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Asphyxia induced by umbilical cord occlusion alters glutamatergic and GABAergic synaptic transmission in neurons of the superior colliculus in fetal rats

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ABSTRACT

Using optical recordings, we studied the effects of asphyxia on intracellular Cl^- and Ca^{2+} concentrations ($[Cl^-]i$; $[Ca^{2+}]i$) in the superior colliculus of fetal rats, which were connected *via* the umbilical cord to the dam. Acute asphyxia was induced by umbilical cord occlusion. The number of fetal superior colliculus neurons showing GABA-mediated increases in $[Cl^-]i$ (leading to hyperpolarization) following local synaptic electrical stimulation had decreased by 3 h post-asphyxiation, while the number showing GABA-mediated decreases in $[Cl^-]i$ (leading to depolarization) increased. $[Ca^{2+}]i$ rise, which occurred after acute asphyxiation, was antagonized by both non-NMDA and NMDA receptor antagonists. The increase in $[Ca^{2+}]i$ following focal superior colliculus stimulation was markedly attenuated at 3 h post-asphyxiation.

These findings suggest that asphyxia induced by umbilical occlusion induces changes in glutamatergic and GABAergic synaptic transmission in the fetal brain.

1. Introduction

We have developed an *in vivo* preparation that allows us to record electrical activity (Sakaguchi and Nakamura, 1987; Nakamura and Sakaguchi, 1990; Nakamura et al., 1995; Sakata et al., 1998) and intracellular Cl^- and Ca^{2+} concentrations ($[Cl^-]i$; $[Ca^{2+}]i$) in the fetal brain (Sakata et al., 2006). This model, in which rat fetuses remain connected to the dams by an intact umbilical cord that is then clamped with a fine surgical clip, provides a means by which to study the effects of asphyxiation on *in vivo* electrical activity, $[Cl^-]i$, and $[Ca^{2+}]i$ in the fetal brain (Sakata et al., 2000).

Using *in vivo* optical recordings, we have shown that both excitatory and inhibitory synaptic transmission occur prenatally in the superior colliculus (Sakata et al., 2006). Focal superior colliculus stimulation induces increases in $[Cl^-]i$, mediated by $GABA_A$ receptors, and in $[Ca^{2+}]i$, mediated primarily by NMDA receptors.

Because there are no *in vivo* studies reporting the effects of asphyxia on synaptic transmission in the fetal brain, we performed the present study to see whether asphyxia induced by umbilical cord occlusion alters glutamatergic and GABAergic synaptic transmission of the fetal superior colliculus.

2. Materials and methods

2.1. Preparation of animals

Fifty-seven pregnant rats (Sprague–Dawley, three months old) were housed in separate cages for the duration of gestation, starting on the first day they were sperm-positive (embryonic day 1: E1). Fetuses were studied at E22. Pregnant rats were initially anesthetized with urethane (1.2–1.4 g/kg, i.p.), given as often as was necessary during the experiment. If required, the anesthetic (0.01 mg/g, i.p.) was injected directly into the fetus. The fetus was then paralyzed with gallamine triethiodide (0.01 mg/g, i.p.). Two or three fetuses were used from each dam. The body temperature of the dams was maintained at $37 \pm 1\,^{\circ}C$ by a heating pad. Warm saline ($\sim 37\,^{\circ}C$) was frequently poured onto the surface of the skin and uterus to maintain an appropriate body temperature and humid environment for the fetuses. In the present experiments, body temperature of fetuses was not measured during umbilical occlusion. It has been shown that during umbilical cord occlusion, no significant change was observed in brainstem temperature in E22 fetuses (Sakata et al., 2002). However, the possibility cannot be excluded that the present results of the fetal superior colliculus may have been confounded by iatrogenic hypothermia. The experiments were reviewed by the Committee of the Ethics on Animal Experiments at Yamaguchi University Graduate School of Medicine and carried out under the Guidelines for Animal Experiments at Yamaguchi University Graduate School of Medicine, in accordance with Japanese Federal Law (No. 105) and Notification (No. 6).

The method used for fetal rat head fixation to a conventional stereotaxic apparatus has been described elsewhere (Sakaguchi and Nakamura, 1987; Nakamura and Sakaguchi, 1990, 1995; Sakata et al., 1998, 2000, 2006). Briefly, pregnant rats were laid in an open box made of acrylic boards with a heating pad installed at the bottom. The uterus was exposed by Cesarean section and partially cut to expose the fetus. The fetal body was placed in a syringe tube (~18 mm diameter) and fixed to a stainless

Abbreviations: $[Cl^-]i$, intracellular Cl^- concentration; $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; NMDA, N-methyl-D-aspartate; E, embryonic day; MEQ, 6-methoxy-N-ethylquinolinium iodide.

