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Research report

Effect of perinatal asphyxia on tuberomammillary nucleus neuronal density and object recognition memory: A possible role for histamine?

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HIGHLIGHTS

- Perinatal asphyxia reduces performance in the object recognition task.
- Reduced performance in the object recognition is reverted by an H3 antagonist.
- Perinatal asphyxia reduces the number of ADA-immunoreactive neurons.
- Perinatal asphyxia reduces the expression of HDC in the hypothalamus.
- The performance in memory task is correlated with ADA-immunoreactive neuronal density.

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ABSTRACT

Perinatal asphyxia (PA) is associated with long-term neuronal damage and cognitive deficits in adulthood, such as learning and memory disabilities. After PA, specific brain regions are compromised, including neocortex, hippocampus, basal ganglia, and ascending neuromodulatory pathways, such as dopamine system, explaining some of the cognitive disabilities. We hypothesize that other neuromodulatory systems, such as histamine system from the tuberomammillary nucleus (TMN), which widely project to telencephalon, shown to be relevant for learning and memory, may be compromised by PA. We investigated here the effect of PA on (i) Density and neuronal activity of TMN neurons by double immunoreactivity for adenosine deaminase (ADA) and c-Fos, as marker for histaminergic neurons and neuronal activity respectively. (ii) Expression of the histamine-synthesizing enzyme, histidine decarboxylase (HDC) by western blot and (iii) thioperamide an H3 histamine receptor antagonist, on an object recognition memory task. Asphyxia-exposed rats showed a decrease of ADA density and c-Fos activity in TMN, and decrease of HDC expression in hypothalamus. Asphyxia-exposed rats also showed a low performance in object recognition memory compared to caesarean-delivered controls, which was reverted in a dose-dependent manner by the H_3 antagonist thioperamide (5–10 mg/kg, i.p.). The present results show that the histaminergic neuronal system of the TMN is involved in the long-term effects induced by PA, affecting learning and memory.

Abbreviations: ADA, adenosine deaminase; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; HDC, L-histidine decarboxylase; PA, perinatal asphyxia; TMNv, tuberomammillary nucleus, pars ventral; TMNd, tuberomammillary nucleus, pars dorsal.

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1. Introduction

Perinatal asphyxia (PA) is a severe condition associated with obstetric complications during labour and delivery, leading to high mortality among affected newborns. Survivors can suffer from severe to mild neurological disabilities, such as cerebral palsy, mental retardation, increasing further the risk factors for developing neuropsychiatric disorders and cognitive disabilities [1–4]. Models of PA have demonstrated that affected animals, show cognitive disability when evaluated by different experimental paradigms at adulthood, such as deficits in working memory, object recognition and spatial memory [1–3], which can be explained by impairments in telencephalic brain regions, such as neocortex, basal ganglia, hippocampus, and amygdala, or by deficits primarily affecting ascending pathways from the brainstem [4,5]. Indeed, deficits in dopamine release have been reported in adult PA-exposed animals [6], in agreement with the hypothesis that deficits in monoamine pathways are a signature for neuropsychiatric disorders, such as schizophrenia and depression, implying detrimental activity of cognitive abilities, including learning and memory [7,8]. The tuberomammillary nucleus (TMN) of the ventral posterior hypothalamus innervates widespread regions of the telencephalon, demonstrated by retrograde tracing and immunohistochemical studies [9]. The TMN is characterized by containing magnocellular neurons which synthesize and produce histamine in the brain, the only source of histamine in the brain. Histamine has been shown to play a critical role in learning, memory and cognitive processes [10]. To our knowledge, however, the effect of PA on histamine systems has not yet been explored.

We hypothesized that PA affects the histamine system and the alteration over this neuromodulator contributes to the alteration in learning and memory observed in asphyxia-exposed rats. To test this hypothesis, we have investigated here the effect of PA on the density and neuronal activity of TMN neurons by using ADA-immunoreactivity, as a histaminergic marker and c-Fos immunoreactivity as a neuronal activity marker. The expression of histidine decarboxylase (HDC), the histamine-synthesizing enzyme was also evaluated in the hypothalamus, as well as the performanceon an objects recognition memory task at adulthood. Thioperamide, a H3 autoreceptor antagonist [11], was used in order to increase histamine release and improve the cognitive impairment observed in PA animals.

