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Research report

Changes in neural dendrites and synapses in rat somatosensory cortex following neonatal post-hemorrhagic hydrocephalus

Chen Xin, Huang Xin, Li Bin, Zhao Zhenyu, Jiang Lei, Huang Chengguang*, Lu Yicheng

Department of Neurosurgery, ChangZheng Hospital, Second Military Medical University, Shanghai 200003, China

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ABSTRACT

Neonatal post-hemorrhagic hydrocephalus is associated with cognitive decline and a serious deterioration in the patient's quality of life. The underlying impairments to neurons are not well understood. Here, we used the method described by Cherian et al. to construct a model of hydrocephalus after intra-ventricular hemorrhage and then observed the subsequent pathological changes in the morphology of neurons labeled by enhanced green fluorescent proteins (EGFP) using the *in utero* electroporation technique. Injection of venous blood into the lateral ventricles of 7-day-old rats in the operation group caused marked enlargement of the ventricles in 60% (9/15) of the rats after 2 weeks and in 53.3% (8/15) of the rats after 3 weeks. Compared with the control group, the length of the neural dendrites in the somatosensory cortex was shortened and the number of both neuron dendrite branches and synapses was significantly decreased. There was no evidence of cerebral cortical neuron death as shown by positive EGFP cell counting which suggest that neurological dysfunction after intra-ventricular hemorrhage-induced hydrocephalus may be related to the shortening of neural dendrites and the decreased number of synapses in somatosensory cortex and thus provides a possible neurological cause for hydrocephalus-induced cognitive decline and motor dysfunction.

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1. Introduction

The motor, cognitive and sphincteral dysfunctions that can occur during hydrocephalus following intra-ventricular hemorrhage seriously threaten the patient's quality of life and the prognosis [7,8,20]. Even when the hydrocephalus is relieved by surgery, residual motor, cognitive and sphincteral neurological dysfunctions may persist in some patients [5,8,13,22,27]. The deleterious effect of hydrocephalus to brain is dependent on magnitude and duration of ventriculomegaly, also modified by the age of onset [8]. Rapid increase in head circumference is often as a symptom of hydrocephalus in children but not adult patients. Symptoms of hydrocephalus patients varied significantly from individual to individual, but the patients always associated with neurological dysfunctions [7,19,20].

Higher neurological functions depend on the construction of large numbers of neural circuits and projections [12]. Neurotransmitter delivery across synapses is the basis of all neural circuits. Neural function depends on neurotransmission [1]. Any change in cortical neural structures, especially degeneration of dendrites and a decrease in the number of synapse, alters the formation of neural circuits and projections and, thus affects cortical movement, sensation and other higher neurological functions [18,29]. Leggio et al. has demonstrated that there is a clear correlation between dementia and a decreased number of cortical synapses [16]. Using immunohistochemistry, Li et al. have found the presence of degeneration of cortical and hippocampal neurons in their animal model of brain trauma [18]. Swann et al. have also found degeneration of cortical neurons and decrease in the number of synapses after epilepsy [26].

Hydrocephalus after intra-ventricular hemorrhage can induce cognitive and motor dysfunctions. However, the neuropathological changes underlying these dysfunctions are still unknown. Using the method described by Cherian et al., we constructed a model of hydrocephalus after intra-ventricular hemorrhage using rat pups in which a sub-population of cortical neurons was labeled with EGFP [4]. At postnatal day 21 and 28, we examined the pathologic changes in neural dendrites and synapses in the somatosensory cortex after hydrocephalus. We found that the length of dendritic growth was shortened, and the number of neural dendrites and synapses in the somatosensory cortex was decreased, which might be the cause of the cognitive decline and motor dysfunction induced by hydrocephalus after intra-ventricular hemorrhage.

* Corresponding author at: Department of Neurosurgery, Changzheng Hospital, No. 415 FengYang Road, Shanghai 200003, China. Tel.: +86 2181885679; fax: +86 2163586116.
E-mail addresses: drhuangcg@sina.com.cn, drhuangcg@sina.com (H. Chengguang).

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2. Materials and methods

2.1. Animal surgery

All animal care and experimental procedures were licensed and carried out according to institutional guidelines that are in compliance with related national and international laws and policies. Citrated rat blood was injected stereotactically into the lateral cerebral ventricles, as previously described [6]. In brief, mixed-sex 7-day-old Wistar rat pups were anesthetized by intraperitoneal injection of 50 mg/kg tribromoethanol (Sigma, Stockholm, Sweden). After the postnatal 7 days (P7) pups were deeply anesthetized, 80 mL of citrated rat blood was injected into the lateral ventricles alternately. Thirty pups out of 60 animals from 6 litters were injected with blood, while 30 littermates served as normal (non-injected) controls, and a needle was inserted into the ventricles without injecting any fluid. The needle was left *in situ* for the same period as was employed for the injection group, as described by Cherian et al. [6]. The pups were reared to different postnatal ages, 50% of which were reared to postnatal 21 days and the other 50% were reared to postnatal 28 days. Under deep anesthesia, rats were perfused transcardially with normal saline and then 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. At each time point, 30 pups (15 for control and 15 for blood injection group) from 3 pregnant rats were used for data analysis in each set of experiments. Only cases in which electroporated neurons occurred exclusively in the somatosensory cortex were included.

