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Trophic and proliferative perturbations of in vivo/in vitro cephalic neural crest cells after ethanol exposure are prevented by Neurotrophin 3

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ABSTRACT

Neural crest cells (NCCs), a transient population that migrates from the developing neural tube, distributes through the embryo and differentiates into many derivatives, are clearly involved in the damage induced by prenatal exposure to ethanol. The aim of this work was to evaluate alterations of trophic parameters of in vivo (in ovo) and in vitro NCCs exposed to teratogenic ethanol doses, and their possible prevention by trophic factor treatment.

Chick embryos of 24–30 h of incubation were treated during 10 h with 100 mM ethanol, or 40 ng/ml Neurotrophin 3 (NT3), or 10 ng/ml Ciliary Neurotrophic Factor (CNTF), or ethanol plus NT3 or CNTF, or defined medium; then the topographic distribution of NCC apoptosis was assessed using a whole-mount acridine orange supravital method. Cultures of cephalic NCCs were exposed to the same ethanol or NT3, or CNTF treatments, or ethanol plus one of both trophic factors, or N2 medium. A viability assay was performed using the calcein-ethidium test, apoptosis was evaluated with the TUNEL test, and proliferative capacity after BrdU labeling.

After direct exposure of embryos to 100 mM ethanol for 10 h, a high level of NCC apoptosis was coincident with the abnormal closure of the neural tube. These anomalies were prevented in embryos exposed to ethanol plus NT3 but not with CNTF. In NCC cultures, high cell mortality and a diminution of proliferative activity were observed after 3 h of ethanol treatment. Incubation with ethanol plus NT3 (but not with CNTF) prevented NCC mortality as well as a fall in NCC proliferation.

The consequences of direct exposure to ethanol expand data from our and other laboratories, supporting current opinion on the potential risk of alcohol ingestion (even at low doses and/or during a short time), in any period of pregnancy or lactation. Our in vivo/in vitro model encourages us to examine the pathogenic mechanism(s) of the ethanol-exposed embryo as well as the use of trophic factors for the treatment and/or prevention of anomalies induced by prenatal alcohol.

1. Introduction

Neural crest cells (NCCs) form an embryonic cell population which arises from the dorsal neural tube, migrates long distances through particular pathways in the embryo and gives rise to several derivatives, such as neurons and glia of the peripheral nervous system, pigment cells of the skin, craniofacial components and some endocrine cell types (Le Douarin and Kalcheim, 1999). Moreover, NCCs have major clinical relevance since they are involved in both inherited and acquired developmental human abnormalities (Antony and Hansen, 2000; Dunty et al., 2001; Dunty et al., 2002; Wentzel and Eriksson, 2009), globally known as the Neurocristopathy family (Bolande, 1997). It is also well known that NCCs exhibit a high proliferative rate (Paglini and Rovasio, 1994a,b, 1999) and intrinsic (physiological) apoptotic behavior (Cartwright and Smith, 1995b; Cartwright et al., 1998; Graham et al., 1993; Hirata and Hall, 2000), which makes this cell population potentially labile (Rovasio and Battiato, 2002).

Among the exogenous agents harmful to NCC development, ethanol occupies a key role in teratological studies since prenatal ethanol exposure results in a pattern of anomalies known as Fetal Alcohol Syndrome (FAS) (Jones and Smith, 1973). This embryopathology involves varying degrees of growth retardation, brain and craniofacial malformation, as well as mental health dysfunction, and it is known that many anatomical/functional anomalies of FAS clearly involve cranial NCC-derived tissues (Giles et al., 2008; NIAAA, 2000; Sulik, 2005).

Animal models have played a significant role in determining the phenotypic characteristics and biological consequences of prenatal alcohol exposure. FAS has been reproduced in mammals (Sulik, 2005), frogs (Nakatsuji, 1983) and chickens (Cartwright and Smith, 1995a;

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Rovasio and Battiato, 1995, 1996, 2002). However, there is limited and indirect data or speculative conclusions about the factors involved in the prenatal mechanism of action of ethanol at cellular and molecular levels (Dunty et al., 2001, 2002; Goodlett and Horn, 2001; Kumada et al., 2006, 2007), even though a well-documented effect of ethanol is the inhibition of cell proliferation in the brain (Bonthius and West, 1991; Miller, 1995), cerebellar neurons (Goodlett and Horn, 2001), and neural tube of chick embryos (Giles et al., 2008).

