Original Research

Intermittent hypoxia stimulates formation of binuclear neurons in brain cortex—A role of cell fusion in neuroprotection?

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Abstract

Oligodendrocyte fusion with neurons in the brain cortex is a part of normal ontogenesis and is a possible means of neuroregeneration. Following such fusion, the oligodendrocyte nucleus undergoes neuron-specific reprogramming, resulting in the formation of binuclear neurons, which doubles the functional capability of the neuron. In this study, we tested the hypothesis that the formation of binuclear neurons is involved in long-term adaptation of the brain to intermittent hypobaric hypoxia, which is known to be neuroprotective. Rats were adapted to hypoxia in an altitude chamber at a simulated altitude of 4000 m above sea level for 14 days (30 min increasing to 4 h, daily). One micrometer sections of the left motor cortex were analyzed by light microscopy. Phases of the fusion and reprogramming process were recorded, and the number of binuclear neurons was counted for all section areas containing pyramidal neurons of layers III–V. For the control group subjected to sham hypoxia, the density of binuclear neurons was $4.49 \pm 0.32\,mm^2$. In the hypoxia-adapted group, this density increased to $5.71 \pm 0.39\,mm^2$ ($P < 0.04$). In a subgroup of rats exposed to only one hypoxia session, the number of binuclear neurons did not differ from the number observed in the control group. We suggest that the increased content of binuclear neurons may serve as a structural basis for the neuroprotective effects of the adaptation to hypoxia.

Keywords: Adaptation to hypoxia, cell fusion, neuron, oligodendrocyte, brain regeneration

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Introduction

Adaptation to hypoxia (AH) has been shown to improve cognitive function[1] and to be neuroprotective in experimental epilepsy,[2–4] alcohol withdrawal,[5] Alzheimer's disease,[6] experimental[7] and clinical parkinsonism,[8] and clinical schizophrenia.[3] However, the molecular and cellular mechanisms of these protective effects are poorly understood.

Earlier, we observed fusions of neurons with oligodendrocytes in the rat cortex during normal ontogenesis.[9,10] In the heterokaryons formed by this process, the oligodendrocyte nucleus undergoes neuron-specific reprogramming.[10] This reprogramming results in the transformation of heterokaryons to dikaryons, i.e., binuclear neurons. We suggested that the formation of binuclear neurons could contribute to regeneration, due to the increased number of processes and synapses and, thus, higher communicative capability of binuclear cells.[10] The emergence of binuclear cells may compensate for the loss of neurons and synapses in the process of ontogenesis or disease. The role of binuclear neurons in brain regeneration was demonstrated by studying experimental hemorrhagic stroke in the motor cortex. Rats with the shortest time for recovery of motor function had the highest content of binuclear cells in the cortex surrounding the lesion.[10]

The aim of the current study was to investigate hypoxia-induced neuronal fusion as a potential neuroprotective effect of adaptation to intermittent hypoxia. Specifically, we tested the hypothesis that the neuroprotective program of AH stimulates the formation of binuclear neurons in the rat brain cortex.

Materials and methods

Animals

Experiments on male Wistar rats aged 2 months and weighing 280–310 g at the beginning of the study were performed according to the current standards (National Standard of Russian Federation GOST P 53434-2009 and the National Research Council Guide for the Care and Use of Laboratory Animals). The protocol of the study was approved by the Institute of General Pathology and

Pathophysiology Animal Care and Use Committee. The rats were divided into two groups, the adaptation group ($n=7$) and the control group ($n=7$) that was subjected to sham hypoxia.

Adaptation to hypoxia

The adaptation group of rats was adjusted to hypoxia in an altitude chamber at a simulated altitude of 4000 m above sea level. The total duration of the adaptation was 14 days. On the first day, rats were ''elevated'' to 1000 m and the session lasted for 30 min; on the second day—2000 m for 1 h; on the third day—3000 m for 1.5 h; on the fourth day—4000 m for 2 h; on the fifth day—4000 m for 3 h; and on the sixth and subsequent days—4000 m for 4 h. The velocity of the ''elevation'' to the simulated altitude did not exceed 15 m/s. All experiments were performed at the same time of day, starting at 10 : 00. The rats were anesthetized and sacrificed on day 15 of the study, 24 h after the last hypoxic or sham session.

