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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

were stained overnight in a solution containing 1% toluidine blue. Intense staining was partly removed using successive alcohol solutions 50% and 70%. The external granular layer (EGL) width was measured in lobules IV–VI using the Image Pro Plus software (Media Cybernetics). Five different optic fields from each tissue block were measured (10 measurements of the width in regular intervals for each optic field) under the 20× objective magnification, by two independent observers, blindly. The difference between the two scorers was always lower than the standard error of the measurements.

2.3. Western blot analysis

Western blot analysis was used to determine the levels of non-phosphorylated and phosphorylated NF subunits, as well as the levels of estrogen receptor beta (ER-β). At sacrifice, cerebella were immediately frozen in liquid nitrogen and stored in −80 °C until use. The tissue was homogenized by sonication in ice cold buffer (100 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.5 mM DTT, 0.1% SDS and 1 mM PMSF) and centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatants were used for Western blot analysis, as previously described [17]. The following primary antibodies were applied overnight at 4 °C: a monoclonal antibody against a common epitope of the three subtypes of non-phosphorylated neurofilaments (1:500, Dako A/S, Denmark), a monoclonal antibody against the phosphorylated subunits pNF-H and pNF-M (1:1000, Millipore Corporate, Billerica, MA, USA), a polyclonal antibody against estrogen receptor beta (B1N1, 1:1000, kindly provided by Dr. M.N. Alexis, [5]) and a monoclonal antibody against b-actin (1:10,000, Chemicon International Inc., Temecula, CA, USA). Appropriate biotinylated secondary antibodies were used and the immunoreactivity signal was visualized by the use of 3,3-diaminobenzidine tetrahydrochloride (Dako A/S, Denmark).

For quantification of the signal, the membranes were scanned and the optical density (OD) of each band was divided by the respective b-actin band OD reading, to correct for protein loading. A fixed square frame was used to capture each protein band and after the background signal was subtracted by selecting a background area of the membrane, the OD of each band was calculated from pixel density using Image Pro Plus software. Each sample was run three times in order to obtain reliable density data. To normalize the measurements among different membranes, the same control sample was included in all membranes.

2.4. Statistics

Two-way analysis of variance (ANOVA), with AraC treatment and sex (Phase I) or AraC treatment and testosterone treatment (Phase II) as the independent factors, followed by the *Bonferroni* post hoc test when appropriate, was used to evaluate significant effects on EGL width, and on the levels of pNF-H, pNF-M and ER-β. Furthermore, the effect of AraC and sex (or testosterone treatment) on the three neurofilament subunits was analyzed by repeated two-way ANOVA with subunit as the within-subject factor. Significance was set at $p < 0.05$.

3. Results

3.1. Effects of AraC in the cerebellum of male and female pups

3.1.1. Histological observations

A significant effect of AraC was evidenced on the width of the external granular layer ($F_{(1,23)} = 45.285$, $p < 0.001$) (Fig. 1e). Sixteen-day old AraC-treated male and female rats had decreased to undetected EGL width, compared to the control groups [($F_{(1,10)} = 19.205$, $p = 0.002$ and ($F_{(1,12)} = 27.569$, $p < 0.0001$) for males and females, respectively].

Abundant spindle-shaped granular cells migrating from the external to the internal granular layer were detected within the molecular layer in the control groups (Fig. 1a and c). These cells were scarcely observed or even absent in some lobules of AraC-treated animals (Fig. 1b and d). AraC treatment did not significantly affect the formation of Purkinje and internal granular cerebellar layers.

3.1.2. Effect on NF subunits and phosphorylation

AraC treatment differentially affected NF subunits in the cerebellum of 16-day old male and female rats (Fig. 2a and b). More specifically, repeated two-way ANOVA for the three subunits revealed a significant effect of subunit ($F_{(2,44)} = 7.953$, $p = 0.001$), as well as a significant subunit × AraC interaction ($F_{(2,44)} = 4.643$, $p = 0.015$). When analyzed per sex, the effect of AraC was statistically significant in females ($F_{(1,10)} = 6.032$, $p = 0.034$), but not in males. An interaction of sex × AraC was also significant ($F_{(1,22)} = 4.727$, $p = 0.041$). Post hoc analysis for each subunit showed an increase of NF-M levels in the female AraC group, compared to control females ($p = 0.015$) and to AraC males ($p = 0.039$). Increased levels of NF-L were also detected in female AraC group compared to both female control and male AraC groups ($p = 0.011$ and $p = 0.006$, respectively). No significant changes were observed for the NF-H subunit. No effect of sex, AraC treatment or their interaction was detected in the levels of phosphorylated subunits (Fig. 2c).

