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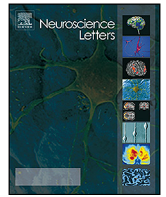

Research article

The effect of carmustine on Bergmann cells of the cerebellum

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HIGHLIGHTS

- Administration of carmustine to pregnant mice induces hyperlocomotion in the offspring.
- Layer disorganization and dysplasias were detected in cerebellum.
- Bergmann glial cells presented a reduced number of protrusions as a consequence of carmustine treatment.

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ABSTRACT

Administration of the alkylating agent carmustine to pregnant mice induces hyperlocomotion in the offspring. Motor performance was evaluated by the rotarod task, which revealed that these animals have diminished Grab Frequency and a higher Performance Index, whereas Error of Latency and Latency to Fall were unaffected. Considering the recently revealed role of Bergmann cells of cerebellum in the control of motor activity, we used the transgenic mice GFAP-GFP to explore the impact of carmustine on the organization of these glial cells. Multiple examples of cell layer disorganization were detected; many soma of Bergmann cells were displaced to the external cell layer, and their processes were not well defined until young adulthood. In addition, the roof of the fourth ventricle was convoluted. These observations suggest that the exacerbated locomotion induced by carmustine may be due, in part, to the altered organization of the cell layers of cerebellum.

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1. Introduction

Early brain development is susceptible to chemical agents that may result in abnormalities that alter the cortex layers, *i.e.*, cortical dysplasia (CD) [1]. These, in turn, are frequently linked to cognitive disabilities and focal epilepsies. Administration of the alkylating agent carmustine (bis-chloroethylnitrosourea) to pregnant rats induces effects similar to those found in patients with CD, [2,3]. This experimental model of CD has been widely validated in histological and behavioral tests [2,4–7].

Carmustine binds nonspecifically to DNA thereby interfering with DNA replication and delaying the cell-cycle while the DNA sequence remains unaltered. This compound is used as an anticancer therapy [8,9]. When administered in a timely manner, carmustine has effects on the rapidly dividing cells that differentiate into neurons, leading to the formation of multiple cortical layers and heterotopias. Cortical dysplasia and heterotopias induced by carmustine have been found in the hippocampus, cerebellum, and brain cortex of rats treated during the critical period of fetal neurogenesis. The offspring of carmustine-treated rats showed impaired short-term working memory but intact long-term aversive memory, whereas their spontaneous motor activity and anxiety-like responses were normal [2,4–7].

It is well established that the glial cells of the brain are fundamental for proper balance of the nervous system, playing a central role in the control of neural transmission [10–12]. However, little is known about how the organization of glial cells is altered in experimental CD. The aim of this study was to assess whether administration of carmustine during the peak day of gliogenesis during mouse development could affect glial organization. Initial observations of mice treated with carmustine suggested altered motor activity; thus, this work describes the effect observed on the organization of cerebellar Bergmann cells (BCs), cells now known to have a role in motor coordination [13]. The major focus was on the roof of the fourth ventricle, formed by the X lobule (*nodulus*).

Abbreviations: BCs, Bergmann cells; BCNU, bis-chloroethylnitrosourea; CD, cortical dysplasia; GFAP, glial fibrilar acidic protein; GL, granular layer; ML, molecular layer; PCL, Purkinje cellular layer; IV, fourth ventricle.

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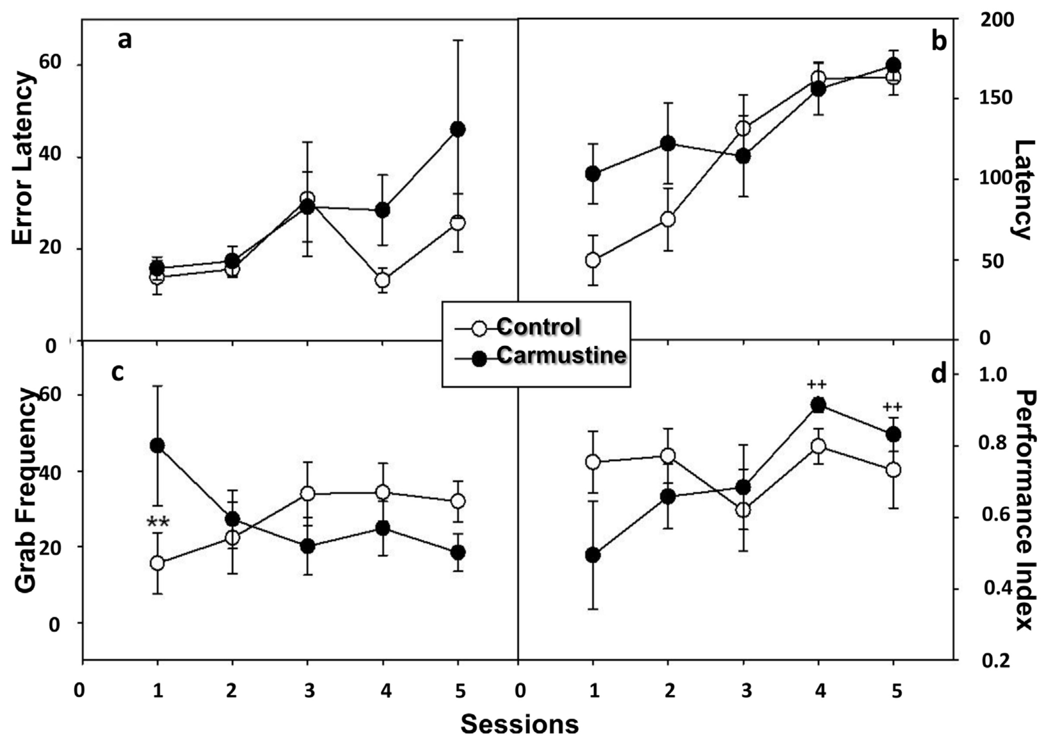