Previous studies from our laboratory have demonstrated that, after direct treatment with ethanol, embryos showed malformations of the neural tube, reduced numbers and impaired distribution of NCCs, as well as significant and permanent structural and dynamic in vitro changes (Rovasio and Battiato, 1995, 1996, 2002). Ethanol exposure is also associated with the increased apoptosis of NCCs (Cartwright and Smith, 1995a,b) and neural tube cells (Giles et al., 2008) in a dose- and time-dependent manner. Experimental data has shown that some cell types can be rescued from apoptosis and other cell perturbations when ethanol is administered together with trophic factors (Heaton et al., 2003, 2004; Kilburn et al., 2006; Luo et al., 1997; McAlhany et al., 2000; McGough et al., 2008). It is also known that Neurotrophin 3 (NT3) null mice are deficient in skin innervation and NCC-derived Merkel cells, displaying significant cell loss associated with immunocytochemical and ultrastructural apoptotic changes (Halata et al., 2005). On the other hand, it has been shown that cutaneous overexpression of NT3 selectively rescues most of the skin sensory innervation in NT3 knockout mice (Krimm et al., 2000). Ciliary neurotrophic factor (CNTF) is also a well-known trophic molecule that promotes survival of neural (Kassen et al., 2009) and non-neural cells (Rezende et al., 2007). Taken together, these data emphasize the importance of a strategy aimed at preventing/rescuing ethanol-induced NCC perturbations by trophic factor treatment.

The purpose of this study was to determine the action of ethanol on the apoptosis pattern in early chick embryo development stages as well as on in vitro NCC viability and proliferative parameters, and the possible role of trophic factor supply. We found that the ethanol-induced increase in apoptosis, as well as the low proliferative capacity of NCCs, may be reverted by simultaneous treatment with NT3.

2. Materials and methods

2.1. Whole embryo culture, determination of apoptosis and morphology

The topographic distribution of in vivo NCC early apoptosis was assessed by acridine orange supravital intake (Cartwright and Smith, 1995b; Graham et al., 1993), on fertile Cobb line chick eggs pre-incubated for 24–30 h (stages 7–8 HH) (Hamburger and Hamilton, 1951) at 38 °C and 80% humidity, and cultured in a modified Auerbach's shell-less culture system (Battiato et al., 1996; Rovasio and Battiato, 1995). Briefly, the whole egg was transferred to a bowl, and a flat ring of 4% agarose[1] in phosphate buffer solution (PBS) (20-mm outer diameter, 10-mm inner diameter; 2 mm thick) was placed over the blastoderm, forming a small container in which 200 µl of treatment solution was poured. Groups of embryos were treated with: (1) 200 µl of N2 basal medium (Bottenstein and Sato, 1979) (50% DMEM and 50% F12 media, plus 15 mM sodium bicarbonate, 15 mM Hepes buffer, 50 IU/ml G sodium penicillin and 50 µg/ml streptomycin sulfate). (2) 200 µl of N2 medium with a final concentration of 100 mM ethanol. (3) 200 µl of N2 medium with a final concentration of 40 ng/ml of NT3, or 10 ng/ml of CNTF. (4) 200 µl of N2 medium with both 100 mM ethanol and 40 ng/ml of NT3, or 10 ng/ml of CNTF (protection experiments). In all groups, re-incubation was performed to complete 40–50 h (stage 11–12 HH) of embryo development. As a control, after the initial egg pre-incubation time, one group was uninterrupted following the in ovo incubation without treatment; no differences were observed between in ovo and shell-less cultured control groups. For an in ovo alternative experimental group, other eggs were injected with 100 µl of PBS or ethanol in N2 medium into the yolk, estimating an equivalent final concentration of ethanol in relation to total egg volume (Rovasio and Battiato, 1995); no significant differences were observed in the parameters studied between shell-less and in ovo ethanol treatments.

At the end of the experimental time, a sample of 100 µl of fluid was taken from the immediate surrounding milieu of the embryo and the concentration of ethanol was verified by a head-space gas chromatography as explained (Pueta et al., 2011). Then, each embryo was excised, washed with warm PBS, and incubated with 1 ml of N2 medium containing 0.5 µg of acridine orange, at 37 °C during 30 min. After washing twice with PBS, each embryo was whole-mounted between slide and coverslip using anti-bleaching medium (Molecular Probes, Eugene, OR), and was observed and recorded using an Olympus BX-50 microscope (Olympus Corp., Shinjuku-ku, Tokyo, Japan) with fluorescence filter for rhodamine (excitatory filter = 510–550 nm and barrier filter = 590 nm).