3.1.3. Effect on ER-β levels

The levels of ER-β in the female cerebellum were higher than in male, irrespective of AraC treatment (Fig. 2d and e). More specifically, a significant effect of sex on the levels of ER-β was evidenced in our study ($F_{(1,19)} = 37.628$, $p < 0.001$). Subsequent analysis showed increased ER-β levels in control females comparing to males ($p = 0.002$) and in AraC-treated females comparing to their male counterparts ($p = 0.003$).

3.2. Impact of perinatal androgenization on AraC effect

In Phase II experiment we investigated the possible impact of early androgenization of female pups on AraC-induced alterations in NFs and ER-β expression. The success of testosterone masculinization was verified by

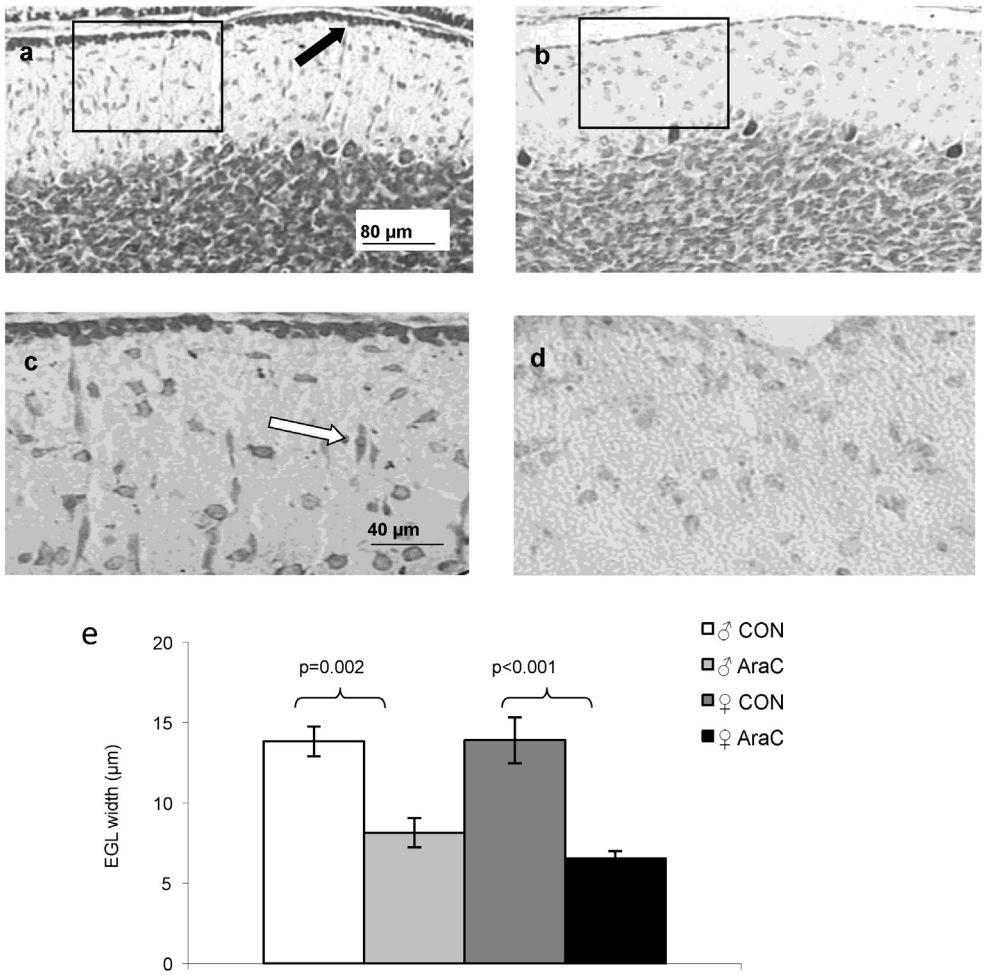

Fig. 1. (a–d) Representative bright field photomicrographs of Nissl stained cerebellum sections from control (a, c) and AraC treated (b, d) male rats. A prominent decrease in EGL width could be witnessed in the AraC treated animal comparing to the control. The black frame in (a) [10× objective magnification] corresponds to the area magnified in (c) [20× objective magnification] and accordingly the black frame in (b) corresponds to the area magnified in (d). Black arrow in (a) indicates EGL location and white arrow in (c) indicates migrating granular cells, respectively. (e) Effect of AraC treatment on EGL width in the developing cerebellum of male and female rats. Bars represent mean ± SEM of EGL width. Significance was set for $p < 0.05$.

measurements of the urogenital distance of pups at sacrifice. The mean distance was 1.01 ± 0.04 cm in males, 0.59 ± 0.02 cm in intact females and 0.77 ± 0.04 cm in testosterone-treated females.