Fig. 1. Rotarod tests. a. Error of Latency. After six sessions, no significant differences were observed between control and carmustine-treated mice. b. Latency to Fall. Mice treated with carmustine fell faster in the first and second sessions, but became adapted after the fourth session. The two-way ANOVA (F[4,79] = 9239 $p < 0001$) indicated significant differences in performance for the first two sessions; however, a *post hoc* Duncan test indicated a difference only in the first session. c. Grab frequency. No differences were observed between control and treated animals except in the first session, when the carmustine group spent more time on the rod (Two-way ANOVA (F [4,79] = 0201 $p > 0,05$)). d. Performance Index. No significant differences were observed between groups (Two-way ANOVA F[4,79] = 1516 $p > 0,05$)); in contrast, differences in performance among carmustine-treated individuals were observed in sessions 4 and 5, whereas control mice did not show this trend (One-way ANOVA (F[4,39] = 0.592, $p < 0.05$).

2. Material and methods

2.1. Animal handling and tissue processing

All protocols and procedures were approved by the bioethics committee in the INB-UNAM, (ID:INEU/SA/CB089). Transgenic eGFAP-GFP [14] pregnant female mice ($n = 8$) were given carmustine, 20 mg/kg in a 5 % glucose solution by a single intraperitoneal injection [6]. Control animals were injected with 5 % glucose. At least two male pups from each pregnant mouse were selected on postnatal day (P) 5, P10, and P25 for tissue processing; the rest were used for behavioral tests. Mice were anesthetized with pentobarbital (30 mg/Kg) and intracardially perfused with saline solution and 4% formaldehyde in phosphate buffered saline (PBS), pH 7.2; cerebella were cryoprotected in graded sucrose solutions (10, 20, and 30% in PBS), and 40-μm coronal slices were obtained. Cell nuclei were labeled with propidium iodide, and the preparations were processed for imaging in a confocal microscope (Zeiss, LSM-780®). 15- to 20-μm *z* stacks were obtained with 10X and 25X objectives.

2.2. Behavioral tests

Mice were tested on a rotarod apparatus (Series 8, IITC Life Science) on six consecutive days [15]. On day one, mice received one habituation trial of 120 s (acceleration from 0 to 40 RPM in 120 s). On day two the rotation speed was increased to 40 RPM after 60 s, and this speed remained constant for 180 s. The trial began when the animal was placed on the rod and ended when any of the following events occurred: (1) the mouse fell down or remained over the rod for more than 180 s. Data scored were: (1) Error Latency (time until the mouse stopped moving for the first time); (2) Fall Latency (Time until the subject fell down); (3) Grab Frequency (number of times the subjects clung to the rod without falling down); (4) Performance Index (Fall Latency – Error Latency/Fall Latency). In addition, differences in the exploration time of novel objects was evaluated by the novel object recognition test [16] that is presented as Supplementary information. All data were analyzed and plotted with SIGMAPLOT software.

2.3. Golgi Staining

Mice were intracardially perfused with 4% paraformaldehyde under pentobarbital anesthesia, and 3-mm thick slices were cut from lobe X of the cerebellum. Impregnation was carried out with the rapid Golgi procedure [17,18]. The tissue was progressively dehydrated and embedded in collodion, and 120-μm coronal sections were cut with a microtome.

Samples were observed under a light microscope (Olympus Ckx41) and photographed using a 40X objective. Several images were obtained from a single cell to visualize all the cell processes. Images that include all the details of a single cell were processed with the Clone Stamp tool of Adobe Photoshop. Images were processed with ImageJ v.1.47 software. The diameter of the soma, soma area, number of processes, length of process, and the absolute and relative extension of processes with bushy protrusions were evaluated as previously described by Hanke & Reichenbach [19]. Seven cells from wild type mice and 10 cells from carmustine-treated mice were analyzed by the U- Mann–Whitney test.

3. Results

Animals treated with carmustine were born after 19 days of gestation, in contrast to the regular 21 days. Mice treated with carmustine were underweight (3.1 g ± 0.26 g carmustine *vs.*

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