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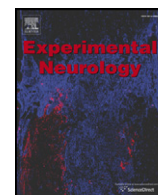

Research Paper

Reversible recovery of neuronal structures depends on the degree of neuronal damage after global cerebral ischemia in mice

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It has been observed by *in vivo* imaging that damaged neuronal structures can be reversibly restored after ischemic insults with the application of timely therapeutic interventions. However, what degree of neuronal damage can be restored and the time frame for reversible recovery of neuronal structures remain unclear. Here, transcranial two-photon imaging, histological staining and electron microscopy were used to investigate the reversible recovery of neuronal structures from dendrites to soma after different durations of global cerebral ischemia in mice. Intravital imaging revealed that the damage to dendritic structures was reversible when ischemia time was <1 h, but they became difficult to restore after >3 h of ischemia. Data from fixed YFP brain slice and Golgi staining indicated that the damage of dendritic structures progressively extended to deeper dendritic shafts with the extension of ischemia time. Furthermore, longer duration of ischemia caused an increasing number of degenerating neurons. Importantly, significant chromatin margination and karyopyknosis of neuron were observed after 6 h of ischemia. These data suggested that neuronal structures could be reversibly restored when ischemia time was <1 h, but irreversible and progressive damage to neurons occurred with longer duration of ischemia. Consistently, behavioral performance of post-ischemic animals experienced an ischemia time-dependent recovery. Taken together, our data suggested that recovery of neuronal structures following ischemia was dependent on the duration of ischemia, and prevention of neuronal loss is a key target for therapeutic interventions in ischemic stroke.

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1. Introduction

Cortical neurons are vulnerable to ischemic insult. However, the most vexing problem is that it is difficult to reconstruct the injured neural circuits once ischemia results in the death of neurons (Brown et al., 2010). One of the concerns about stroke treatment is that cortical neurons are terminally differentiated cells and generally lack the ability to regenerate (Bjorklund and Lindvall, 2000; Nguyen et al., 2002). Thus, it is crucial to restore the damaged neuronal structures and to prevent neuronal loss for acute stroke treatment. Fortunately, through *in vivo* two-photon imaging, we and others have previously shown that the damaged neuronal structures could be restored after ischemia if reperfusion occurred soon enough (Li and Murphy, 2008; Murphy et al., 2008; Zhang et al., 2005; Zhu et al., 2016). However, it is still unknown what degree of neuronal damage could be restored and what affects the time frame for reversible recovery of neuronal structures.

So far, the most effective treatment for this disease is timely intervention within the first few hours after onset of a stroke (Hacke et al., 2008). It is commonly believed that the therapeutic time window for effective application of thrombolytic agents is within 3–6 h following stroke in clinical and animal experimental studies, and many researchers are working on expanding the time window for treatment of acute ischemic stroke (Del Zoppo et al., 2009; Fisher and Albers, 2013; Kim et al., 2005; Liang et al., 2015; Wang et al., 2015). Most of the results regarding the time window for stroke treatment were concluded from magnetic resonance imaging, computed tomography, fixed brain tissue sections or cognitive and behavioral outcomes. Until now, the time-lapse changes of neuronal structures after different durations of ischemia were still not fully understood. What happens to neuronal structures during the critical time window (3–6 h) for stroke treatment and what determines the therapeutic time window remain unclear. However, it is difficult to achieve time-lapse observation of neuronal pathological changes after acute ischemic stroke in human, and it has become a necessary choice to investigate the time frame of neuronal damage and restoration after ischemia by establishing an animal model.

Here, we used *in vivo* two-photon imaging combined with electron microscopy (EM) and histological staining to examine the damage and recovery of neuronal structures after different durations of global cerebral ischemia in mice. The results showed

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that the recovery of neuronal structures from dendrites to soma was critically dependent on the duration of ischemia, coinciding with the critical 3–6 h therapeutic time window for acute ischemic stroke (Hacke et al., 2008; Ringleb et al., 2002; Wang et al., 2015; Yoon et al., 2015). Our results indicated that the reversible recovery of neuronal structures was associated with the degree of neuronal damage, and the therapeutic time window for acute ischemic stroke would be based on the reversible recovery of neurons. How to restore the damaged neuronal structures and prevent neuronal loss has become the ultimate target for acute stroke treatment (Neher et al., 2013). The time frame for recovery of neuronal structures from dendrites to soma after different durations of ischemia has provided new insight for clinical treatment of ischemic stroke.

2. Materials and methods

2.1. Mice

Transgenic mice with yellow fluorescent protein-labeled layer 5 cortical pyramidal neurons (Thy1-YFP line H, JAX stock No. 003782) were obtained from the Jackson Laboratory and housed in animal facilities at School of Basic Medical Sciences of Lanzhou University. A total of ninety transgenic mice and twenty C57BL/6 mice aged one month of both sexes were used in these experiments. All procedures for *in vivo* imaging were carried out in accordance with the institutional guidelines and all animal experiments were approved by the Ethics Committee of Lanzhou University.