The altitude chamber was equipped with an external compressor which evacuated air from the chamber to reduce the chamber pressure. The simulated altitude was measured by an altimeter and the chamber pressure was manually adjusted. The chamber was large enough to allow rats to move freely.

The control group ($n=7$) was sham-adapted in the chamber at atmospheric pressure. During AH or sham conditioning sessions, the rats did not show any signs of distress and often slept. For sham AH, the 14-day AH protocol was followed, except that the chamber was maintained at atmospheric pressure. AH was not associated with weight loss or any visible changes in appearance and behavior of adapted or sham-adapted rats.

Morphological analysis

The rats were anesthetized with ether. A catheter was introduced into the left ventricle for transcardial infusion of 15 ml of isotonic sodium chloride followed by 10 ml of 4% paraformaldehyde solution in phosphate-buffered saline (PBS) to fix the brain. The skull was opened and the brain was isolated and stored in 4% solution of paraformaldehyde in PBS. Three 1–1.5 mm^3 tissue samples from the left motor cortex (S1FL) were embedded in epoxy resin and studied morphologically. One micrometer sections were cut with an ultramicrotome (Leica EM UC6, Austria) and analyzed with an Olympus BX51 light microscope equipped with a Color View II camera (Olympus, Japan). Photography and image analysis were performed using Imaging Software for Life Science Microscopy Cell F (Olympus, Japan). Six to nine nonserial sections of each sample were studied. The cortex structure was examined, and cortical binuclear cells were counted. The number of di- and heterokaryons was counted over the entire section area containing pyramidal neurons of layers III–V, and the section area was measured with the Cell F software. The motor cortex was studied since we had previously observed fusions of neurons with oligodendrocytes in the rat motor cortex,[1,9,10] and because many of the reported benefits of AH likely involve this region of the brain.[2–8]

For each rat, the numbers of binuclear cells observed in all sections were summed and divided by the sum of all areas to compute the density of binuclear cells. These values were subjected to statistical analysis to determine whether AH affected the density of binuclear cells. The density of binuclear cells in each section was also computed by dividing the number of binuclear cells by the section area. From these data, a coefficient of variation was computed, and these coefficients were averaged to yield a measure of overall intra-animal variability. When the number of binuclear neurons was counted in another nine sections from the same animal, this value did not differ from that from the first nine sections by more than 10%.

Statistical analysis

Density of binuclear neurons in control and AH rats were analyzed with an unpaired *t*-test (SigmaPlot 11.0), after having passed tests for normality and equal variance. Values are presented as means ± SEM. P values <0.05 were considered statistically significant.

Results

No histological differences, other than the number of binuclear neurons, were found between cortex samples from the control and the AH groups. All found binuclear cells were pyramidal neurons, based on the fact that these cells were the largest, were pyramidal in shape, and usually had apical dendrites.

Binuclear pyramidal neurons with morphologically different nuclei (heterokaryons) were observed in cortex sections from both groups. One heterokaryon nucleus was always the nucleus of the neuron, but the structure of the other nucleus in the heterokaryon was variable (Figure 1). When difference between nuclei was the greatest, the second nucleus was considerably smaller than the neuronal nucleus, did not have a nucleolus, and did not contain almost any euchromatin. This second nucleus was similar to the oligodendrocyte nucleus in its dimensions, shape, and chromatin structure (Figure 1a,b). In other instances, this similarity was less obvious. Then the second nucleus had distinct hetero- and euchromatin zones (Figure 1c). Often this second heterokaryon nucleus was more sharply divided into hetero- and euchromatin zones with a considerably larger euchromatin zone than the oligodendrocyte nucleus. Such nuclei did not contain a nucleolus, and their chromatin structure was intermediate between oligodendrocyte and neuron nuclei (Figure 1d). Frequently, binuclear neurons with two identical neuronal nuclei (dikaryons) were observed (Figure 2).