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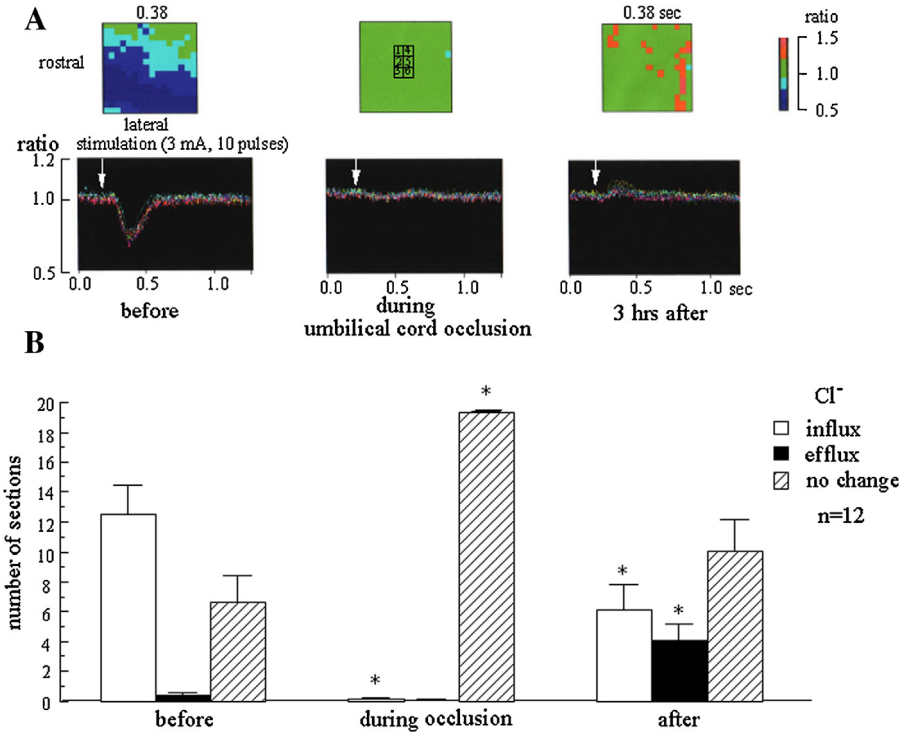

Fig. 1. Effects of asphyxia on changes in $[Cl^-]i$ induced by focal stimulation of the fetal superior colliculus. (A) Upper panels: optical imaging of changes in $[Cl^-]i$ before, during, and 3 h after acute asphyxia induced by umbilical cord occlusion. Changes in the fluorescence ratio ranged from 0.5 to 2.5 as indicated by a color scale (rightmost panel). A decrease (<1.0) and increase (>1.0) in fluorescence ratio indicates an increase and decrease in $[Cl^-]i$, respectively (see Section 2); 'blue' colors indicate stronger increases in $[Cl^-]i$ (before occlusion, left panel), while 'red' colors indicate stronger decreases in $[Cl^-]i$ (3 h after occlusion, right panel). Lower panels: The six colored lines show the time course of changes in fluorescence ratio, which were obtained from the six numbered square areas in the upper middle panel. Focal superior colliculus stimulation was induced at 0.2 s (shown by white arrows). Before occlusion, each superior colliculus region responded to stimulation with rises in $[Cl^-]i$ (downward reflection; fluorescence ratio, <1.0) (left panel). During occlusion, superior colliculus stimulation did not induce any changes in $[Cl^-]i$ (middle panel). At three h after asphyxia, focal superior colliculus stimulation induced decreases rather than increases in $[Cl^-]i$ (upward reflection; fluorescence ratio, >1.0) (right panel). (B) Superior colliculus sections categorized by overall direction of stimulation-induced Cl^- flux before, during, and three h after acute asphyxia induction. Note that at three h post-asphyxia, the number of sections showing superior colliculus stimulation-induced Cl^- influx had become significantly lower than the pre-asphyxia level, while the number showing Cl^- efflux had become significantly higher.

steel board by adhesive tape. The umbilical cord was passed through a small opening at the bottom of the syringe tube and covered with cotton. The fetal head was attached to the stereotaxic apparatus with a simple device made from a small stainless steel tube. After the skin over the cranium was removed, the rostral portion of the skull was glued directly to the wire with dental cement and cyanoacrylate glue. Care was taken to ensure that the skull was positioned horizontally between the bregma and lambda and that the head was not tilted to one side.

