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Effect of cytosine arabinoside on cerebellar neurofilaments during development: A sexual dimorphism

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ABSTRACT

Previous reports suggest that the resistance of neuronal cytoskeleton to drug toxicity may vary with age and gender. The aim of the present study was to assess the impact of cytosine arabinoside (AraC) treatment on neurofilament (NF) levels and phosphorylation status in the developing cerebellum of male, female and testosterone propionate (1.25 mg/rat)-androgenized female rats. AraC (200 mg/kg bw) was administered from postnatal day (PND) 14–16 and changes in the level and phosphorylation of NFs were detected at PND 16 by Western blot analysis. The drug had no effect in male pups, while it increased the non-phosphorylated NF subunits of medium and low molecular weight in females. Androgenization of females prevented the AraC-induced increase in NF subunits. The levels of estrogen receptor beta (ER-β), known to mediate neuroprotective actions of estrogens in the brain, were significantly higher in the developing female cerebellum, as compared to males and androgenized females.

These data show that the neurofilament cytoskeleton in the developing rat cerebellum exhibits resistance to AraC that appears sexually dimorphic. In young males the resistance is exemplified by a lack of responsiveness, whereas in juvenile females it is presented by an androgenization-sensitive NF upregulation.

1. Introduction

Cytosine arabinoside (AraC) is a cytostatic drug widely used in the treatment of leukemia. The anticancer property of AraC is based on its ability to inhibit DNA replication and topoisomerase II mediated DNA repair [6,36]. In non-dividing cells, like postmitotic neurons, AraC is implicated in the generation of reactive oxygen species that trigger DNA strand breaks [12]. The cerebellum is vulnerable to AraC toxicity and complications affecting its function are ranging from transient and mild to permanent ones [40]. Neurofilaments (NF), the most abundant proteins of neuronal cytoskeleton, are essential for the maintenance of cell structure and organelles' transport along the axes [29]. These proteins are common targets of neurotoxic agents [21,28]. Accumulation of neurofilament aggregates has been found in perikarya of postmortem cerebella of AraC-treated individuals [38], as well as in a number of neurological disorders including motor neuron disease and dementias, suggesting a filamentous degeneration process in these disorders [20].

NFs are heteropolymers of three main subunits (NF-H 200 kDa, NF-M 170 kDa, NF-L 68 kDa) whose balance and phosphorylation state is particularly important for the

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formation and integrity of the neuronal cytoskeleton. Majority of axonal NFs is highly phosphorylated and constitute a static pool integrated to the axis cytoskeleton, while a minor, less phosphorylated, fraction is involved in axonal transport [7]. Increased phosphorylation of NF-M and NF-H side arm domains is associated with slower transport rates [2]. On the other hand, phosphorylated NFs are less susceptible to proteolytic degradation [7], implying the importance of phosphorylation dynamics in NFs' integrity and function.

We have previously shown that AraC administration in adult rats leads to a decrease of NFs in the molecular layer of the cerebellum that is accompanied by motor deficits [16]. Among the three NF subunits, NF-H exhibited an increased susceptibility to AraC treatment [17].

During the first two weeks of life in rodents, cerebellum is considered more sensitive in AraC toxicity due to the increased permeability of the blood brain barrier, comparing to adult [37]. During this period, the impact of AraC administration on this area has been extensively investigated [27,26,32], and severe deficits in glia, neuronal migration and cerebellar cortex formation have been demonstrated in drug-treated animals. However, there is paucity in the literature concerning AraC toxicity at the beginning of the third postnatal week. At this stage, precursors of granular cells continue their mitosis in the external granular layer (EGL) and migrate through the molecular zone to form the internal granular layer, guided by radial glia [14]. The presence of dividing cells in the EGL along with postmitotic but still maturing Purkinje cells, render the developing cerebellum a good model for the study of AraC effects on heterogeneous neuronal populations [1]. We selected the third postnatal week as a timeframe for the examination of AraC impact on cerebellar development due to the combination of dividing and non dividing neurons that occurs during the selected period. These neuronal populations would have a distinct response to AraC. The maturation of Purkinje cells with the development of dentritic synapses also takes place during the third postnatal week [25]. In contrast, postpartum human cerebellum histology already resembles the adult one, with EGL having already disappeared. Hence, despite the fact that AraC is widely administered during leukemia treatment in young children, there is little direct clinical relevance between the third postnatal week rodent cerebellum and that of a young human.

The aim of the present study was to investigate the effect of AraC on the neurofilament component of cytoskeleton in the rat cerebellum during the third week of life. Since there are studies suggesting that neuroprotection is biased by the gender [4], we studied animals of both sexes. According to the literature, the adult female brain appears more resistant to toxins owing to the neuroprotective action of estrogens [4]. Upon neuronal damage, estrogen treatment has been shown to attenuate proteolytic degradation of NFs [33]. In a rat model of head injury, NF-M in the adult female hippocampus was more resistant to degradation and a significant increase of this subunit was observed during recovery [18]. To elucidate the nature of the observed sex differences in response to AraC, we further included in the study females that were neonatally androgenized during the critical period of brain sexual differentiation. The levels of estrogen receptor beta (ER-β) were also determined as a possible mediator of neuroprotection. ER-β has been implicated in the regulation of cytoskeleton proteins, including NFs [11,31]. In the developing cerebellum, ER-β-mediated actions may help the growth process for which cytoskeletal proteins are indispensable [15].

2. Materials and methods

2.1. Animal groups and experimental design

Sixteen day-old Wistar rats were used in this study. The animals' genitors were purchased from the Hellenic Pasteur Institute (Athens, Greece) and acclimated in our vivarium (lights on from 06:00 to 18:00 h) before breeding, having free access to food and water. The experimental design consisted of two phases.

In Phase I, both male and female rats were used. On postnatal day (PND) 14, the pups within each litter were randomly assigned per sex to either the AraC or the Control group (6–7 per group). Pups in the AraC group were s.c. injected with 200 mg/kg bw of AraC (Aracytin, Pharmacia), a dose capable to penetrate the blood brain barrier and reach the brain [26]. In our former experiment in adult rats [16,17] we used a higher dose 400 mg/kg bw administered intraperitoneally, which appeared to cause significant systematic toxicity during a pilot study we performed with developing rats. As a result we decided to use a lower dose subcutaneously which proved to penetrate the blood brain barrier (as demonstrated by its impact on dividing cells in the EGL). The suggested human low s.c. dose is $2 \times 10\,mg/m^2/d$ for a 14-day scheme but at this dose the drug is unlikely to cross the blood brain barrier [41]. Control pups were injected with normal saline. Injections were applied once per day (at 10 am) from PND 14 to 16 and adjustment of the drug dose to body weight was made when appropriate. Upon injections, the pups were returned to their mothers in the home cage. On PND 16, the animals were decapitated 6 h post injection.

In Phase II, only female pups were used. On PND 3, the animals were s.c. injected with either sesame oil or testosterone propionate (1.25 mg/rat in 50 μl of sesame oil), to induce brain androgenization [19,34,39]. On PND 14, the pups were randomly assigned to either the AraC or the Control group and treated thereafter as in Phase I.

All animal treatments were performed according to the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) on the ethical use of animals and the experimental protocol was approved by the Ethical committee of the School of Medicine, Athens University.

2.2. Histology

Nissl staining was applied to examine the effect of AraC treatment in cerebellum architecture and tissue morphology. Formalin-fixed paraffin-embedded cerebella halves from Phase I experiment were used. Six micrometer thick midline sagittal sections were collected onto silane-coated slides. Upon deparaffination and rehydration the sections