3.2.1. Impact on neurofilament alterations

Early androgenization prevented the AraC-induced increases in NF that were observed in 16-day old female pups (Fig. 3a and b). Statistical analysis showed that AraC treatment had an initial significant effect ($F_{(1,20)} = 6.624$, $p = 0.018$) on each neurofilament subunit. Subsequent per subunit analysis showed an increase of NF-M levels in AraC-treated females, compared to control females ($p = 0.025$). However, AraC-treated androgenized females did not differ from their control counterparts. NF-L levels were also higher in the AraC females, as compared to the controls ($p = 0.010$), whereas those of testosterone-treated group did not differ. No significant changes were observed for the NF-H subunit. Androgenization had no effect on the impact of AraC in the status of NF subunit phosphorylation (Fig. 3c).

3.2.2. Impact on ER-β levels

Perinatal androgenization reduced ER-β levels in testosterone-treated females, irrespective of AraC treatment (Fig. 3d and e). Statistical analysis revealed an overall significant effect of testosterone treatment on the levels of ER-β ($F_{(1,19)} = 11.484$, $p = 0.004$). Subsequent analysis showed reduced ER-β levels in testosterone-treated females, compared to their non-androgenized female counterparts ($p = 0.039$ for controls and $p = 0.048$ for AraC-treated pups).

4. Discussion

The main finding of this study is that the NF component of the cytoskeleton in the cerebellum of 16-day old rats exhibits resistance to AraC in a sexually dimorphic pattern. In contrast to the reported AraC-induced reduction of NFs