For the control group, the density of binuclear neurons was $4.49 \pm 0.32\ mm^2$. In the hypoxia-adapted group, this density increased to $5.71 \pm 0.39\ mm^2$ ($P < 0.04$). Binuclear cells were distributed unevenly in brain sections of both groups (Figure 3). The average coefficient of variation for density of binuclear cells among intra-animal sections was 0.42 ± 0.02 for control rats and 0.32 ± 0.03 for AH rats ($P < 0.05$).

A separate group of five similar rats was studied after exposure to only one hypoxia session. The number of

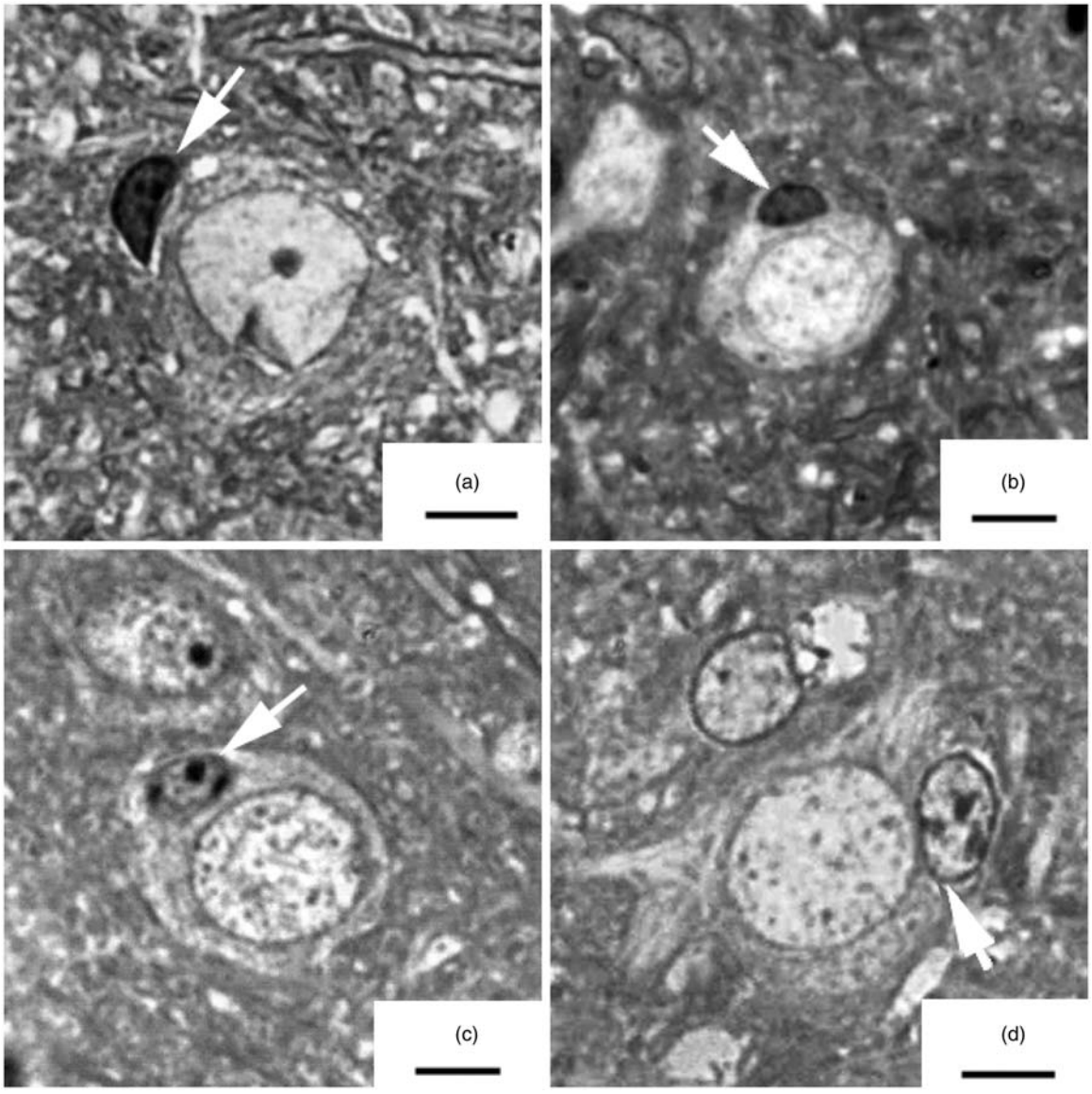

Figure 1 Variations of oligodendrocyte nucleus structure in heterokaryons. The scale bars are 15 μm. (a) The oligodendrocyte (arrow) is located next to the neuron. The oligodendrocyte nucleus is small and contains evenly hyperchromic chromatin; separation of hetero- and euchromatin is not pronounced. (b) Heterokaryon. The oligodendrocyte nucleus (arrow) is located in the neuronal cytoplasm. This oligodendrocyte nucleus is identical to the nuclei of unfused oligodendrocytes in size and chromatin structure. (c) Heterokaryon. The nucleus indicated by the arrow does not differ from oligodendrocyte nuclei in the size. However, as distinct from (a) and (b), chromatin is visibly separated into hetero- and euchromatin. (d) Heterokaryon. The nucleus indicated by the arrow is significantly larger than oligodendrocyte nuclei. In this nucleus, heterochromatin is visible as a narrow belt along the nuclear membrane with three clusters in the center. Euchromatin is darker than in the neuronal nucleus, and it occupies a significant part of the nuclear space

cortical binuclear neurons counted in these rats did not differ from the number observed in the control group.

The actual number of fusions was certainly greater than was visible in the semi-thin sections analyzed here, because binuclear neurons were identified only when both nuclei of a dikaryon or a heterokaryon were observed in the section plane. If one nucleus of a binuclear cell was below or above the section plane, that cell would have been classified erroneously as mononucleate. Figure 4 illustrates this case. The section shows a neuron containing a non-neuron nucleus. Therefore, this cell is not a mononuclear neuron but, in fact, is a heterokaryon with the neuronal nucleus lying outside the section plane.

