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Long lasting cerebellar alterations after perinatal asphyxia in rats

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ABSTRACT

The developing brain may be particularly vulnerable to injury before, at and after birth. Among possible insults, hypoxia suffered as a consequence of perinatal asphyxia (PA) exhibits the highest incidence levels and the cerebellar circuitry appears to be particularly susceptible, as the cellular makeup and the quantity of inputs change quickly during days and weeks following birth.

In this work, we have used a murine model to induce severe global PA in rats at the time of birth. Short-term cerebellar alterations within this PA model have been previously reported but whether such alterations remain in adulthood has not been conclusively determined yet. For this reason, and given the crucial cerebellar role in determining connectivity patterns in the brain, the aim of our work is to unveil long-term cerebellum histomorphology following a PA insult.

Morphological and cytological neuronal changes and glial reaction in the cerebellar cortex were analyzed at postnatal 120 (P120) following injury performed at birth. As compared to control, PA animals exhibited: (1) an increase in molecular and granular thickness, both presenting lower cellular density; (2) a disarrayed Purkinje cell layer presenting a higher number of anomalous calbindin-stained cells. (3) focal swelling and marked fragmentation of microtubule—associated protein 2 (MAP-2) in Purkinje cell dendrites and, (4) an increase in glial fibrillary acidic protein (GFAP) expression in Bergmann cells and the granular layer.

In conclusion, we demonstrate that PA produces long-term damage in cellular histomorphology in rat cerebellar cortex which could be involved in the pathogenesis of cognitive deficits observed in both animals and humans.

1. Introduction

The developing brain may be particularly vulnerable to maternal stress and to other environmental insults before, at and after birth (Kinney et al., 2008; McEwen, 2007). Among these insults, hypoxia suffered as a consequence of perinatal asphyxia (PA) appears to be one of the most important stressful factors.

Brain injury induced by PA is one of the most frequent causes of morbidity and mortality in term and preterm neonates, accounting for 23% of neonatal deaths globally (Lawn et al., 2005). Following PA, approximately 45% of newborns die and 25% have permanent neurological deficits including motor and cognitive alterations of variable severity, such as seizures, spasticity, eyesight and hearing impairment, attention deficit, hyperactivity, mental retardation and other neuropsychiatric syndromes with delayed clinical onset (de Haan et al., 2006; du Plessis and Volpe, 2002; Kaufman et al., 2003; Odd et al., 2009; Titomanlio et al., 2011; Van Erp et al., 2002; Vannucci and Hagberg, 2004). PA thus interferes with neonatal development, generating long-term mental and neurological deficits whose underlying mechanisms have not been fully elucidated yet.

Histologically, brain injury occurring early during development results in significant damage in different areas of the central nervous systems (CNS). In particular, hypoxia/ischemia as consequence of PA causes damage in cerebellum, hippocampus, neostriatum and substantia nigra (Capani et al., 2009). The

Abbreviations: PA, perinatal asphyxia; PCs, Purkinje cells.

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mechanisms for brain injury after hypoxia/ischemia care thought to include energy failure, free radical damage, cytokine release and excitotoxicity, and caspase-dependant cell death (Barks and Silverstein, 1992; Cheng et al., 1998; Hagan et al., 1996; Liu et al., 1996; O'Lone et al., 2007).

The type and distribution of human brain lesions differ markedly between premature and term babies, likely as a consequence of differences in brain maturation and regional vulnerability, as described in different previous studies (Miller and Ferriero, 2009; Verger et al., 2001a,b; Yager and Thornhill, 1997). The immaturity of a particular brain region is highly relevant, as the insult affects the initial plastic changes required for establishing neurocircuitries and synaptogenesis (Anderson et al., 2011). In this sense, the cerebellum is particularly vulnerable because, in humans, it reaches its mature volume within months of birth (Rice and Barone, 2000) and develops throughout pregnancy with rapid growth in the third trimester and in the first postnatal year (Limperopoulos et al., 2007; ten Donkelaar et al., 2003; Zervas et al., 2005). During days and weeks following birth, the cerebellar circuitry is susceptible to injury (ten Donkelaar et al., 2003), as the cellular makeup and the quantity of inputs change quickly (Wang and Zoghbi, 2001). Moreover, it has been demonstrated that brain injury derived from premature birth is associated with cerebellar growth impairment (Limperopoulos et al., 2005).

Traditionally, the role of the cerebellum has been associated to the coordination of voluntary movement, gait, posture, speech and motor functions (Ghez and Fahn, 1985) and has not been considered relevant to the field of psychiatry or the study of brain–behavior relationships. However, growing evidence shows that the cerebellum may play a role in cognition, behavior and psychiatric illness (Glickstein, 2006).

