \(F_{(6,54)} = 2.9; P = 0.6\). Figure 4F depicts the learning curves of the two acclimatized groups (AH, AG) compared with both the C and the H conditioned groups. Here again, the pseudoconditioned group did not present any significant change in the percentage of CRs across the successive training sessions (Fig. 4F, PS group).

Thus conclusions similar to those already indicated for the trace conditioning paradigm can be drawn for the classical eyelink conditioning carried out with the help of a delay paradigm.

Image analysis and quantification of ChAT and AChE immuno-detection. Immunoreaction with the anti-ChAT (α-ChAT) and anti-AChE (α-AChE) antibodies was found in the brain areas included in this study (septum, piriform and visual cortices, and hippocampal CA1 area) in all of the studied groups. Distribution patterns of the two enzymes were different for the three studied areas: in the septum, there were two different populations of cells expressing the two enzymes, with the AChE-expressing ones located more medially, and the ChAT ones located laterally to them (Fig. 5, A–D). In the piriform cortex, the two enzymes appeared uniformly distributed, the same as their colocalizations (Fig. 5E–H). A similar distribution was found in the visual cortex (in areas with a rather reduced pyramidal layer) and with the same bipolar ChAT-expressing neurons located in intermediate layers (Fig. 5, I–L). Finally, in the hippocampal CA1 area, the distribution was similar to that observed in the piriform cortex in the

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somatic line of pyramidal cells (Fig. 5, M–P). In contrast, in the surrounding interneurons, both ChAT and AChE appeared to be colocalized (Fig. 5, M–P). For the septum, one-way ANOVA applied to the AChE data indicated no significant difference between groups \( F(4,69) = 1.3; \) \( P = 0.2 \). For the ChAT data, a significant difference between groups was found \( F(4,130) = 6.05; \) \( P < 0.001 \). A Bonferroni post hoc test found significant differences between the A 0h group and C, H 0h, and H 24h, and a Student’s \( t \)-test showed differences in relative IR area between ChAT and AChE immunoreaction in the H 24h group (Fig. 6A).

With respect to the piriform cortex, significant differences between the A 24h group and the other groups were found in the Bonferroni post hoc test, after the performance of a one-way ANOVA, for ChAT in L \( F(4,261) = 17.8; \) \( P < 0.001 \) and R \( F(4,304) = 15.9; \) \( P < 0.001 \) hemispheres. This test applied to the AChE indicated a significant difference between groups for the R hemisphere \( F(4,275) = 9.54; \) \( P < 0.001 \), but not for the L \( F(4,278) = 2.08; \) \( P = 0.083 \). The Bonferroni post hoc test found significant differences between the A 0h group and C, H 0h, and A 24h, and differences between the H 0h group and H 24h and A 0h were also found. Likewise, a Student’s \( t \)-test showed differences between ChAT and AChE relative IR areas in the two hemispheres of the A 24h group (Fig. 7A).

The quantitative analysis of the visual cortex showed significant differences between groups, after the performance of a one-way ANOVA, for ChAT in R \( F(4,129) = 3.05; \) \( P < 0.05 \) and L \( F(4,127) = 4.9; \) \( P < 0.005 \) hemispheres, although Bonferroni post hoc test only detected differences between H 0h group and C, H 24h, and A 24h, in the L hemisphere. For AChE, differences between groups were detected in the R \( F(4,165) = 7.2; \) \( P < 0.001 \) and L \( F(4,155) = 18.04; \) \( P < 0.001 \) hemispheres; Bonferroni post hoc test showed differences between A 24h group and C and A 0h groups in the R hemisphere, and between A 24h and all the rest of the groups in the L hemisphere. A Student’s \( t \)-test showed differences between ChAT and AChE IR relative areas for the A 24h and H 0h groups in both hemispheres (Fig. 8A).

