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A single episode of neonatal seizures alters the cerebellum of immature rats

Selene Lomoio[a], Daniela Necchi[a], Vladislav Mares[b,c], Elda Scherini[a,*]

[a] Department of Animal Biology, Laboratory of Cell Biology and Neurobiology, University of Pavia, via Ferrata 1, 27100 Pavia, Italy
[b] Institute of Physiology, Academy of Sciences of the Czech Republic v.v.i., Prague, Czech Republic
[c] Faculty of Natural Sciences, University of J.E. Purkinje, Usti n.L., Czech Republic

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KEY WORDS
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Summary
Purpose: To test whether a single episode of early-life seizures may interfere with the development of the cerebellum. The cerebellum is particularly vulnerable in infants, since it is characterized by an important postnatal histogenesis that leads to the settling of adult circuitry.
Methods: Seizures were induced in 10-day-old Wistar rats with a single convulsive dose (80 μg/g b.w., s.c.) of pentylentetrazole (PTZ). Immediately after rats were treated with ^{3}H-thymidine (^{3}HTdR, 2.5 μCi/g b.w, s.c.). Rats were killed 4 h later and paraffin sections of the cerebellar vermis were processed for ^{3}HTdR autoradiography and immunocytochemistry for 2/3 subunits of AMPA glutamate receptor (GluR2/3), glutamate transporter 1 (GLT1) and calbindin.
Results: Seizures reduced the proliferation rate of cells in the external germinal layer. Purkinje cells showed increased GluR2/3 immunoreactivity. However, some Purkinje cells were unstained or lost. Increased GLT1 immunoreactivity was present in glial cells surrounding Purkinje cells. Calbindin immunoreaction confirmed that some Purkinje cells were missed. The remaining Purkinje cells showed large spheroids along the course of their axon.
Conclusions: Data indicate that seizures lead to a loss and alteration of Purkinje cells in the cerebellum of immature rats. Since at 10 days of life Purkinje cells are no more proliferating, the loss of Purkinje cells should be permanent.

Introduction

Neonatal seizures, which affect approximately 3 in 1000 infants, are often associated with an adverse developmental outcome. However, whether seizures are themselves the primary cause of the poor developmental outcome is still a matter of controversy. In fact, other factors, which in turn may have triggered seizures, such as illnesses, brain insult or medication effect may concur to the brain damage.

An animal model study, which eliminates other causes of brain damage, demonstrated that multiple episodes of early-life seizures result in late learning impairment correlated with cell loss and synaptic reorganization in

* Corresponding author.
Tel.: +39 0382 986320; fax: +39 0382 986325.
E-mail address: scherini@unipv.it (E. Scherini).

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the hippocampus (Swann, 2004). On the contrary, other studies indicated that immature rats experiencing a single episode of early-life seizures did not suffer late behavioral alteration nor cell loss and synaptic reorganization (Holmes et al., 1988; Haas et al., 2001). Nevertheless, a report describes hippocampal-dependent memory and synaptic plasticity impairment in rats after a single episode of early-life seizures (Lynch et al., 2000) and, recently, neuronal degeneration has been described in the hippocampus following lithium-pilocarpine status epilepticus in 12 days old rats (Druga et al., 2010).

So far, the majority of studies have been focused on hippocampus, the key brain region for memory, and little attention has been directed to other brain areas and, in particular, to the cerebellum. The cerebellum may be especially vulnerable in infants, since it is characterized by an important postnatal histogenesis leading to the settling of adult circuitry. In addition, a recent study indicates the cerebellum as a region of seizure focus (Carmody and Brennan, 2010) and cerebellar dysplastic lesions can be epileptogenic (Vander et al., 2004). Several studies indicate a role for α-amino-3-hydroxy-5-methyl-D-aspartate glutamate receptors (AMPAR) in long lasting sequelae after early-life seizures (Sanchez et al., 2001; Cornejo et al., 2007; Friedman et al., 2007). Moreover, AMPAR antagonists, but not NMDA receptor antagonists or GABA agonists, administered within the first 48 h after seizures, attenuated seizure-induced neuronal injury in the hippocampus (Koh et al., 2004). AMPARs mediate fast synaptic transmission and in the cerebellum, they are highly expressed at both climbing and parallel fiber synapses on Purkinje cells (Zhao et al., 1998). AMPARs are a tetrameric assembly of GluR1—4 subunits. In the adult cerebellum, the Ca^{2+}-permeable GluR1 and GluR4 subunits are exclusively expressed in glial cells, while the Ca^{2+}-impermeable GluR2 and GluR3 subunits are present on Purkinje cell membranes (Douyard et al., 2007). During the cerebellar development, these subunits undergo profound changes in their distribution and recently it has been speculated that AMPAR may be involved in stabilizing outgrowing branches of Purkinje cell dendrites (Douyard et al., 2007).