2. Materials and methods

2.1. Perinatal asphyxia

Pregnant Wistar rats on the last day of gestation (G22) were euthanized by cervical dislocation and hysterectomized. One or two pups were immediately removed and used as sibling non-asphyxiated caesare-delivered controls (C). The uterine horns containing the remaining fetuses were immersed into a water bath at 37 °C for 21 min to induce severe asphyxia, thereafter the uterine horns were excised, the pups delivered and stimulated to breath. Asphyxia-exposed and control animals were kept on a warming pad for 1 h, and then assigned to surrogate dams for nursing, pending further experiments [12]. After weaning, thirty adult male rats (PA = 18; C = 12), weighing 270–350 g were kept in separate cages (3 per cage) at controlled temperature (21–24 °C) and 12/12 h light/dark schedule. Water and food were supplied *ad libitum.* All experiments were carried out in compliance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996), minimizing the number of animals used and their suffering. Local Institutional Bio-Safety and Bio-Ethical Committee at the Medical Faculty, University of Chile (CBA #0212 FMUCH), approved all experimental protocols.

2.2. Animals groups

Eighteen rats were used for behavioral analysis 12 of them (6C and 6PA) were used for object recognition task and immunohistochemistry analyses. Six PA rats were used for object recognition and pharmacologic blocking of H3 histamine receptor. Twelve animals (6C and 6 PA) were used for HDC Western Blot analysis.

2.3. Object recognition task

The object recognition task was performed in an 80 × 80 × 60 cm box (with a black floor) placed in a noise isolated experimental room. Behavior was recorded with a video camera in an overhead shot/view position. The animals were first habituated to the box for 15 min in the absence of any object for two consecutive days and then exposed to two identical plastic figures for 5 min letting the animals freely exploring and familiarizing with the objects. After 90 min, each animal was tested for object recognition memory by putting the animal in the box with one of the previously exposed objects, and a new one for 5 min. Offline analysis of the video recording evaluated the time spent by the animal to explore the new object, compared to that exploring the previously exposed object, as an indicator of memory recognition. This measure was recorded with an automated video-tracking Matlab routine using (Mathworks, Inc, USA).

2.4. Pharmacological treatment

A group of six PA animals was injected with saline, or the H3 histamine receptor antagonist, Thioperamide (5 mg/kg, 10 mg/kg i.p.) at different sessions, performed with the same order with one week interval among the experiments. Ten minutes after each treatments, the rats were tested for the object recognition task as described above.

2.5. Immunohistochemistry

One hour after the behavioral test, rats were anesthetized with chloral hydrate (350 mg/kg; i.p.), transcardially perfused with 300 ml of saline (NaCl, 0.9%) followed by 500 ml a phosphate buffered (PB, pH 7.4, 0.1 M) paraformaldehyde (4%) solution. The brain was then removed and postfixed in the same solution for two hours, and transferred to 30% sucrose with 0.02% sodium azide in phosphate buffered saline (PBS) until saturated and sunk. Brains were cut in the coronal plane, 50 μm thicknesses, using a sliding frozen microtome. The sections were then processed for Nissl staining and immunohistochemistry. For immunohistochemistry, free-floating sections were incubated with the primary antibody (rabbit anti-Fos polyclonal antibody, Ab-5, Oncogene, San Diego, CA, diluted 1:20,000), overnight at room temperature. Then, the samples were rinsed and incubated with the secondary antibody (Biotin-SP- conjugated AffiniPure goat anti-rabbit IgG H+L; Jackson ImmunoResearch, PA, diluted 1:1000) for 1 h, rinsed and incubated in Vectastain ABC Elite Kit (Vector Laboratories, CA, diluted 1:500), for 1 h. Finally, the sections were revealed with 0.05% 3-3′diaminobenzidine hydrochloride (DAB) and nickel chloride to get an enhanced dark blue nuclear reaction product for c-Fos. Selected, already immunostained sections were subjected to a second immunostaining, to identify ADA-ir neurons in the TMN, with rabbit anti-ADA, polyclonal antibody, (diluted 1:5000, Chemicon, CA). Revealed with DAB without nickel intensification, yielding a brown cytoplasmic precipitate, contrasting with the dark blue nuclear DAB-nickel labeling for the c-Fos-reaction. The specificity