During the analysis of the immunoreaction in the hippocampal CA1 area, the ANOVA showed differences for ChAT in both the L \( F(4,70) = 7.9; \) \( P < 0.001 \) and R \( F(4,73) = 9.5; \) \( P < 0.001 \) hemispheres. The Bonferroni post hoc test detected differences between the A 0h and C, H 0h, and H 24h groups, and between A 24h and H 0h groups for both hemispheres. In the case of AChE, one-way ANOVA showed differences in both L \( F(4,75) = 2.73; \) \( P < 0.05 \) and R \( F(4,75) = 4.9; \) \( P < 0.01 \), and the Bonferroni post hoc test showed differences between A 0h and H 0h in the L hemisphere, and between A 0h and both A 24h and H 0h in the R hemisphere. As in the case of the piriform cortex, the Student’s \( t \)-test showed differences between ChAT and AChE relative IR areas for the A 24h group (Fig. 9A).

**DISCUSSION**

In a previous study, we showed there was no improvement of cognitive abilities in altitude-acclimatized animals, only restoration of the impairment generated under hypobaric hypoxic conditions (17). However, it was still necessary to further check the effects of acclimatization to altitude because of the considerable controversy (regarding its potential benefits or damage) the issue continues to generate. For that, we have...
made new behavioral approaches that could contribute to clarifying this question. Thus we modified four conventional cognitive tasks (object recognition, operant conditioning, eight-arm radial maze, and classical eyelock conditioning) to increase their complexity. The aim was to obtain submaximal scores that could be surpassed by those animals with improved capabilities, it already having been seen that, when looking at maximal performances, it was not possible to detect subtle presumed differences. It is appropriate here to explain that, when we refer to submaximal performances or scores, we are not making a comparison with submaximal exercise, since this last term is used to define not a score, but a level of effort during the practice of certain sports (3, 14).

In this study, the results of the behavioral tasks have demonstrated an improvement of the acclimatized animals tested under ground level conditions (AG group), although not before 24 h, in the three tasks used to test explicit memory (object recognition, operant conditioning in the Skinner box, and eight-arm radial maze). Specifically, and with regard to the object recognition task, it is necessary to point out that preference indexes were calculated comparing the preference for a known familiar object shown in the previous session and the preference for a novel one. In other words, we were comparing preference indexes; no preference between objects showed in no adjacent sessions. Furthermore, the present results showed that the retention capabilities were improved in the AG group for an interval of 24 h because, in this experiment, we were testing the preference for a novel object 1, 12, and 24 h (36 h minus 12 h) after the exposition to a known one.

Present results also indicated an improved performance from the first conditioning session in the classical conditioning task, a test useful to evaluate implicit memory. Nevertheless, there is controversy in the literature concerning the issue, and we should be cautious in interpreting these results. They do show, in conjunction, that, under our experimental conditions, acclimatization to altitude seems to improve cognitive performance. It is important to emphasize this point because, as described elsewhere, hypobaric hypoxia impairs spatial memory in an elevation-dependent fashion (33, 35), and the positive effects of acclimatization to altitude have been detected only at low or moderate altitudes, or during short times of exposure to high altitudes (15, 36, 38, 41, 43). In fact, a study on the “dose” of hypoxic exposure for an optimal level of performance during the classical “live high-train low” altitude training for humans proposed an altitude of 2,000–2,500 m as the best one (40), coinciding with Muza et al. (26), who recommend a continuous residence of 1–2 days at 2,200 m as preexposure altitude inducing acclimatization. On the other hand, individual variations in acclimatization to altitude, measured as an increase in erythropoietin concentration, have also been reported (5). In other studies (16), high altitude suppresses training-dependent cognitive advantages. Those results do not disagree with the present ones, since we evaluated, in different conditions, the cognitive performance of subjects not trained previously. In any case, it will be interesting to evaluate the suppression of training advantages, or the suppression of CRs, in subjects previously trained, or conditioned, after different times of acute exposition, or acclimatization, to altitude. With regard to the acquisition of conditioned reflex responses, and as in the present work, an acceleration of the generation and degree of retention of conditioned reflex responses in animals adapted to altitude hypoxia has been reported before (21), although, interestingly, in male, but not female, rats (22).