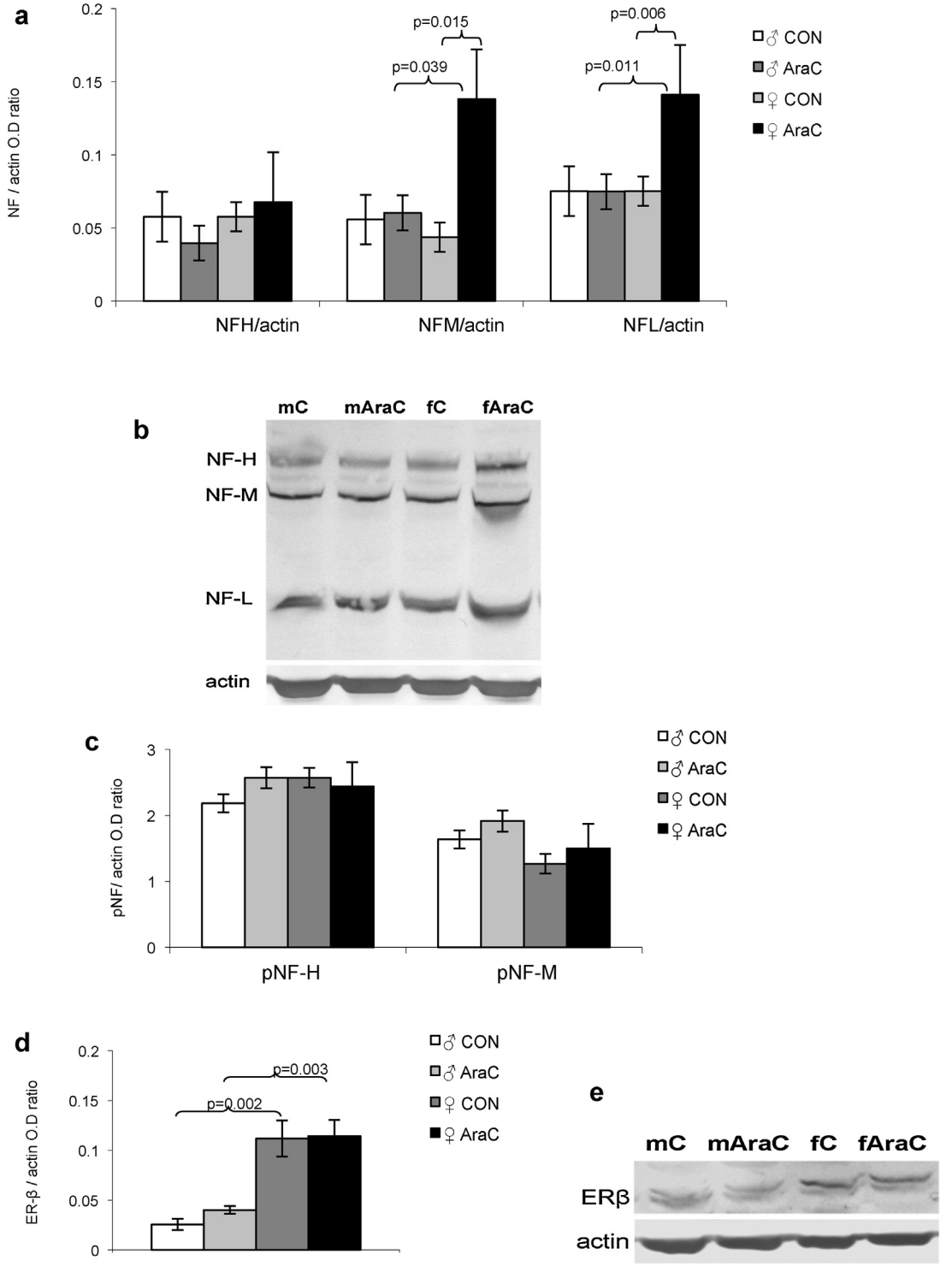

Fig. 2. Effect of AraC on NFs and ER-β of male and female rats. (a) Effect of AraC on the levels of non-phosphorylated neurofilament subunits (NF-H, NF-M, NF-L) in the developing cerebellum of male and female rats. Bars represent mean ± SEM of OD ratio of each NF subunit/actin band in each sample. (b) Representative Western immunoblot for the NF subunits in experimental groups. (c) Effect of AraC on the levels of phosphorylated subunits (pNF-H, pNF-M) in the developing cerebellum of male and female rats. Bars represent mean ± SEM of OD ratio of each pNF subunit/actin band in each sample. (d) Effect of AraC on the levels of estrogen receptor beta (ER-β) in the developing cerebellum of male and female rats. Bars represent mean ± SEM of OD ratio of ER-β/actin band in each sample. (e) Representative Western immunoblot for ER-β in experimental groups. Significance was set for $p < 0.05$.