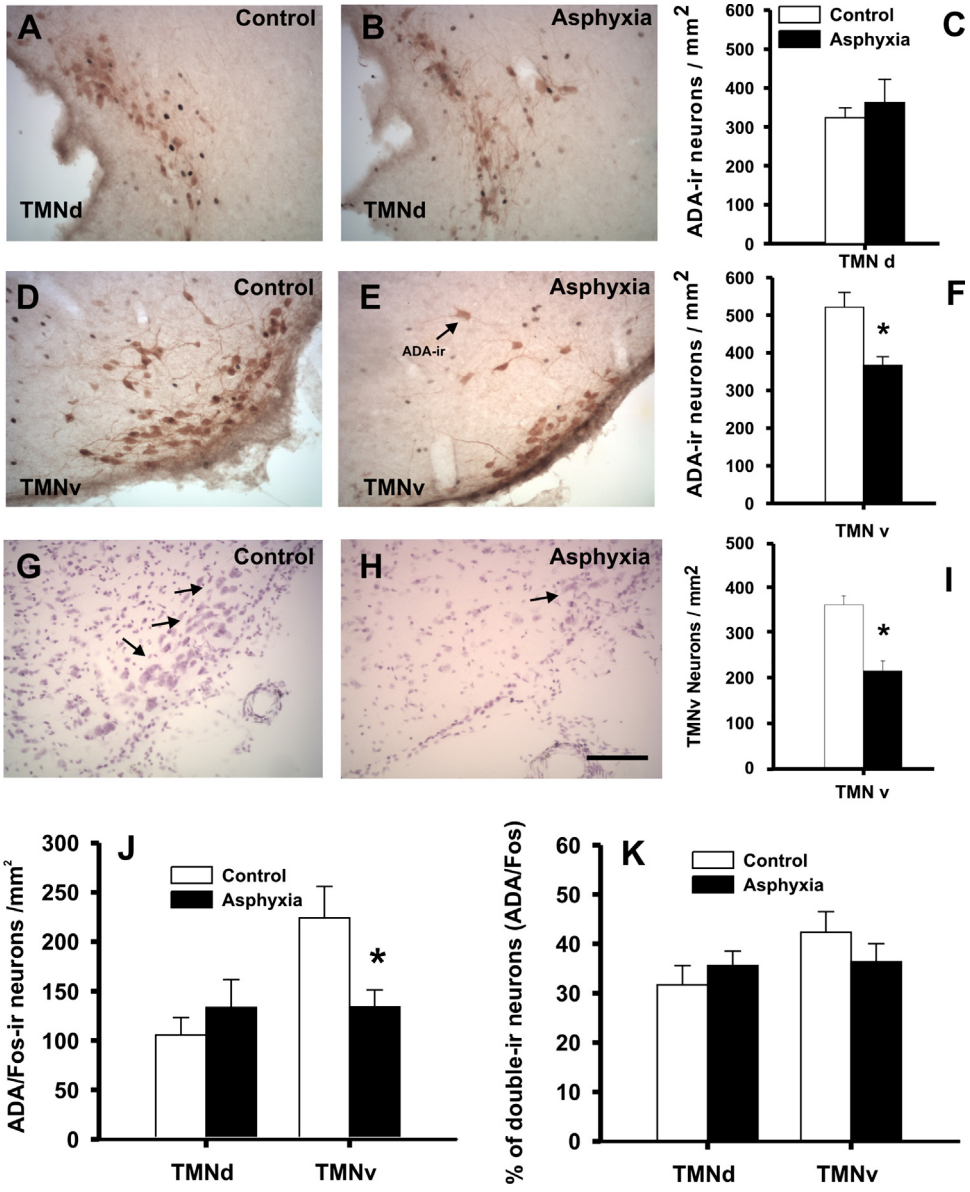

Fig. 1. Effects of PA over neuronal density and activity in the TMN. Photomicrographs showing ADA-immunoreactive (ADA-ir, arrow) neurons in ventral TMN (TMNv) of control (A) and perinatal asphyxia-exposed (PA) (B) animals. In C, quantification of the number of ADA-immunoreactive neurons/mm^2 in TMNv, in sections from control (open columns) and PA (filled columns) rats (n = 6 for each group; *p < 0.01, *t*-test). In (D) and (E) photomicrographs showing ADA-immunoreactive neurons in dorsal TMN (TMNd) of control and PA animals respectively. In (F), quantification of the number of ADA-immunoreactive neurons/mm^2 in the TMNd. In (G) and (H) photomicrographs, showing magnocellular nuclear foci in Nissl staining in the TMNv of control and PA animals respectively. In (I), quantification of the number of nuclear foci (arrows) of Nissl staining neurons/mm^2 in TMNv. In J, number of double-immunoreactive neurons (ADA/Fos) per area through the TMNd to TMNv. In K, the corresponding percentage of double-immunoreactive neurons. Scale bar, 100 μm. *p < 0.05 respect to control, *t*-test.

of the antibodies was tested and reported previously [13]. The number of ADA, c-Fos-immunoreactive neurons and magnocellular nuclei in the Nissl staining, were estimated by counting individual neurons in a rectangular grid (0.25 × 0.5 mm) of 3 (150 μm) evenly spaced coronal sections of the TMN per rat, as was previously described in detail by Valdes et al. [13]; using the ImageJ software (NIH, Bethesda, ML, USA). The number of c-Fos-, ADA-, and double-labelled neurons was expressed as a unit of area and percentage of double-labeled neurons (ADA-c-Fos) over total ADA-immunoreactive neurons. All cell counting was conducted by three blinded researchers.