Discussion

The major finding of this study was that AH was associated with the increased formation of binuclear neurons. Cells can become binuclear in two ways: (a) nuclear division without cytoplasmic division and (b) cell fusion. Nuclear division cannot explain the increase in binuclear cells after AH since neurogenesis is absent in the cortex.[11–13] Furthermore, in many binuclear cells, the nuclei were clearly different (Figure 1), indicating that they originated from different cells, i.e., a neuron and an oligodendrocyte. The presence of two differing nuclei in the same neuron can only be explained by cellular fusion.[10]

No predictive signs of forthcoming cell fusion were seen. Fusion became evident only after the appearance of a second nucleus in the neuronal cytoplasm. For this reason, we could not use cytoplasmic or membrane markers to identify the cellular parent of the second nucleus. It was only possible to identify the second cell participating in the fusion by morphological features of the second nucleus after the fusion has occurred. In cases of maximum morphological difference, the second nucleus of a heterokaryon could be identified with certainty as an oligodendrocyte nucleus since nuclei of two other potential candidate participants in the fusion, astrocytes[14] and synantocytes,[15–17] differ from the oligodendrocyte nucleus by the presence of nucleolus and lower heterochromatin content.

When a heterokaryon nucleus differed from an oligodendrocyte nucleus, the most likely explanation is pro-neuronal reprogramming of the oligodendrocyte nucleus within the neuron. The morphological similarity between the former oligodendrocyte nucleus and the neuronal nucleus as well as the presence of neuronal markers in and around the former oligodendrocyte nucleus[10] supports this explanation. Similar reprogramming of a small cell nucleus after the fusion with a large Purkinje neuron was reported by Weimann *et al.*[18]

In the current study, AH produced an increased number of cortical binuclear neurons. Reprogramming of the nucleus from the fused cell would improve the ability of the cortex to withstand ischemic insult.[10] Importantly, AH has been shown to reduce brain ischemic damage[19,20] as well as to improve cognitive function[1,21] and to be