Normal/abnormal morphology of whole embryo was double-blind evaluated under a high power stereoscopic microscope, using as a normal pattern the external features described and illustrated in the Hamburger and Hamilton series (Hamburger and Hamilton, 1951).

A group of embryos was submitted to immunolabeling of NCCs with NC-1 antibody (Vincent et al., 1983). Briefly, after fixation for 3 h with 4% paraformaldehyde in PBS and prior piercing of the ectoderm with a microneedle, whole mounted chick embryos were rinsed with PBS and incubated in a wet chamber at room temperature with blocking solution (1% bovine serum albumin and 1.5% Glycine in PBS) 3 times, 1 h each. They were then incubated with NC-1 monoclonal antibody-containing ascitic fluid diluted 1/100 with PBS for 12 h, washed in blocking solution 3 times, 1 h each, and incubated with secondary anti-mouse IgGAM antibody conjugate with fluorescein isothiocyanate (FITC) for 12 h at 4 °C. After PBS washing, preparations were mounted with coverslip using anti-bleaching medium and observed with a fluorescence filter for FITC (excitatory filter = 450–480 nm and barrier filter = 515 nm). Recordings were performed using T-MAX or Ektachrome 400 ASA (Kodak, Rochester, NY) films, then digitized with a SnapScan e50 scanner (Agfa Gevaert, NV, Mortsel, Belgium). Alternatively, images were captured using a Hamamatsu C2400 (Hamamatsu Photonics, Hamamatsu City, Japan) video-camera and submitted to image analyses with the SigmaScan-Pro (SPSS, Chicago, IL) software, according to previous descriptions (Rovasio and Battiato, 2002). To insure the homogeneity of results, the embryos of different experimental groups were simultaneously immunolabeled and mounted between glass and coverglass applying the same protocol technique. Briefly, the last step of the whole embryo mounting was performed under a stereomicroscope, placing strips of coverglass of equivalent thickness to that of the embryo as lateral barriers, in order to assure a homogeneous height and to avoid geometrical distortions of the specimen. Then, whole embryos were imaged, maintaining the uniformity of optical parameters, without further computational handling. Some embryos were also submitted to conventional study techniques with transmission electron microscopy.

2.2. Cultures of neural crest cells and treatments

To characterize the trophic and survival parameters of in vitro ethanol-exposed cells during the early migratory stage, primary cultures of cranial NCCs were performed according to descriptions elsewhere (Rovasio and Battiato, 2002; Rovasio et al., 1983). Briefly, fertile chick eggs were incubated as described above to obtain 10 to 13 somite-pair embryos (stages 10–11 HH). Blastoderms and the surrounding membranes were then excised and washed with PBS,

[1] All chemicals were from Sigma Chem. Co. (St Louis, MO), except when another source is stated.

throwing away the vitelline membrane. After cutting and opening the ectoderm, mesencephalic NCCs were carefully obtained by microdissection from the mass of NCCs bilateral to the neural tube, transferred to 25-mm round coverslips pre-coated with fibronectin (Rovasio et al., 1983) and incubated in petri dishes with 200 µl of N2 medium plus 10% fetal bovine serum during 20–24 h at 37 °C, 5% CO_2 in air and humidified atmosphere. To assess the purity of the cell population, an immunodetection of NC-1 positive cells was performed (Rovasio et al., 1983; Vincent et al., 1983). Applying the careful microdissection technique described above, the degree of purity of NCC cultures was constantly near to 100%, without neural tube, ectoderm and/or mesoderm contaminants. If some culture contained tissue contaminants, they were detected by phase contrast microscopy and NC-1 immunolabeling, and consequently discarded. Before any treatment, the culture-containing coverslip was transferred to a perfusion chamber (Sykes and Moore, 1957) and incubated for 3 h with 2 ml of one of the following media: (1) N2 defined medium (N2 basal medium plus 5 µg/ml insulin, 100 µg/ml transferrin, 20 nM progesterone, 100 µM putrescine and 30 nM selenium in 100 ml of medium) (Bottenstein and Sato, 1979) (2) Ethanol 100 mM in N2 defined medium; (3) Trophic factors NT3 [40 ng/ml] or CNTF [10 ng/ml] in N2 defined medium (final concentrations); (4) Ethanol 100 mM plus trophic factors NT3 [40 ng/ml], or CNTF [10 ng/ml], in N2 defined medium. At the end of the experimental time, a sample of 100 µl of the incubation media was taken and the ethanol concentration was evaluated by gas chromatography as explained (Pueta et al., 2011).