2.6. Protein extraction and Western blots

For total protein extraction, adult rats (250 g) were euthanized, hypothalamus sampled and homogenized in ice-cold RIPA buffer containing a protease inhibitor Cocktail Set III, EDTA- Free (Calbiochem, Darmstadt, Germany). Supplemented with 0.5 mM DTT, 0.1 mM Na3VO4, 100 mg/ml PMSF, 2 mg/ml leupeptin, 2 mg/ml aprotinin and 0.05% Triton X-100. The homogenates were incubated 25 min on ice, and centrifuged for 20 min at 10,000*g* at 4 °C, recovering the supernatant. Protein level was determined by the bicinchoninic acid (BCA) method (Pierce, BCATM Protein Assay kit). Western Blots were performed according to Neira-Pena et al., [14], using polyclonal guinea pig anti-HDC (110, 66, 48, 36 kDa, 1:500, Progen); Mouse anti-β-actin (42 kDa,1:3000, Chemicon). Membranes were washed and incubated with the HRP-conjugated secondary antibody (1:10.000 anti-guinea pig; 1:10.000 anti-mouse, Thermo) in Tris-buffered saline (TBS) for one hour. The immune complexes were visualized with an enhanced chemiluminescent substrate, according to the instructions of the manufacturer (Perkin Elmer Life Sciences, Boston, MA), captured by

a ChemiScope 3400 (Clinx Sciences Instruments Co, Ltd.). Reactive bands were quantified by densitometry with Image-J software.

2.7. Statistic

All behavioral and histological data are expressed as means ± SEM. Comparisons were tested with Student's *t*-test for histological analysis. One-way repeated measures ANOVA, followed by the Holm-Sidak posthoc test in the case of pharmacological experiments and Mann-Whitney test for Western blot analysis. Statistics was conducted using a Sigma-Stat software, setting a level of $p < 0.05$ for statistical significance. Regression analysis was performed using the least square method.