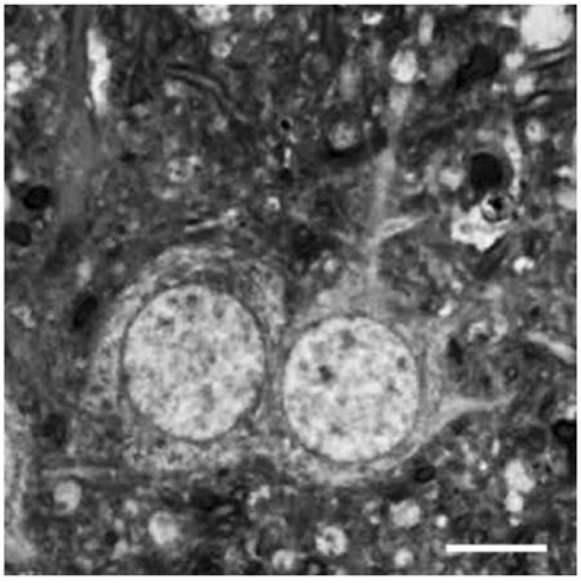

Figure 2 Structure of a dikaryon. The binuclear neuron contains two nuclei, which are similar in size and identical in nuclear chromatin structure. The scale bar is 15 μm

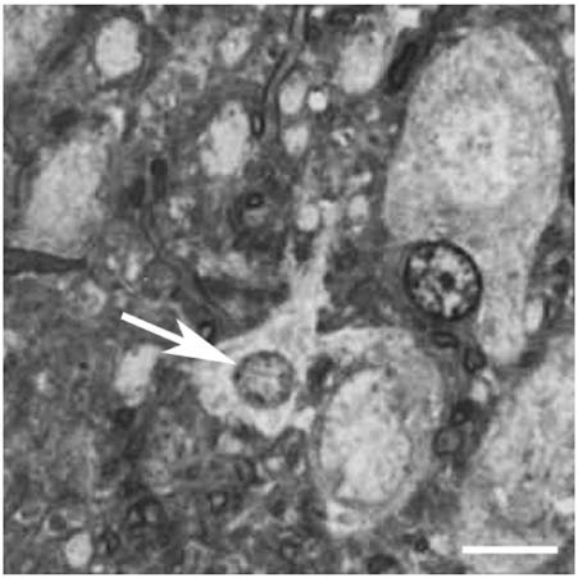

Figure 4 Heterokaryon. The arrow shows a non-neuronal nucleus which appeared in the section of binuclear pyramidal neuron. The scale bar is 15 μm

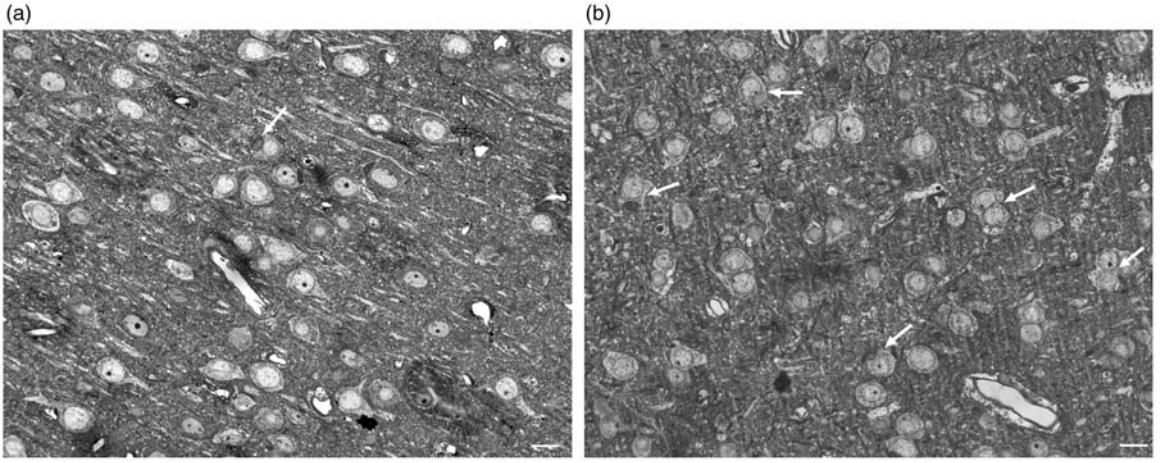

Figure 3 Distribution of binuclear neurons in rat cortex. (a) Cortical section from a control rat. The arrow indicates a single binuclear neuron (heterokaryon). (b) Cortical section from a rat adapted to intermittent hypoxia. The arrows indicate five binuclear neurons (two dikaryons and three heterokaryons). The scale bars are 30 μm

neuroprotective in experimental epilepsy,[2–4] alcohol withdrawal,[5] Alzheimer's disease,[6] experimental[7] and clinical parkinsonism,[8] and clinical schizophrenia.[3] It is tempting to ascribe these benefits, at least in part, to increased functional potential, i.e., formation of an increased number of cortical binuclear neurons and additional neuronal spines and synapses, of an increased number of cortical binuclear neurons, but proof of this theory requires more definitive research.