2.3. Viability test

Cell survival from control and ethanol conditions was analyzed by the Live/Death Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). Cultures of cranial NCCs were washed with PBS and incubated with 100–150 µl of 4 µM ethydium H-1 plus 2 µM calcein AM in PBS during 15 min at room temperature, then mounted with 10 µl of the same fresh work solution, and observed with a fluorescence filter for FITC. As a positive control, NCC cultures were incubated with sodium azide. Cell images were obtained as explained above and the viability index was calculated in a double blind manner as the proportion of live cells (calcein-positive cells = green) and dead cells (ethydium-positive cells = red) in the total number of cells, over all microscopical fields corresponding to the NCC halo of growing explants. Whole mount embryos and NCC cultures were also evaluated for apoptotic cells using a commercial TUNEL kit (Tdt-FragEL DNA Fragmentation Detection Kit, Calbiochem, San Diego, CA) as described by the manufacturer.

2.4. Proliferation assay

NCC proliferative capacity was assessed after incorporation of 5-bromo-2′-deoxyuridine (BrdU). Briefly, each culture was incubated with a final concentration of 10 µM BrdU for 3 h at 37 °C and 5% CO_2 besides the corresponding treatment medium (see above). After washing with PBS at room temperature, the cultures were fixed with 4% paraformaldehyde in PBS, for 10 min, washed in PBS 3 times, 1 min each, and permeabilized with 0.2% Tritón X-100 in PBS for 10 min. The cultures were rinsed in PBS (3 × 1 min) and the DNA was denatured with 2 N HCl for 2 h at room temperature. After washing with 0.1 M borate buffer, pH 8.5 (2 × 5 min), cultures were washed in blocking solution (3 × 5 min), then incubated with 1:100 dilution of anti-BrdU primary antibody in a wet chamber for 20 h at room temperature, washed again with blocking solution (3 × 10 min) and post-incubated with FITC-conjugate secondary antibody for 1 h at 37 °C. After washing with blocking solution (3 × 1 min), samples were mounted with anti-bleaching solution. As negative control, the same procedure was performed omitting the primary antibody. Cell images were obtained as explained, and cell counts were made on a microscope with phase optic/FITC filter. The proliferation index was calculated in a double blind manner as the proportion between the number of BrdU-positive cells and the total cell number in random fields comprising almost all the surface of NCC outgrowth.

2.5. Statistical analysis

Each experimental condition was replicated 3 or more times; the number of embryos, explants and cells were evaluated as indicated in the Results Section. The proportion of abnormal embryos in different experimental groups was compared using the z-test with Yates' correction. The mean values of each experimental condition were compared with the Student "t" test. Both viability and proliferation indexes were submitted to arcsine-square root transformation and then evaluated accordingly by the one-way ANOVA test comparing the groups with the Tukey assay, or by the Kruskal–Wallis test comparing the groups with the Dunn assay, using the SigmaStat (SPSS, Chicago, IL) software.

3. Results

Changes in trophic parameters induced by a teratogenic exposure to ethanol in an early embryo model (in vivo/in ovo and in vitro NCCs), and possible protection by exogenous growth factors, were evaluated. Topographic distribution of in vivo apoptosis, as well as in vitro cell viability and proliferative response allowed us to observe direct effects of ethanol on this embryo system.