3. Results

3.1. Perinatal asphyxia reduces density and neuronal activity of TMN neurons

ADA-positive neurons were counted in selected regions of the TMN, dorsal (TMNd) and ventral (TMNv) regions, as described in methods. The total amount of ADA-immunoreactive neurons was different in control and asphyxia-exposed subjects, with a significant decrease of positively labeled neurons in TMNv of PA (366.76 ± 22.71, n = 6, neurons/mm^2), versus C (521.21 ± 39.22, n = 6) animals (Fig. 1A–C). No differences were observed in the TMNd, between PA (362.45 ± 59.82, n = 6, neurons/mm^2) and C (323.72 ± 25.36, n = 6, neurons/mm^2) groups (Fig. 1D–F). Because a reduction in the number of ADA-ir neurons in the TMNv could be explained by a decrease in the expression of the enzyme but not by the number of cells, the number of magnocellular nuclear foci was also assessed in the adjacent Nissl staining of the same animals. As shown in Fig. 1H and I, the number of nuclear foci was also significant decreased in the TMNv of PA (218.54 ± 54.07 neurons/mm^2) versus C (364.56 ± 39.29 neurons/mm^2) animals. Similarly, the density of double (ADA and c-Fos) stained neurons was lower in TMNv of PA ($TMNv_{asphyxia}$ = 133.88 ± 17.17 double-stained neurons/mm^2, n = 6) compared with C animals ($TMNv_{control}$ = 224.19 ± 31.72 double-stained neurons/mm^2, n = 6). Not significant difference was found in the TMNd of PA ($TMNd_{asphyxia}$ = 133.41± 28.25 double-stained neurons/mm^2, n = 6), compared to that observed in C ($TMNd_{control}$ = 105.67 ± 17.67 double-stained neurons/mm^2, n = 6) animals (Fig. 1J). To determine whether a lower density of ADA neurons implied a higher c-Fos activity, as a possible compensatory mechanism, the percentage of double-immunoreactive (ADA/Fos) respect to the total of ADA-immunoreactive neurons was estimated. As shown in Fig. 1K, no significant differences were found in any of the explored regions.

Regression analysis was performed considering behavior during the object recognition memory test as dependent variable, and the density of ADA, ADA/c-Fos neurons, or the percentage of double-stained neurons, as independent variable (see Table 1). A positive and significant correlation were observed between behavior and density of ADA- neurons/mm^2 in TMNv (r^2 = 0.523, p = 0.008, see Table 1).

3.2. Perinatal asphyxia reduces the expression of HDC

To determine wheter the reduction of ADA or Nissl neurons in the TMN corresponded to a histaminergic deficit the level of the histamine-synthesizing enzyme, HDC, was analyzed by Western blots. Fig. 2 shows representative immunoblots from hypothalamus extracts from PA and C rats, 110-36 kDa bands for HDC as previously reported [15,16] and 42 kDa for β – actin as house-keeper. Densitometry shows a decrease of the total labeled bands for HDC in PA versus C animals (Fig. 2B), specifically on band 66 and 48 kDa (Fig. 2C).

Table 1
Linear regression between percentage of object exploration and TMN neuronal density. All control (n = 6) and asphyxia-exposed (n = 6) animals were included in the analysis. ADA: all ADA-immunoreactive neurons, ADA/Fos: Double-immunoreactive neurons for ADA and Fos, % (ADA/Fos)/ADA: percentage of double-immunoreactive neurons (ADA/Fos) over the total of ADA-immunoreactive neurons. TMNv: Tuberomammillary nucleus ventral part, TMNd: Tuberomammillary nucleus dorsal part, m: animal groups, n: number of rats.