2.2. Stroke model

Bilateral common carotid artery ligation (BCAL) model was used in all experiments to induce different durations of global cerebral ischemia (Wakita et al., 1994; Zhu et al., 2016). Mice were intraperitoneally anesthetized with a mixture of ketamine hydrochloride (150 mg/kg body weight) and xylazine (25 mg/kg body weight) (K/X). Body temperature was maintained at 37 $\pm$ 0.5°C using a heating pad (Taimeng) during surgical operation. The bilateral common carotid arteries (BCCA) of the mice were carefully separated from carotid sheath, and then loosely encircled with surgical sutures. Surgical sutures around the BCCA were tied tightly to induce ischemia. The decrease of blood flow and appearance of dendritic beadings of all animals used in this study were evaluated through a thinned skull during ischemia by *in vivo* two-photon microscopy or epifluorescence microscopy. Only animals with obvious decrease of blood flow and dendritic beadings within 10 min after BCAL were considered as successful ischemia model and chosen for ischemia group. The animals were subjected to ischemia for various durations from 20 min to 6 h, and then the surgical sutures were loosened to realize reperfusion. Sham control group received the same surgical procedure (thinning of the skull and exposure of BCCA) without ligation of the BCCA. The mice were in poor condition after global cerebral ischemia, especially those with long durations of ischemia. To support their recovery, mice were put on a heating pad after ischemia, and were intraperitoneally injected with 100 μl phosphate buffer saline (PBS) containing 5% glucose every hour before they were fully awake. Mice were then put into separate cages. For animals with poor feeding behavior, liquid diets were given to the cages in addition to normal diet, and PBS containing 5% glucose were injected every 2–3 h to animals without eating ability.

2.3. Two-photon imaging

Two-photon *in vivo* imaging was used for real-time observation of the reversible recovery of dendritic structures after different durations of ischemia. The surgical procedure for transcranial imaging has been described in detail previously (Yang et al., 2010; Zhang et al., 2005; Zhu et al., 2016). Briefly, after deep anesthetization of the mouse with K/X, a 4 mm^2 region of the skull was carefully thinned using a high-speed drill (Fine Science Tools) over the right somatosensory cortex. The mouse was placed on the platform of two-photon microscope (Olympus FV1000). The excitation wavelength for YFP was tuned to 920 nm, and then the dendritic structures over thinned area were transcranially imaged using a 25×/1.05 numerical aperture (NA) water-immersion objective (Olympus). Repeated 1024 × 1024 image stacks were collected by a step size of 0.75 μm.

2.4. Histology

For histological analysis of dendritic changes in deeper layer of cortices, YFP expressing transgenic mice which were respectively subjected to 20 min, 1 h, 3 h and 6 h of ischemia were transcardially perfused with PBS, followed by 4% paraformaldehyde (PFA), and then the brains were dissected out and post-fixed with PFA for 24 h. Coronal brain sections (50 μm) were cut on a vibrating microtome (Leica) and then examined and photographed with a laser scanning confocal microscope (Olympus FV1000).

2.5. Golgi-Cox staining

For Golgi staining, animals which were subjected to different durations of ischemia were transcardially perfused with PBS. The brains were quickly removed and placed in Golgi-Cox solution (Gibb and Kolb, 1998). The brains were stored in the dark for 2 days and then transferred into fresh Golgi-Cox solution for 14 days. After that, Golgi-Cox solution was replaced with 30% sucrose, and then coronal sections (200 μm) were obtained on a vibrating microtome (Leica). Sections were treated with ammonium hydroxide for 30 min, followed by Kodak Fix Solution for 30 min. After dehydrating in graded ethanol, the sections were transferred into a mixed solution of chloroform, xylene and 100% alcohol (1:1:1) for 15 min. The sections were mounted in neutral balsam, examined and photographed using a 100×/1.25NA oil immersion objective (Olympus).

2.6. Fluoro-Jade C (FJC) staining

To evaluate the effects of different durations of ischemia on neurodegeneration, FJC (Chemicon) was used to stain degenerating neurons (Schmued et al., 2005). C57BL/6 mice were anesthetized and transcardially perfused with PBS at 24 h following reperfusion after different durations of ischemia, followed by 4% PFA. Brain sections (30 μm) were made using a vibrating microtome and mounted onto gelled slides. The slides were transferred into 0.06% potassium permanganate solution for 10 min with gentle shaking on a shaker (protected from light). After rinsing in distilled water, the slides were transferred into 0.0001% FJC staining solution (in 0.1% acetic acid) for 10 min (protected from light). The slides were rinsed and dried, and then dehydrated fully in 100% ethanol followed by three changes of xylene and mounted with DPX (Sigma). The FJC-stained neurons were examined and photographed with an Olympus BX51WI microscope equipped with a DP72 CCD camera.

2.7. Electron microscopy

To investigate ultrastructural changes of neuronal nucleus after different durations of ischemia, a total of 15 mice divided into four groups (sham group $n = 4$ mice, 20 min of ischemia group $n = 5$ mice, 20 min of ischemia/3 h of reperfusion group $n = 4$ mice, and 6 h of ischemia group $n = 2$ mice) were used for electron microscopy. Anesthetized mice were transcardially perfused with PBS, followed by mixed fixative (2.5% glutaraldehyde and 2%

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