3.1. NCCs apoptosis in in vivo early migratory stage

To determine the distribution of programmed cell death during the early stages of NCC migration along the neural tube of whole embryos, apoptosis expression was studied by using the supravital intake of acridine orange, which proved adequate for visualizing patterns of apoptosis in whole mounted embryos of insects (Abrams et al., 1993), fishes (Furutani-Seiki et al., 1996), mice (Chen and Behringer, 1995) and birds (Cartwright and Smith, 1995a; Graham et al., 1993). In normal embryos (in ovo controls or shell-less culture, without treatment), the apoptosis pattern in 26–29 h chick embryos (stage 8 HH) was diffusely expressed as few and scattered spots along the neural folds, at the cephalic end and the notochord (data not shown). In later stages (40–45 h of development, stage 11 HH), a more definite apoptosis pattern was seen in NCCs emerging along the neural tube (Fig. 1B, arrows), as well as at the 3 rd and 5th rhombomere levels (Fig. 1B, arrowheads), and in presumptive NCCs at the neural folds prior to fusion (Fig. 1B, P). Parallel specimens assayed with TUNEL exhibited analogous appearance to the acridine orange group (data not shown). Also, cross-sections at different neuraxis levels treated with the TUNEL method confirmed the appearance already described of apoptotic images in NCCs at the neural tube closure (Fig. 2, A) (Graham et al., 1993). Moreover, electron microscope images allowed us to verify with strict morphological criteria the typical endocytic engulfing of apoptotic NCCs by another cell of the same cell population (Fig. 2, B) (Rovasio and Battiato, 2002; Sanders and Wride, 1995; Wyllie et al., 1980). Embryos of the same stage, whole immunolabeled with NC-1 antibody, showed a topological association between early migratory NCCs and the apoptotic phenomena during neural tube closing (Fig. 1A), indicating that most of the apoptotic cells corresponded to NCCs. Thus, NCCs were observed emerging from the closing neural tube (Fig. 1A, arrows), as well as in the early migratory stage toward the ocular field (Fig. 1A, O), the future branchial arch pathways (Fig. 1A, Ba) and the somite regions (Fig. 1A, S), and presumptive NCCs were seen in the neural folds at the caudal end of the embryo (Fig. 1A, P).

When the embryos were exposed for 10 h to 100 mM ethanol, the proportion of abnormal embryos was significantly higher than in the

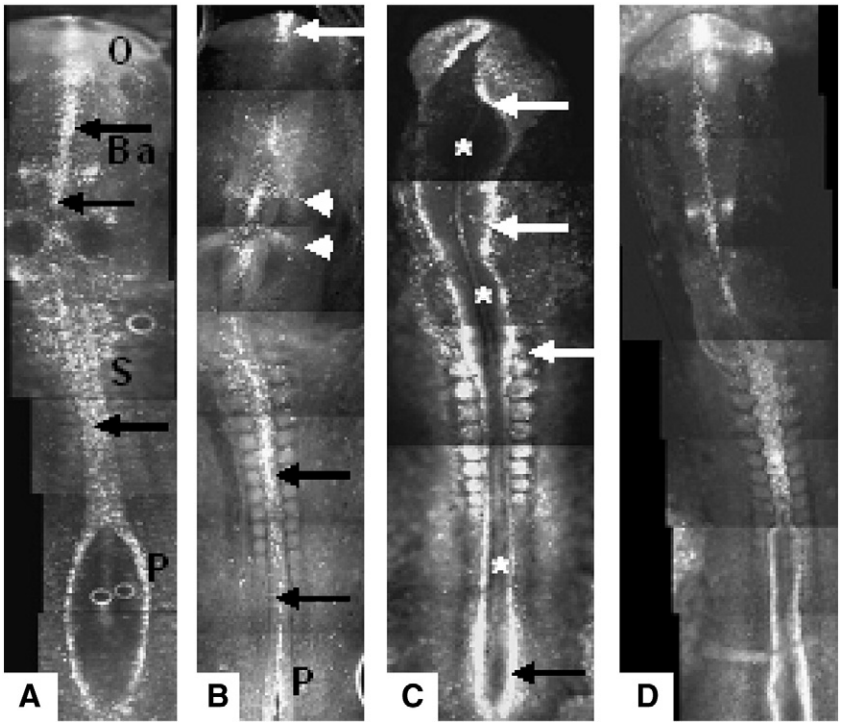

Fig. 1. Whole-mounted chick embryos. (A) Control embryo (stage 11 HH) after immunolabeling of NCCs which emerge from different levels of the closing neural tube (arrows) and migrate toward the ocular field (O), branchial arches (Ba) and somitic region (S). Presumptive NCCs are also seen at the neural folds (P). (B) Control embryo at stage 11 HH. After acridine orange staining, the normal pattern of apoptosis is seen in NCCs emerging from the closing neural tube (arrows), at 3 rd and 5th rhombomere levels (arrowheads) and as presumptive NCCs at the neural folds (P). (C) Shell-less cultured embryo after 10 h of 100 mM ethanol treatment. A higher level of apoptosis (arrows) and cranio-rachischisis (*) is observed. Acridine orange staining. (D) Shell-less cultured embryo after 10 h exposure to 100 mM ethanol plus 40 ng/ml of NT3. Morphology and NCC apoptosis patterns are equivalent to the control embryos (compare with Fig. 1A, B). Acridine orange staining. In the last two conditions, the embryos were incubated for the same time (40–45 h), allowing the controls to arrive at stage 11 HH.