Linear regression	r^2	p	m,n
% of object exploration time v/s			
ADA (TMNv/mm^2)	0.523	0.008	2,6
ADA (TMNd/mm^2)	0.0003	0.957	2,6
ADA/Fos (TMNv/mm^2)	0.182	0.166	2,6
ADA/Fos (TMNd/mm^2)	0.210	0.512	2,6
% (ADA/Fos)/ADA (TMNv)	0.483	0.127	2,6
% (ADA/Fos)/ADA (TMNd)	0.024	0.436	2,6

3.3. Low performance in object recognition memory is reverted by H3 histamine antagonist

As shown in Fig. 3A, animals that had undergone PA exhibited an impaired performance in the object recognition task compared to the controls. Percentage of the new object exploration respect to the total exploration time was PA = 41.67% ± 6.34 (n = 6); C = 69.39% ± 7.36 (n = 6). Thus behavioral data, histological and Western blot experiment suggest that PA induce an alteration in the histamine system, explaining the memory deficits. To demonstrate that hypothesis, additional PA animals (n = 6) were injected with vehicle (saline, 1 ml/kg, i.p.) and thioperamide (5, 10 mg/kg, i.p.) and tested for object recognition memory (10 min after each treatment), with a proper washout interval between each experiment (1 week). As shown in Fig. 3B, the thioperamide treatment (10 mg/kg, i.p., n = 6) was able to prevent the behavioral deficit induced by PA, Fig. 3A.

4. Discussion

It is well documented that antihistaminic agents can induce memory and learning disabilities. Increasing histamine levels, by intracerebroventricular administration of histidine, histamine or systemic administration of histamine H_3 receptor antagonists has been shown to increase learning and memory assessed by different experimental paradigms [17–19]. Acute injection of histamine into CA1 immediately after training, but not later, enhanced consolidation of memory in an inhibitory avoidance memory paradigm [20]. The intracerebral injection of an H_3 receptor inverse agonist (which also increases histamine release) enhanced memory retention [21]. Conversely, pharmacologically blocking of histamine receptors by diphenhydramine or pyrilamine, first generation H_1 antagonists [22], as well as H_1 receptor knockout mice [23] produced impairment of memory and learning.

Regarding neural plasticity, histamine can promote long-term potentiation (LTP) in the hippocampus. It has also been suggested that histamine acts on NMDA receptors, via an allosteric binding site, debilitating the binding of Mg^{2+} and potentiating glutamatergic synapses in the hippocampus, even without the presence of tetanic electric stimulation [24,25].

Current evidence suggests that PA causes neurotoxicity by increasing excitatory neurotransmitter levels together with oxidative stress, leading to imbalance between neurotransmitter systems, an imbalance that can be sustained up to adulthood. Indeed, it has been observed that glutamate and aspartate levels are still elevated three months after perinatal asphyxia in hippocampus and striatum [6], perhaps together with histamine levels in