In recent years, we have used a murine model to induce severe global PA in rats at the time of birth, as this model offers three key advantages: first, it mimics relevant aspects of human delivery in an adequate way; second, as it is a non-invasive procedure, it allows for the study of both short and long-term effects; third, it is easily reproducible across laboratories (Capani et al., 2001). Short-term cerebellar alterations within this model of PA have been previously reported (Biran et al., 2011, 2012) but whether such alterations remain in adulthood has not been conclusively determined yet. For this reason, and given the crucial cerebellar role in determining connectivity patterns in the brain, the aim of this paper is to unveil long-term cerebellum morphologycal alterations following a perinatal asphyctic–ischemic insult. To this end, modificacions in the structural organization of the cerebellar cortex were analyzed in young adulthood rats (P120) following injury performed at birth (P0).

2. Material and methods

2.1. Animals

All procedures involving animals were approved by the Institutional Committee of Animal Care and Use at the University of Buenos Aires (CICUAL, School of Medicine) and conducted according to the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

Female Sprague-Dawley rats in the fifteenth day of pregnancy were placed in individual cages and maintained on a 12:12 h light/dark cycle in a temperature- ($21 \pm 2\,^{\circ}C$) and humidity-controlled ($65 \pm 5\%$) environment. Animals had access to food (Purina chow) and tap water ad libitum and were divided in 3 groups: (1) surrogate mothers ($n = 3$), (2) mothers of control pups ($n = 3$) and (3) mothers assigned to PA ($n = 4$).

2.2. Induction of asphyxia

The induction of asphyxia was carried out as previously described (Saraceno et al., 2010). At the time of delivery, a first spontaneous birth was allowed, dams were rendered unconscious by CO_2 inhalation and rapidly killed by decapitation and subjected to a caesarean section and hysterectomy (Dorfman et al., 2006). The uterus horns containing the fetuses were immediately immersed in a water bath at 37 °C for 19 min (sub-severe PA) (Bjelke et al., 1991; Capani et al., 2009; Van de Berg et al., 2003). Following asphyxia, the uterus horns were rapidly opened and the male pups removed, cleaned and then resuscitated by intermittent tactile breathing stimulation for a few minutes until regular pulmonary breathing was established. The umbilical cord was then ligated and animals were left to recover for 1 h under a heating lamp. They were then given to surrogate mothers and mixed in with male control pups (10 pups total per surrogate mother) until the end of the study.

Asphyxia was administered for 19 min, as 21 or more minutes in this condition results in survival rates under 3% (Capani et al., 2009).

Animals born by natural delivery were used as controls to avoid alterations produced by the short time of exposure to CO_2, which may be similar to the injury produced by asphyxia.

2.3. Histological examinations

Four-month-old rats were anaesthetized (Nembutal 50 mg/kg body weight, i.p.) and perfused transcardially with 30–50 ml of 0.1 M phosphate buffered saline (pH 7.4) (PBS) containing 4% paraformaldehyde. Cerebella were removed and post-fixed in the same fixative solution for 2 h at room temperature, and then immersed in 0.1 M phosphate buffer (pH 7.4) containing 20% sucrose at 4 °C overnight. Cerebella were then paraffin-embedded, cut in 4-μm-thick sagittal sections, dewaxed by xylene and hydrated by successive immersion in ethanol 100%, 96%, 70% and water.

2.4. Immunohistochemistry

After antigen retrieval with 0.01 M citrate buffer pH 6, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in 60% methanol. Non-specific binding sites were blocked with 5% bovine serum albumin. Sections were incubated overnight at 4 °C with a rabbit polyclonal anti-Glial fibrillary acidic protein antibody (anti-GFAP, 1:500; Sigma Louis, MO, USA), anti-microtubule-associated protein 2 (MAP-2 1:250; Sigma St. Louis, MO, USA), anti-neuron-specific nuclear protein (anti-NeuN, 1:100; Millipore, Chemicon, CA, USA) or anti- calcium-binding protein calbindin-D28k (anti-calbindin D28K, 1: 250; Santa Cruz Biotechnology, Inc.). After several washes, sections were incubated with secondary antibodies (biotinylated anti-rabbit IgG or biotinylated anti-mouse IgG, Vector Laboratories Inc., Burlingame, CA, USA) for 2 h at room temperature.

To reveal the sites of antigen/antibody binding, an avidin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) and the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA) were used.

For control staining, some sections were incubated with PBS instead of the primary antibody and no immunoreactivity was detected. Sections were dehydrated in ethanol, cleared in xylene and mounted in Canada balsam, and then photographed using Nikon Eclipse 80i microscope and Visiopharm Integrator System software.