Research report

Neonatal prefrontal cortex lesion using CO$_2$ laser technique

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Accepted 23 August 2002

Abstract

Prefrontal cortex (PFC) is a large area of the brain and its neonatal lesion with ibotenic or kainic acid is used to study the early abnormalities in neurodevelopment that lead to behavioral changes linked to schizophrenia. However, these excitotoxic drugs produce a large and asymmetric damage in the PFC. We produced the bilateral lesions of the dorsal part of the PFC of neonatal Sprague-Dawley rats (postnatal day 7, P7) at the anteroposterior +2.5 mm and mediolateral ±0.4 coordinates by the new laser technique that employ the confined radiation of the CO$_2$ laser in the pulsed mode. The laser was used because its coherent radiation can be focused in a very small spot and as small as of several tens of micrometers in diameters. The CO$_2$ laser radiation is strongly absorbed by water that is present in any soft tissue. Thereafter, the configuration of the heated zone and, consequently, that of the lesion does not depend on the morphological non-homogeneity of particular structures. We obtained the symmetric, conical in shape and small-size bilateral lesions of the PFC. The size of the lesion depended on the beam spot-size and could be as small as several dozens of micrometers in diameter. Our data suggests that the laser technique will be used for the anatomical-functional studies of the PFC in the brain.

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Theme: Disorders of the nervous system

Topic: Developmental disorders

Keywords: Prefrontal cortex; Schizophrenia; Animal model; Laser; Neurodevelopment

1. Type of research

1. Neurodevelopmental studies in rats.
2. Laser-induced lesions to frontal cortex.
3. Pyramidal neuron death.

2. Time required

2.1. Surgical procedure

The surgical procedure of bilateral prefrontal cortex (PFC) laser application takes approximately 30 min per animal. This includes the preparation of the surgical field by assuring the area is sufficiently sterile to mitigate the probability of sepsis, induction of unconsciousness with anesthesia by hypothermia, making a 0.5 cm incision, recording of initial stereotaxic coordinates, bilateral application of confined laser radiation during several seconds, closing the skin with veterinary tissue adhesive at conclusion of procedure, and postoperative monitoring of animals for proper warmth.

2.2. Tissue preparation

At postnatal day 64 (P64), rats were anesthetized with sodium pentobarbital (75 mg/kg, i.p.) and brains were rapidly removed, frozen in isopentane maintained at −40 °C, and stored at −80 °C until use. Frozen brains
were sectioned at 50 μm thickness on the coronal plan using a Leica cryostat. Sections at the level of frontal cortex were collected on cleaned, gelatin-coated microscope slides (4 sections/slide) and then stored at −80°C until the day of stain. Sections were stained with 0.5% cresyl violet and examined under microscope where lesions were visualized. Perfusion and brain removal took 20 min per animal, cryosectioning took 30 min per animal and staining took 3 h.

3. Materials

3.1. Animals

Sprague–Dawley dams with 3-day-old pups were obtained from our animal facilities and litters were culled to 8–10 male pups. At postnatal day 7 (P7), pups weighing 15–18 g were included to surgery. After surgery, pups were returned to the dams. Animals were then housed in a climate-controlled colony room at 24°C with a 12 h on/12 h off light cycle. Animals were weaned at P21 and grouped two or three animals per cage, with food and water ad libitum.

3.2. Special equipment

- CO2 laser (Synrad, Model 48-II 28W, USA) equipped with the focusing lens and beam delivery tube.
- Stereotaxic apparatus (Stoelting, 51600, single lab standard stereotaxic).
- Acrylic body platform styled for P7 pups.
- Cryostat (Leica, Model CM1100, Germany, or similar instrument) to obtain 50 μm coronal tissue sections.
- Dremel Mini-mite (Model 750, Racine, WI, USA).
- Microscope (Leica, Model DMLS, Germany) equipped with a CCD video camera (JVC, Model TK 1380 or similar video camera) and QWIN software.

3.3. Chemicals and reagents

- Gelatin (48723, Fluka, Sigma–Aldrich, Buchs, Switzerland).
- Cresyl violet acetate (C-1791, Sigma, St. Louis, MO, USA).
- Potassium phosphate buffer solution (PBS, 0.1 M), pH 4.0.
- 2-Methylbutane (32,040-4, Aldrich, Milwaukee, WI, USA).
- Sodium pentobarbital (Sedalphorte, Salud y Bienestar Animal, Mexico City, Mexico).
- Paraformaldehyde (I-016, Indeq, Puebla, Mexico).
- DPX microscope mountant (2201, Hycel de México S.A de CV, Jalapa, México).