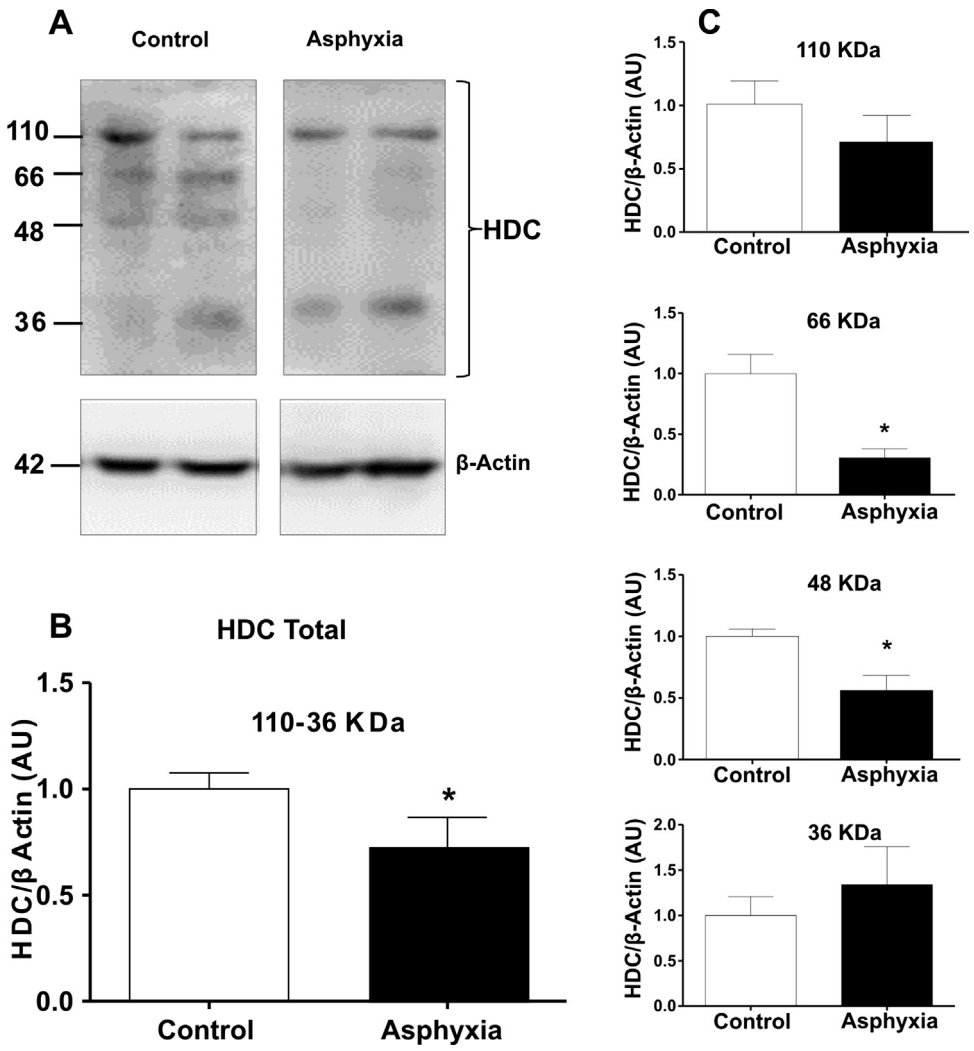

Fig. 2. Effects of PA over HDC expression measured by western blot. (A) Representative immunoblots from hypothalamus extracts of adults control and PA rats of HDC (110-36 kDa) and β-actin (42 kda). (B) Densitometry analysis of the intensity of all protein bands of control (n = 6, open columns) and PA (n = 6, filled columns) rats. (C) Densitometry analysis of the intensity of protein band-by-band of control and PA rats. Results are presented as HDC/b-actin ratio normalized by controls. Data are means ± SEM. *p < 0.05 (Mann-Whitney test).

the hypothalamus [4]. Thus, this data suggests that asphyxia also trigger an imbalance in the histamine system, as discuss above, associated with cognitive disabilities and mood disorders. Similarly, it has been shown that histamine levels are increased by ischemia when induced at adult stages [26]. Increase of histamine levels could provide neuroprotection, decreasing glutamate and dopamine release [27]. Indeed it is proposed that in the brain histamine inhibits [28]. We do not know, however, what is the consequence of increased histaminergic neural transmission following PA or how these changes affect behavior at adulthood.

Histaminergic neurons in the TMN express histidine decarboxylase (HDC) the histamine-synthesizing enzyme and co-express