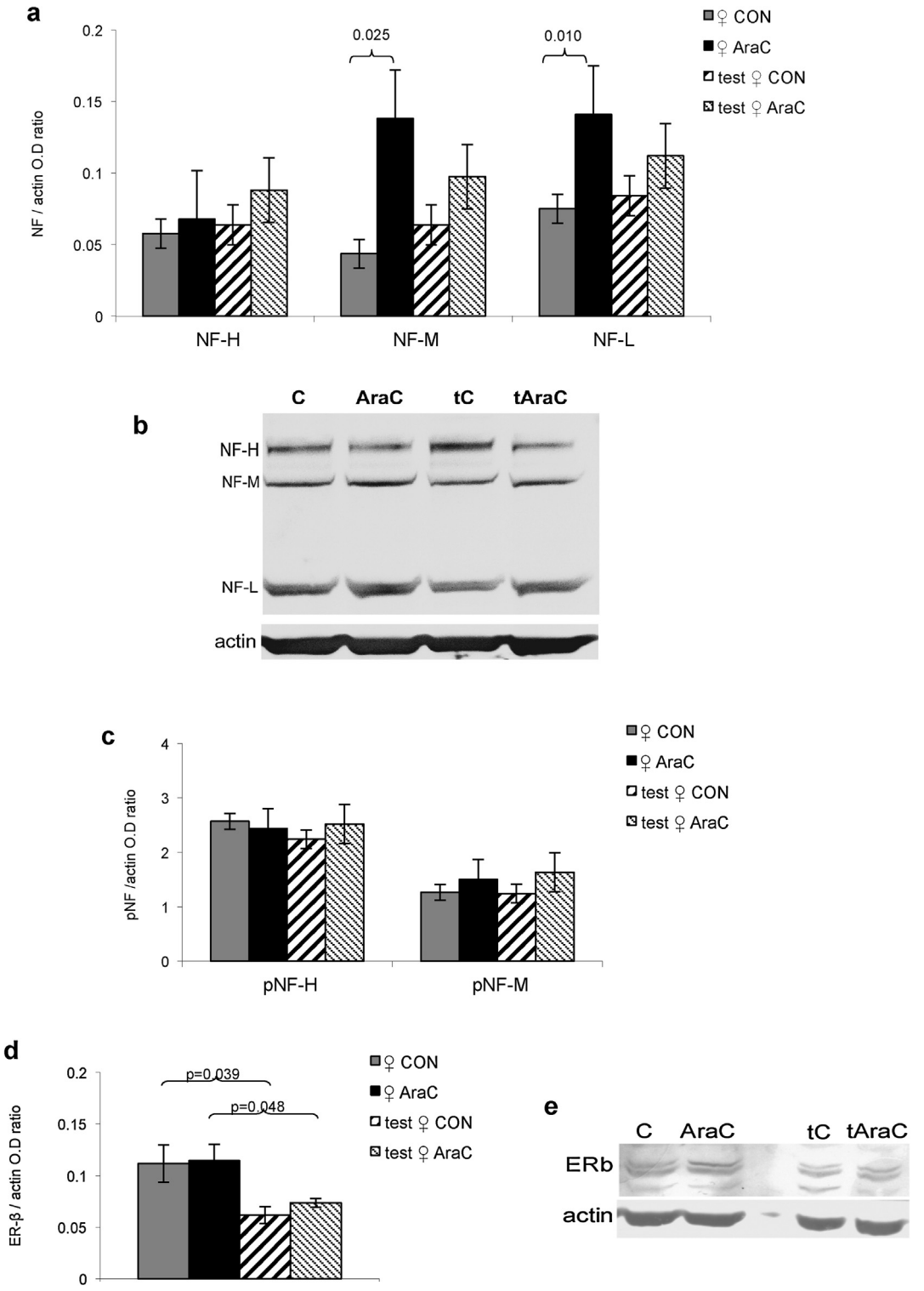

Fig. 3. Effect of AraC on NFs and ER-β of female and androgenized female rats. (a) Effect of AraC on the levels of non-phosphorylated neurofilament subunits (NF-H, NF-M, NF-L) in the developing cerebellum of female and androgenized female rats. Bars represent mean ± SEM of OD ratio of each NF subunit/actin band in each sample. (b) Representative Western immunoblot for the NF subunits in experimental groups. (c) Effect of AraC on the levels of phosphorylated subunits (pNF-H, pNF-M) in the developing cerebellum of female and androgenized female rats. Bars represent mean ± SEM of OD ratio of each pNF subunit/actin band in each sample. (d) Effect of AraC on the levels of estrogen receptor beta (ER-β) in the developing cerebellum of female and androgenized female rats. Bars represent mean ± SEM of OD ratio of ER-β/actin band in each sample. (e) Representative western immunoblot for ER-β in experimental groups. Significance was set for $p < 0.05$.

in the cerebellum of adult rats [17,16], toxin administration in the beginning of the third week of life had either no effect on NF levels (in males) and phosphorylation status (in both sexes) or even increased two of the NF subunits (in females). In the adult cerebellum, reduced NF levels and in particular NF-H form following AraC is considered as a hallmark of degeneration and is accompanied by motor deficits [16]. At this point we should highlight the fact that a direct comparison between adult and neonatal rats is problematic because the dose and exposure time to AraC differed. Moreover, data concerning the impact of AraC on female adult rats are missing.