Our histochemical studies on the cholinergic system in the medial septum, piriform cortex, and hippocampal CA1 area have shown different distribution patterns of the AChE and ChAT enzymes. Thus, while in the septum there is clear evidence of cholinergic neurons distributed laterally to a central band of ChAT-positive neurons, in the piriform cortex there is no obvious lamination, in accordance with earlier descriptions of, at least, ChAT distribution (7). A similar pattern was found in the visual cortex, with ChAT expressing bipolar cells previously described in rats (29). In the hippocampal CA1 area, the distribution of ChAT and AChE seems to be uniform, although, in the adjacent areas of CA1 and other nonpyramidal layers of the hippocampus, such as the regio superior of Cajal, we found neurons expressing both AChE and ChAT enzymes in their perikaryon. Previous descriptions exist of these cells expressing ChAT (8), as well as others expressing AChE (1). In our study, we found colocalization of the two enzymes in the perikaryon of those cells. We cannot confirm whether the cells are either expressing enzymes or have axosomal synapses coinciding in the soma of these cells, which are, in any case, ChAT positive.

The quantification of the immunoreaction has shown, in general, a ChAT/AChE balance in all of the studied areas for the C group, an increase of ChAT in both acclimatized groups, and significant differences with respect to AChE in the piriform and CA1 areas of the A 24h group. An imbalance between ChAT and AChE was also detected in the medial septum of the H 24h group, and between H 0h and A 24h in the visual cortex, but in the contrary sense. All together, and with the exception of the visual cortex, these results can lead us to conclude that, in reference to the cholinergic system, the critical aspect of the injury caused by acute exposure to hypoxia is a ChAT/AChE imbalance due to an increase in the latter, and that the benefits from acclimatization are due to the opposite effect.

To clarify our conclusions, it is necessary to indicate that, when we refer to an ChAT/AChE balance, we are trying to describe that, in contrast to the C group, which was assigned with a relative IR area of 1 for each immunostaining quantification (ChAT and AChE), other groups not only reached values different to the C group, but presented significant differences between ChAT and AChE expressions. Furthermore, when we made the histological study, our intention has been to detect a direct and single effect of altitude on the cholinergic system, so we have worked with animals not submitted to any behavioral task that could have an influence on the collected results. Then, as the difference between the groups C and H, and between AG and AH, is the simulated altitude in which the behavioral tasks were made, it was not possible to map groups of the behavioral and the histological study, since the animals used to the histological study did not carry out any behavioral task. In any case, and only in function of the altitude at which the animals were exposed, could it be assumed that: 1) the two C groups map because they never were submitted to altitude; 2) the acute hypoxic animals (H) of the behavioral task could map with some of the H 0h or H 24h groups, because all of them were submitted only to acute hypobaric hypoxic conditions; and 3) some of the acclimatized animals (AG or AH) could map with the A 0h and/or A 24h groups, because all of them were acclimatized to altitude. Thus
only after the results have been obtained was it possible to detect parallelisms between groups.

All together, acclimatization should have the same effects as the pro-cognitive drugs based on an inhibition of AChE or an increase of ChAT (4, 13, 23, 24, 25, 34). In the cases in which an increase in AChE was detected, a doubt arises concerning what of the types of esterases was augmented. In mammals, the AChE gene produces three types of coding regions through the choice of 3’ splice acceptor sites, generating proteins which possess the same catalytic domain, associated with distinct C-terminal peptides. One of them, the R form, is expressed during development and induced by stress in the mouse brain (20), and is not known to be a functional specie. Thus, it is possible that the AChE increase were due to an increase of the R form, and therefore an increase of a nonfunctional molecule.

In any case, the more stressing situation was the one suffered by the acute hypobaric group perfused at 0h (H 0h), and this group did not present any increase in the AChE levels.

An additional question is the reason for the elevated levels of acetyl choline. A possible explanation (42) is related to the brain levels of erythropoietin (EPO), a molecule produced not only in kidney, and which role in the hematological and ventilatory response to hypoxia has been proposed (9).

It is also interesting to remark that, as detailed above, in most of the cognitive tasks used here, the improvement in the AG group was not detected before 24 h, coinciding with the time in which there was a larger increase of ChAT with respect to AChE. Although the conditions under which the acclimated animals (AG) were kept were not the same as for the A 24h group (the former remained in hypobaric conditions during the whole time in which the tasks were performed), they remained under ground level conditions throughout the time the chamber was used to test all the other groups; thus, at 24 h from the beginning of the tasks, the conditions were almost the same. The conclusion from these results could be, therefore, that the greatest benefits of acclimatization should be gained 24 h after a prolonged stay at altitude.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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