The purpose of the present work was to examine whether a single episode of early-life seizures interferes with the developmental program of the cerebellum and GluR2/3 distribution and expression. These questions were explored by treating 10-day-old rats with a single convulsive dose of pentylentetrazole (PTZ). PTZ is a convulsive drug widely used to induce seizures experimentally. Though the mechanism of action of PTZ is not fully understood, it is generally accepted that part of its action is due to its antagonist binding to the picrotoxin-binding site of the postsynaptic $GABA_A$ receptor (Macdonald and Barker, 1977). In the rat, the 10th day of life is a crucial age for the development of the cerebellum. At 10 days, the generation of granule cells reaches a peak (Altman, 1972) and synaptogenesis of parallel fibers starts on Purkinje cell dendrites (Zhao et al., 1998).

Here we report damage to Purkinje cells, accompanied by inhibition of DNA synthesis and up-regulation of GluR2/3 subunits in the cerebellum of early postnatal rats after a single episode of PTZ-induced seizures.

Material and methods

Animals and treatment

Male 10-day-old Wistar rats (4 animals, date of birth 0) were treated with a single dose of 80 μg/g b.w. of pentylentetrazole (PTZ, Sigma, MO, USA) dissolved in saline, applied subcutaneously. Control animals (4 rats from the same litter) received saline only. Immediately after, the animals were injected subcutaneously with ^{3}H-thymidine (^{3}HTdR, 2.5 μCi/g b.w., specific activity 600 GBq/mM, UVVVR, Prague, CZ), followed by the same dose 1 h later.

All animals were killed by decapitation 4 h after the last ^{3}HTdR injection, brains immediately excised, fixed in Carnoy solution and embedded in Paraplast X-tra (Polysciences Inc., Warrington, PA, USA). Ten-μm thick midsagittal sections were used for autoradiography and immunocytochemistry.

Experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic. Animal care and experimental procedures were conducted in accordance with the guidelines of the European Community Council directives 86/609/EEC.

Autoradiography

Sections were covered with Kodak AR10 stripping film and exposed for 57 days at 4 °C in the dark. Autoradiographs were counterstained with Mayer hematoxylin.

Immunocytochemistry

The following antibodies were used for immunocytochemistry:

- 1:30 rabbit polyclonal anti-α-amino-3-hydroxy-5-methyl-D-aspartate (AMPA) glutamate receptor 2/3 antiserum (GluR2/3, Chemicon, Temecula, CA, USA). The antibody recognizes both GluR2 and GluR3 AMPA receptor subunits;
- 1:5000 guinea pig polyclonal anti-glial glutamate transporter 1 antiserum (GLT1, Chemicon, Temecula, CA, USA). The antibody recognizes the carboxy terminus of GLT1;
- 1:5000 rabbit polyclonal anti-calbindin antiserum (CB38, Swant, Bellinzona, CH). In the cerebellum, this antibody stains uniquely Purkinje cells, even in their thinnest processes, giving Golgi-like pictures.

Sections were processed as follows. After treatment with 3% H_2O_2 in 10% methanol in PBS and with 10% normal goat serum in PBS, the sections were incubated overnight with the primary antibodies. Afterwards the sections were incubated for 30 min in goat anti-rabbit or goat anti-guinea pig biotinylated IgG, followed by streptavidin-HRP complex (Vectastain Elite kit, Vector, CA, USA). The complex was revealed by 3′,5′-diaminobenzidine tetrahydrochloride. For the reaction control, the primary antibodies were omitted.

Sections were photographed with Olympus Camedia C-5050 mounted on Olympus BX51 microscope.

Cell counting

To evaluate the extent of cell proliferation in the external germinal layer, 10 sections per animal (4 PTZ treated rats and 4 controls), submitted to autoradiography were scored. The sections were photographed and the number of ^{3}HTdR labeled cells/100 μm of cerebellar cortex convolution was counted in the external germinal layer. Since it is known that the rate of cell proliferation varies between surface of folia and depth of fissures and among lobes (Mares and Lodin, 1970), the cell counting was performed in