Since *in vivo* optical measurements can be made for brain sites whose surface is exposed, the superior colliculus, whose surface in the fetal rats is not covered with the cerebral cortex but exposed, was chosen to perform optical recordings of intracellular Cl^- and Ca^{2+} concentrations.

2.2. Electrical stimulation

To electrically stimulate the fetal superior colliculus, a bipolar electrode consisting of two insulated stainless steel wires (200 μm diameter) was inserted into the rostral superior colliculus (0.5 mm depth from superior colliculus surface). Focal stimulation of the superior colliculus was made by either a single pulse (lasting 1 ms) or a train pulse (100 Hz, 10 pulses, lasting 100 ms in total), with currents ranging from 0.5 to 3 mA, delivered from an isolator connected to an electronic stimulator (SEN-3301, Nihon Kohden, Japan).

2.3. Induction of acute asphyxia

Acute fetal asphyxia was induced by occluding the umbilical cord with a fine clip for 3.6 min (Fig. 3) or 10 min (Figs. 1, 2 and 4), while subsequent restoration of umbilical blood flow was achieved by removing the clip and subsequent local application of a vasodilator (0.5–1% papaverine hydrochloride, Dainihon Pharm. Ltd., Japan) to the occlusion site.

2.4. Optical measurements of intracellular Cl^- and Ca^{2+} concentrations

$[Cl^-]i$ and $[Ca^{2+}]i$ in the fetal superior colliculus were examined before, during, and 3 h after the induction of acute hypoxia to assess any changes in intracellular concentrations of these ions. These changes registered as small fluctuations in the intensity of fluorescent dyes sensitive to $[Cl^-]i$ and $[Ca^{2+}]i$ detected by an optical Argus-HiSCA recording system (Hamamatsu Photonics, Japan; Sakata et al., 2006). The optical recording system consisted of a high-speed cooled CCD camera (model C6790-81, Hamamatsu Photonics) attached to a microscope (TMD300, Nikon), a wavelength-exchanging apparatus (model C6789, Hamamatsu Photonics), and a data-analyzing apparatus (model C6774, Hamamatsu Photonics). While attached to the dams, the fetuses were placed under a microscope with a x10 objective lens and CCD camera (320,000 pixels). The surface plane of the superior colliculus was adjusted so as to be horizontal. Both the objective and CCD camera were then focused on the superior colliculus. The image sensor of the CCD camera covered a 1.0 mm × 1.0 mm area of the superior colliculus, which comprises 16 × 16 square subsections (62.5 μm × 62.5 μm each) (upper panels in Figs. 1A and 4A), contained within the visual field of the objective lens. To assess the changes in $[Cl^-]i$ and $[Ca^{2+}]i$ in response to focal superior colliculus stimulation, the fluorescence intensity of the dyes was averaged over 20 square subsections (62.5 μm × 62.5 μm each) in the center of the superior colliculus where the maximal response was evoked, except the experiments shown in Fig. 1B. In Fig. 1B, the number of sections showing Cl^- influx or efflux per a total number of 20 subsections in the central superior colliculus of each animal was averaged. All optical data were stored on a magneto-optical disk drive for subsequent analysis.

2.5. Measurement of $[Cl^-]i$

6-methoxy-N-ethylquinolinium iodide (MEQ; Molecular Probes) was used as the Cl^--sensitive fluorescent dye. The fluorescence intensity of this dye decreases as $[Cl^-]i$ increases. MEQ (5 mg) was dissolved in 100 μl of distilled water perfused with 100% N_2. The MEQ was reduced by application of 15 μl of 12% $NaBH_4$ solution for 30 min while N_2 gas was bubbled through it. The MEQ preparation stage was followed by the procedure described previously by Fukuda et al. (1998). Briefly, reduced MEQ, which is highly membrane permeable (Biwersi and Verkman, 1991), was extracted from the reaction mixture as a yellow organic layer using diethyl ether as a solvent. This organic layer was dehydrated using 10 mg $MgSO_4$ for 5 min, and the ether was then evaporated under a stream of 100% N_2 in a glass test tube. A portion of the organic extraction was dissolved in 15 μl ethyl acetate and 0.5 ml saline. The MEQ preparation was performed immediately before each experiment