control (Fig. 3) and the patterns of apoptosis were significantly altered, in both density and distribution (Fig. 1C), with simultaneous neural tube anomalies. Among such malformations, a variety of failures of neural tube closing, mainly craniorachischisis, predominate (Fig. 1C, *). After exposure to ethanol plus NT3, embryo morphology and the expression pattern of apoptosis were similar to those of the control embryos (Figs. 1D and 3). While the morphology of embryos treated with NT3 or CNTF alone showed no significant differences from the control group, the proportion of abnormal embryos in the group treated with ethanol plus CNTF was significantly higher than in controls (Fig. 3).

3.2. Viability of in vitro cranial NCCs exposed to ethanol

The purity of the NCC population obtained from mesencephalic segments was verified with the immunomarker NC-1 antibody (Vincent et al., 1983) as better than 95% (Fig. 4A). After calcein–ethydium test of the control groups, most of the NCCs were alive, showing intracellular green-fluorescent esterase activity (Fig. 4B). In contrast, in the positive control of the method, cultures treated with sodium azide to block the respiratory chain, all NCCs were dead (Fig. 4C).

The exposure of NCC cultures for 3 h to 100 mM of ethanol showed a low number of cells and a high proportion of dead cells (Fig. 4D), with occasional apoptosis-like images such as DNA fragmentation and dead cells endocytosed by live cells of the same population (Fig. 4E; see also Fig. 2B). Quantified data confirmed that the number of live cells and the total number of NCCs was lower after 3 h of treatment with ethanol than in the control group, whereas the number of dead cells was significantly higher than those of the corresponding control (Fig. 5, right axis; $p<0.0003$). Likewise, viability of NCCs expressed as the index of live cells vs. total cells was significantly lower in ethanol-exposed cultures than in controls (Fig. 5, left axis; $p<0.001$). On the other hand, parameters of live, dead and viability index of NCC cultures treated with NT3, or ethanol plus NT3, or CNTF alone were not significantly different from those of controls, whereas the trophism of the ethanol plus CNTF-treated cultures was similar to the ethanol-treated group. NCC cultures from the different experimental conditions treated with the TUNEL method exhibited analogous results to those just described for the calcein–ethydium test, apportioning an equivalent proportion of apoptotic/necrotic cells to the above-mentioned values for dead cells (data not shown).

3.3. Proliferation of in vitro cranial NCCs exposed to ethanol and trophic factors

Since the decrease of the cell numbers of ethanol-treated NCCs, besides their lower viability, may also be due to diminution of their reproductive capacity, the intake and the immunolabeling of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) were used to assess the proliferative activity of NCC primary cultures. In comparison to control conditions (Fig. 6A), the proportion of BrdU-labeled nuclei was clearly lower in the ethanol-exposed cultures (Fig. 6B). In this group, frequent nuclei with apoptotic-like images were also seen (Fig. 6B, inset).

The amount of proliferative behavior as a trophic capacity of the NCCs shown in different experimental conditions, enabled us to see that the value of the proliferation index of BrdU-positive cells was low in ethanol-exposed NCCs compared to controls (Fig. 7, left axis, gray columns; $p<0.0001$). Treatment with NT3 alone induced a significant increase in the proliferative capacity of NCCs (Fig. 7, left axis; $p<0.015$), and cultures submitted to protection conditions, that is, cells incubated in the presence of both ethanol and NT3 showed a significant increase of cell division in comparison to those in ethanol alone treatment (Fig. 7, left axis; $p<0.004$), while the proportion of