Not only was the density of binuclear neurons increased by AH, the intra-animal distribution of binuclear cells was more uniform in the AH group than in the control group, as indicated by a lesser coefficient of variation. We speculate that AH stimulates the formation of binuclear neurons preferentially in regions of the cortex that had fewer such cells pretreatment. This response could account for the more uniform intra-animal distribution of binuclear neurons we observed, and this may amplify the neuroprotective action of AH.

In our previous study in a model of cortical stoke, ischemia stimulated neuronal fusion and increased number of binuclear neurons.[10] The question remains, why should AH stimulate neuronal fusion? AH is clearly a lesser insult than acute ischemia, but this insult has been shown to cause structural and molecular changes in the brain. For example, synaptogenesis[22] and angiogenesis[5,23] are stimulated by AH. Increased synthesis of heat shock proteins is also stimulated by AH.[24,25] The results of the current study demonstrate that the insult of AH was sufficient to stimulate neuronal fusion and increased numbers of binuclear neurons.

In the current experiments, we used a hypoxia conditioning program which has been proven protective against neurodegeneration[23,26] and nerve cell apoptosis.[27] It is true that more prolonged and severe intermittent hypoxia generally used for modeling sleep apnea may induce neurodegeneration[28] and apoptosis.[29] Increased numbers of binuclear cells could, at least in part, account for beneficial neuronal function in previously reported studies of adaptation to intermittent hypoxia.

Findings of dikaryons or heterokaryons with nuclei different from the oligodendrocyte nucleus could be formally explained by neuron fusion with each other or with some cells other than oligodendrocytes. However, a much more likely explanation of our findings is pro-neuronal reprogramming of the oligodendrocyte nucleus captured by the neuron. The neuron cytoplasm is many times larger than the oligodendrocyte cytoplasm. The small oligodendrocyte cytoplasm is invisible in histological preparations; electron microscopy shows that the amount of oligodendrocyte cytoplasm is much less than the size its small nucleus.[30,31] After the fusion, concentrations of oligodendrocyte epigenomic regulators in the combined cytoplasm of binuclear cell sharply decrease. Therefore, after entering in the neuron, the influence of oligodendrocyte transcription regulators on the oligodendrocyte nucleus greatly declines, and oligodendrocyte nucleus increasingly comes under the influence of neuronal regulators. Under these conditions, the oligodendrocyte nucleus cannot remain an oligodendrocyte nucleus; it will inevitably change its transcription program consistent with the action of local, neuronal regulators. Also demonstrated here is the progressively increasing morphological similarity between the oligodendrocyte nucleus and the neuronal nucleus. The third argument is the appearance of specific neuronal markers, NeuN and MAP2, in and around the oligodendrocyte nucleus along with the increased transcription rate in the oligodendrocyte nucleus similar to the neuronal nucleus.[10]

In summary, increased numbers of binuclear cells in rat cortex were observed following exposure of the rats to intermittent, hypobaric hypoxia. These dikaryons or heterokaryons were formed most likely by fusion of oligodendrocytes with neurons. Reprogramming of the oligodendrocyte nucleus and its transformation into the second neuronal nucleus would be neuroprotective and would enhance the functional capacity of these binuclear neurons. Thus, increased numbers of binuclear cells could, at least in part, account for beneficial neuronal function in previously reported studies of adaptation to intermittent hypoxia.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript. AVG, IPD, and SVK conducted the experiments, AAP and EBM wrote the manuscript. HFD analyzed data, edited, and revised the manuscript. AAK directed the work and edited the manuscript.

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