2.2. In utero electroporation

In utero electroporation is one of the most straightforward methods for visualizing neuron morphology, and the major advantage of this method is that it enables transgenic labeling and morphological analysis of a sub-population of neurons. *In utero* electroporation was performed as described previously [25]. Briefly, uterine horns of pregnant rats were exposed at E16, and 1–2 μL EGFP plasmid solution was injected into the lateral ventricles of the embryonic brain with a fine glass micropipette. The embryo was then clamped between 5 mm diameter tweezer-type disc electrodes (CUY650-5; Tokiwa Science), and given five 50 ms, 40 V electrical pulses at an interval of 950 ms using an electroporator (ECM830; BTX). Uterine horns were then placed back into the abdominal cavity to allow the embryo to continue to develop normally.

3. IMH of GFP and GFAP

Cortical sections were washed three times with PBS, fixed with 4% PFA in PBS at 4 °C for 30 min, washed again, incubated with 1% Triton X-100 in PBS for 5 min and blocked with 5% FBS in PBS for 1 h at room temperature. Neurons were probed with GFAP antibody (1:1000, Cell Signaling Technology) or GFP antibody (1:1000, Molecular Probes) at 4 °C overnight and washed three to six times with PBS. The sections were then incubated with Alexa-488 or Alexta-546 conjugated goat anti-rabbit secondary antibody at 4 °C for 1 h (1:2000, Molecular Probes), incubated at room temperature for 1 h and washed three to six times with PBS. All antibodies were diluted with PBS containing 5% FBS. Images were acquired with a fluorescent confocal microscope (LSM510 and Axiovert 200M, Zeiss). Hoechst 33342 was used to validate the morphological identification of the nucleus.

4. Spine density

Neurons were first identified under low magnification (20× objective with a numerical aperture of 0.7). Subsequently, spines were analyzed under a higher magnification (60× oil-immersion objective with a numerical aperture of 1.4). All protrusions were counted as spines if they were in direct contact with the dendritic shaft. The mean spine density (the number of spines per 10 μm of dendritic length) was estimated on the focal plane along the entire apical dendrite and along three basal and oblique dendrites. Because this method has been proved to produce reliable results [3], no attempt was made to introduce a correction factor for hidden spines.

5. Analysis of dendrite length *in vivo*

Transfected P21 or P28 pups were deeply anesthetized and perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The whole brain was then removed, placed into 4% paraformaldehyde in 0.1 M PBS for 12 h, dehydrated in a sucrose gradient (15%, 30%, dissolved with PBS), sliced into 60 μm coronal sections with a cryostat (CM1900, Leica) at −20 °C and immunostained with anti-GFP. Confocal images (50 μm Z stacks) of isolated cells were acquired and 3D reconstruction was made using Neurolucida software to trace dendritic arbors and measure the total dendrite length.

6. Statistical analysis

Statistical results are presented as the mean ± SEM. The significance of differences was determined by Student's *t*-test as compared to control group using Prism 4.0 software. An * indicates $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ in all statistical data.

7. Results

To explain the pathogenesis of hydrocephalus, some animal models were developed which have many histopathological similarities to humans. Here we use the post-hemorrhagic ventricular dilation (PHDV) model in neonate developed as reported [6]. Sixty pups from 6 litters were studied, of which 30 pups were sham-injected and the other 30 pups were given 80 μL injections of citrated rat blood into the lateral ventricles alternately at postnatal 7 days. By Hoechst counterstaining, the morphology of the hydrocephalus brain was clearly distinguished from the sham-injected brain. The cortical mantle became thinner, and the lateral ventricles were markedly enlarged in the blood-injected rats. The position of the hippocampus was relatively normal but slightly compressed. In addition, the fibers of the internal capsule and corpus callosum were incomplete. Two weeks later, the lateral ventricles were markedly enlarged in about 60% (9/15) of the rats injected with blood, while no significant change was observed in sham-operated rats (0/15) (data not shown). At the same time, we also examined the immunostaining for GFAP, a marker of astrocytes, and we found that the fluorescence intensity of GFAP staining was higher in brains of injected rats than in brains of sham-injected controls; this observation agrees with previous reports [28] (Fig. 1).

To see if dendritic growth was affected by hydrocephalus *in vivo*, *in utero* electroporation was used to deliver EGFP-expression constructs into rat cortical neurons [25]. At the embryonic age of 16 days, EGFP vectors were injected into the lateral ventricles and then electroporated into a sub-population of neuronal progenitor cells in the ventricular zone. At P7, about 80 μL of blood was injected into the transgenic pups. Two and three weeks later, the effects of hydrocephalus on neuron morphology were assayed. EGFP-positive neurons derived from transfected progenitor cells were mostly in layers II/III of the P7 somatosensory cortex (Fig. 2A and B). Representative 3D reconstruction of transgenic neurons is shown in Fig. 2C. Compared to neurons from sham-operated rats, neurons from hydrocephalus rats had significantly fewer mature dendritic arbors, as represented by a decrease in the total length of dendrites and the number of branches. We also examined the morphology of cortical neurons in blood-injected rats without ventricle enlargement; the total length of dendrites and the number of branches were decreased slightly (Compared to neurons from sham-operated rats, $P = 0.35$). These findings suggested that dendritic simplification was associated with neonatal post-hemorrhagic hydrocephalus. In addition, the number of cortical neurons expressing EGFP at P21 and P28 was counted. There was no significant difference in the number of EGFP-transfected cortical neurons between blood-injected and sham-operated rats, indicating that there was no increase in neuron death during this period of hydrocephalus.

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