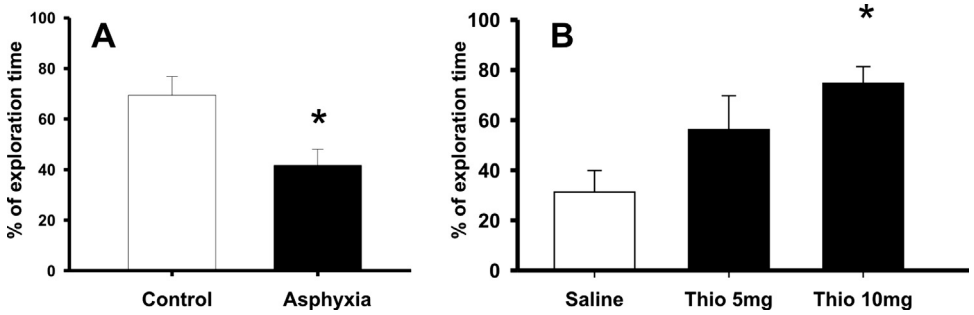

Fig. 3. Effects of PA and H3 antagonist over object recognition memory. In A, percentage of time spent by control (n = 6; open columns) and PA (n = 6, filled column) animals exploring the new object at the object recognition memory task. *p < 0.05 respect to control, Student's *t*-test. In B, percentage of time spent by PA animals exploring the new object at the object recognition memory task, after administration of saline (1 ml/kg, i.p., n = 6) or the H3 histamine receptor antagonist thioperamide (Thio, 5 mg/kg, i.p. n = 6, or 10 mg/kg, i.p., n = 6). * p < 0.05, respect to saline. One-way repeated measures ANOVA, followed by the Holm-Sidak posthoc.

glutamic acid decarboxylase (GAD), [9,29] and adenosine deaminase (ADA) [30–32] the enzyme involved in purine metabolism, degrading adenosine to inosine [33]. Since virtually all ADA neurons in the hypothalamus are also HDC-immunoreactive, except for some spare ADA-immunoreactive neurons outside of the TMN, flanking the arcuate nucleus [30]. Based on these observation, it has been proposed that ADA immunohistochemistry can be used for labeling histamine neurons of the TMN. Histamine neurons have also been described by their electrophysiological signature, with a tonic discharge during high vigilance state [34,35]. Studies with double immunostaining for ADA and c-Fos has demonstrated that ADA-neurons are Fos-immunoreactive during the active period of the behavior of the rat, in agreement with the electrophysiological features of histaminergic cells [36]. Also, histaminergic cells detected by ADA or Histamine-immunoreactivity in primary cultures neurons of TMN, present the same magnocellular morphological characteristics [37].

While adenosine has been proposed as a neuromodulator, based on its selective binding to four G protein-coupled receptor sites [38], no clear evidence exists for inosine as a neuromodulator. Although, it has recently been suggested that inosine can have anti-inflammatory and antinociceptive properties via an A_1 receptor, with potency similar to adenosine [39]. It has also been shown that inosine can induce presynaptic inhibition of acetylcholine release by activation of A_3 adenosine receptor [40]. There is no evidence, however, that inosine is accumulated and released from presynaptic vesicles, no even as a co-transmitter. Thus, the functional relevance for neuronal inosine synthesizing metabolism is not yet established. The discussed evidence supports the idea that ADA and HDC are a marker of the same neuronal population in the TMN.

In summary, we show here that rats that have suffered from PA present a decreased number of neurons in the ventral TMN assessed by Nissl staining and immunohistochemistry using an ADA antibody. Furthermore, we observed a marked reduction in the expression of the histamine-synthesizing enzyme HDC in the hypothalamus. The neuronal reduction in TMNv was correlated with a low performance in an object recognition memory task, reversed, in a dose-dependent manner by systemic administration of thioperamide an H3 histamine receptor antagonist, increasing the histamine release, by blocking an autoreceptor mechanism normally inhibiting histamine release. Further research for determining the cellular mechanism by which PA induces a long-term decrease histaminergic neuronal survival has to be carried out, such as studies evaluating intracellular activation of caspase-dependent mechanism and apoptosis, neuronal cell death, autophagia or necrosis, in histamine cerebral tissues coming from asphyxia exposed animals.

Thus, the present results support the idea that PA is an adverse condition for development, highly correlated with severe damage to the central nervous system. PA might lead to sequelae up to adulthood, without detectable onset signs. Therefore, PA could be a risk factor for developing cognitive disabilities. Since there are few pharmacological treatments for preventing or improving PA-related damage, the present study proposes a novel target based on the idea that histamine system is part of neuromodulatory system involved in cognitive abilities, such as learning and memory, disrupted by PA. Thus, the histamine system in the CNS provides a potential therapeutic target for treating individuals with cognitive problems induced by PA.

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