The lack of changes in the levels of NF proteins in the immature male rat cerebellum is indicative of an increased resistance to AraC toxicity. Interestingly, in female pups AraC treatment increased the levels of NF-M and NF-L subunits. The mechanism and the importance of this upregulation are not clear but it may represent a reactive compensatory mechanism to lesion, favoring local tissue reorganization in the female cerebellum. It is worth noting that NF-M is considered to be a main regulator of radial axonal growth [9] and is the preferred subunit for NF-L co-polymerization. Additionally, a common regulation of these two forms (NF-M and NF-L) is anticipated, as their genes reside in the same chromosome [24].

To assess whether the observed sexually dimorphic responses to AraC could be attributed to the female background, we exposed female pups to androgens during the critical period of brain sexual differentiation, a manipulation known to masculinize developing neural circuits [23]. Our data verified that the observed increases in NF-M and NF-L are dependent on an intact female brain programing.

We hypothesized that the different levels of ER-β receptor between the two sexes may interfere with the sexually dimorphic outcome of AraC on neurofilaments in the developing cerebellum. We did detect that ER-β levels in female pups were five-fold higher compared to their male counterparts and that they were significantly reduced in androgenized females. These findings suggest that ER-β could be involved in the sexually dimorphic alterations of NF levels induced by AraC. However, apart from ER-β, additional mediators are probably participating in this female-specific increase of NF subunits following AraC and further studies are required to clarify the underlying mechanism.

The observation of this study that cerebellar NFs are less susceptible to neurotoxins during development is supported by previous findings in the rat hippocampus. Lopez-Picon et al. [22] showed that NF subunits in the hippocampus of adult, but not immature rats are vulnerable to degradation following kainic acid administration. In the same study, all three non-phosphorylated NF subunits underwent a substantial increase in the hippocampus of 9-day old kainic acid-treated pups (not separated by sex) that was normalized one day later. This observation is quite similar with the finding of the present study and implies the existence of common cellular responses of cytoskeleton components to neurotoxic insults during development.

Phosphorylated NFs are less susceptible to proteolytic degradation [13] and conversely dephosphorylation might represent an early step of the loss of NF integrity. Accordingly, the observed absence of dephoshorylation following AraC in our study implies that the neuronal cytoskeleton at this age may be more resistant to AraC-induced degradation. In support of this, we could detect a significant downregulation of phosphorylated NF-H in AraC-treated adult rats (Koros and Kitraki, unpublished data), that coincides with the enhanced vulnerability of this subunit to degradation in adulthood [17]. Notably, this developmental resistance of NFs to changes in the phosphorylation status was observed in both sexes, suggesting that AraC in immature rats does not affect neurofilaments' stability, but rather the cytoskeleton reactive response to insult that is sexually dimorphic. In agreement with our observations, Lopez-Picon et al. [22], did not detect alterations in the phosphorylation of NF-H in the developing hippocampus following kainic acid administration.

At this point it should be noted that despite the lack of alterations in NF phosphorylation status following AraC, the rat cerebellum remains vulnerable to AraC toxicity during the third postnatal week. Our data showed that AraC administration in the beginning of the third week significantly reduced the width of external granule layer that comprises of dividing precursors of granular cells. This is in accordance with previous studies using other toxic agents, like phenytoin and cisplatin during the same developmental period [10,30]. It has been suggested that this EGL reduction could impair normal migration of precursor cells to form the internal granular layer [3]. Disorganization of the guiding radial glia could also add to this normal growth aberration, as it has been the case for other neurotoxins as well [8,35]. Despite the fact that EGL width reduction is not directly relevant to NF expression alterations, we used it as a marker of AraC toxicity in the developing cerebellum in order to prove accumulation and toxic action of the drug in the cerebellum.

5. Conclusions

Our data support the option that the developmental stage and the gender can influence the resistance of neuronal cytoskeleton to toxic agents. Neurofilaments appear more resistant to AraC treatment in young male rats comparing to the effects reported in male adults although the dose and exposure time differed. In young females, an androgenization-sensitive reactive upregulation of NF-M and NF-L subunits was observed in the cerebellum following AraC that could implicate ER-β participation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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