3.4. Policy issues

The procedures described herein have been conducted in compliance with the policies of the Society for Neuroscience for conducting neuroscience research and with the guidelines of the Laws and Codes of Mexico in The Seventh Title of the Regulations of the General Law of Health Regarding Health Research.

4. Detailed procedures

4.1. Laser applied

Laser installation for making lesions is shown schematically in Fig. 1. We employed a CO2 cw laser (1) operating at λ=10.6 μm. The particular model was Synrad 48-II 28W. The maximum power rating of this laser was 10 W. The beam was 3.6 mm in diameter and of divergence 4 mrad. The laser was mounted on the metallic base, which was tilted at an angle of 90° to the plane of the horizon. The base was fixed to the rail, which served for the mounting of all other units of the installation. The laser radiation was focused by the ZnSe convergent lens (2) of F=38 mm. The lens was mounted on the translation stage (3) (Thor Labs, MDT611). This stage had three translation axes (x, y, z). The lens was equipped with a mounting seat for the connection of the tubular delivery system (4). The termination of the delivery system tube was in contact with

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Fig. 1. Laser installation for making lesions of the neonatal medial prefrontal cortex in rats.
the animal head at right angle to it and against the aperture made in the animal’s skull.

The delivery system was a hollow circular metallic tube (a stainless steel needle of the medical syringe). It was of inner diameter 1.2 mm, outer diameter 1.8 mm, and 80 mm long. The tube mount was concentric with the needle axis and fitted the mounting seat of the lens (3). The relatively large length of the tube facilitated the mixing of the laser beam. In addition, the substantially rough inner surface of the tube contributed to the diffuse reflection and, hence, to intense beam mixing. This permitted to deliver the confined laser radiation of quasi-uniform density distribution across the beam to the object. The tube was not subjected to any special processing, e.g., polishing. However, each time before use, the tube’s interior was cleaned mechanically and then treated with alcohol and phosphoric acid in sequence. The stainless steel tube featured relatively high attenuation per unit length at 10.6 μm wavelength as compared to some specially designed infrared waveguides [12,9,13,5,15]. However, because of the relatively short length of delivery system and excess of the optical power available from the laser, the tube attenuation was of almost no importance in the present installation. These considerations and the low syringe cost determined our choice in favor of this delivery system.

The tilt of the laser and the delivery tube at an angle of ~90° with respect to the plane of the horizon eliminated the possibility of the tube clogging by some biological substance. In our experience, small quantities of blood of the wound of the experimental animal may penetrate into the tube aperture during the exposition of animal to laser radiation. The tilt of the delivery tube presented an important practical improvement with respect to the previous design [11] that featured the horizontal delivery tube.

Finally, each PFC received the laser radiation in the form of a series of pulses. Each series comprised three pulses of 1 s duration each; the interval between the pulses was 5 s.

4.2. Perfusion, fixation and tissue preparation

(i) At P64, each rat was anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (75 mg/kg) and perfused by intracardially with a solution of 0.9% isotonic saline following by 4% formaldehyde in phosphate buffer. The brains were removed and placed in 4% formaldehyde with recovery of behavioral deficits when tested as adults [6,2,4,7]. In contrast, some recent reports show that neonatal PFC lesions induced by ibotenic acid produce a certain neurochemical and behavioral changes after puberty [1,3]. However, the neonatal PFC lesion was asymmetric, big and in some cases, the lesion extended to the adjacent area of the ventral or dorsal part of the PFC, this is because it is too difficult to control the diffusion of the
According to our knowledge, there is no report on any study related to discrete lesions of each of these regions and their correlation to the subsequent behavioral changes. However, it is clear from the lesion studies that there are at least two distinct prefrontal regions, including (1) a dorsal part of the prefrontal cortex, which is made up of medial precentral and dorsal anterior cingulate cortex [7], and (2) a ventral part of the prefrontal cortex, which is made up of the prelimbic cortex and infralimbic cortex [7]. Our results clearly show that the dorsal part of the PFC may be lesioned in the small areas and divided in functional subregions, as suggested our unpublished behavioral results.

Neonatal PFC laser-induced lesions resulted in the formation of a cavity of a relatively small size in all cases seen at P64. While the neonatal lesions produced by ibotenic acid (seen at PD60) produced occasionally small cavities apparent only under the microscopic inspection [1,3]. This permits to suggest that the CO2 laser-induced lesions to the PFC do not feature any anatomical plasticity while neurotoxical lesions feature the anatomical plasticity [3,8]. In addition, the neonatal PFC lesions produced with CO2 laser feature the bilateral symmetric conic cavity with small extension after puberty. While other techniques: mechanical, electrical or neurotoxical damage to the neonatal PFC result in lesions of irregular shape with small cavities and large extension [1,3,8].

6.1. Troubleshooting

First, anesthesia by hypothermia was obtained by keeping the pup on wet ice for 15 min (larger periods resulted in increased mortality). Second, 20 min of anesthesia was obtained as the result. In this period of time the pup was positioned and taped on a platform, an incision was made over the skull. The bilateral holes were made in the bone at the level of prefrontal cortex with the portable drill at low speed. Then, the CO2 laser radiation was applied. Under
the appropriate preparation of instruments, etc., and after some practice, we could do all this procedure in less than 20 min. Following the precautionary steps described above, and keeping the pups warm, both during and after surgery, and monitoring their respiration after surgery, it was possible to maximize the pup survival rate.

6.2. Alternative and support protocols

6.2.1. Laser lesion

The exposition time of the PFC to the CO₂ laser radiation is critical. As described in detail in Section 4.1, expositions longer than 10 s decreased the animal survival rate, this may be due to the excess heating of the brain [11]. However, this problem was overcome by using a series of pulses of laser radiation. Each series comprised three pulses of 1 s duration each; the interval between the pulses was 5 s. This technique increased the pup survival rate closely to 100%.

6.2.2. Excitotoxic lesion

Ibotenic and kainic acid are the most common drugs used to produce excitotoxic damage of the cortex. The volume and concentration of the excitotoxic drugs are critical. More than 300 μl per side into the neonatal brain rat, increase its mortality [3]. The lesion size depends on both, concentration and diffusion of the drug. However, the major problem with this drugs is that the lesion size in the neonatal rats is not really small and symmetric with a irregular shape. Other secondary problem is the mortality of the pups, in special with the kainic acid.

6.2.3. Classical aspiration lesion

The aspiration pressure in the neonatal cortical lesion is critical. High pressure in the aspiration cause big damage in the neonatal brain [2,14]. The blood volume in the neonatal rat is low, so small breeding is another problem with this procedure. Furthermore, the lesion size is not consistent, with lack of symmetry between sides with irregular shape in comparison with the laser procedure [2,14]. However, the aspiration procedure is uncomplicated and not expensive technique.

7. Quick procedure

7.1. Laser lesion

(i) Prepare surgical field so that the area is sufficiently sterile so as to mitigate the probability of sepsis.  
(ii) Securely attach the acrylic body platform for P7 pups.  
(iii) Anesthetize the P7 pup by keeping it for 15 min on wet ice, then position the animal on the body platform with head presentation well-centered in a horizontal plane and snout positioned in a proper proximity to nostril.

(iv) Carefully make a 1-mm incision in the pup's scalp to expose the skull. Locate bregma and note the initial stereotactic coordinates.  
(v) Use the following coordinates: AP -2.5 mm and ML ±0.4 [1,3].  
(vi) Apply the CO₂ laser radiation.  
(vii) Mark pups for later identification, then place the pups on head pad. After recovery, return pups to dam.  
(viii) Wean the pups at P21, and then separate into groups of three per cage with free access to food and water.

7.2. Perfusion and stain

(i) At P64, previously anesthesia induced by sodium pentobarbital (100 mg/kg), transcardially perfused with a primary flush of 50 ml of phosphate-buffered saline (PBS, 0.1 M, pH 7.4) followed by 20 min of 4% paraformaldehyde–PBS via gravity drip.  
(ii) Immediately brain was removed and stored at −80 °C until the sectioning day.  
(iii) Frozen rat brains were sectioned at 50 μm thicknesses on the coronal plane using a cryostat. Sections at the level of the PFC were collected on gelatin-coated microscope slides and stored at −80 °C until the day of staining.  
(iv) Violet cresyl staining procedure (as described in detail in Section 4.3) was used to assess the neural loss.

8. Essential literature references

Original papers: [1,3,11].

Acknowledgements

This study was supported in part by grants from CONACyT (30675-M, 35001-A), UNAM (IN113799) and Fundación Mexicana para la Salud AC. We are grateful to Dr. Carlos Escamilla for his help and suggestions related to keeping of animals. We thank Juan J. Ramírez and Jesus Sánchez for correcting the manuscript. L.S.-H., R.C. and A.S.-G. acknowledge the CONACYT for the studentship. G.F. and S.N.K. acknowledge the National Research System of Mexico for membership.

References