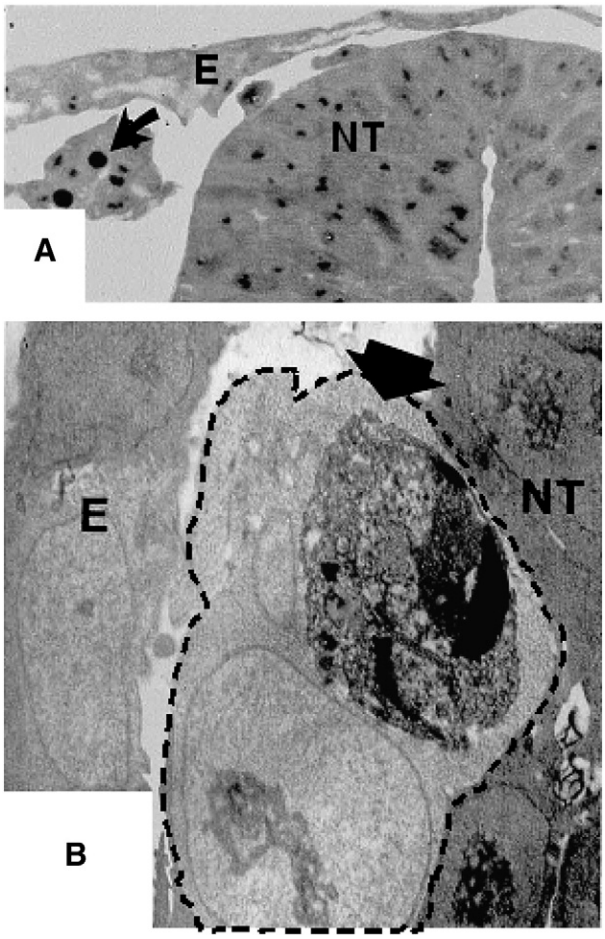

Fig. 2. Light and electron microscope images of NCC apoptosis. (A) Transverse section at the trunk level of ethanol-treated chick embryo after the in situ end-labeling (TUNEL) method, showing apoptosis figures of NCCs (arrow). 60×. (B) Electron microscope image of trunk transversal section of ethanol-treated embryo showing typical endocytoses of early migratory apoptotic NCC (arrow) by another cell of the same cell population (broken line). 6000×. E, ectoderm. NT, neural tube.

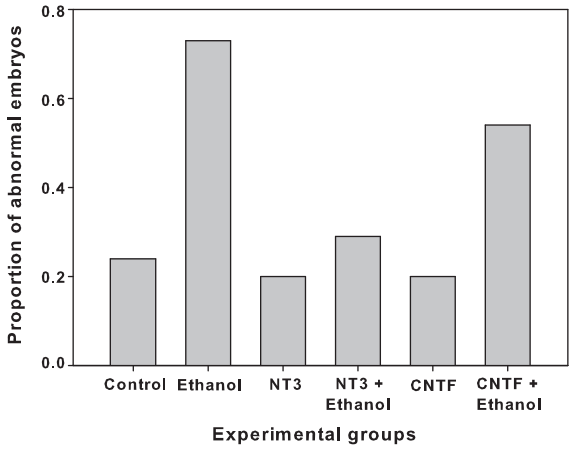

Fig. 3. Proportion of abnormal chick embryos after different treatments in shell-less cultures. Results from in ovo incubated embryos or eggs injected into the yolk with PBS were not different compared with the control shell-less cultured embryos. No significant difference was observed either between shell-less and in ovo ethanol treatments. Ethanol vs. Control: $p<0.001$; CNTF + Ethanol vs. Control: $p<0.001$; CNTF vs. CNTF + Ethanol: $p<0.001$; NT3 or NT3 + Ethanol or CNTF vs. Control: NS. Z-test with Yates correction. Total number of embryos in Control: 85; Ethanol: 89; NT3: 60; Etanol + NT3: 55; CNTF: 49; Ethanol + CNTF: 62. NS: no significant difference.

proliferating cells in the ethanol plus NT3 group showed no significant differences with the control or the NT3 alone groups (Fig. 7, left axis). With CNTF treatment, incubation with this growth factor was followed by low cell proliferation in relation to the control group, although it was higher than in the ethanol group (Fig. 7, left axis; $p<0.005$), and the mixture of ethanol plus CNTF showed a diminution of proliferative capacity compared with the CNTF alone treatment (Fig. 7, left axis; $p<0.05$).

A complementary estimation of trophic characteristics of a cell population takes into account the total number of cells per explant. Assuming that, operatively and based on embryo age, the initial size of the explant could be considered constant, we calculated that the cell number per explant was similar at the start of each experimental run. Moreover, at the end of the different treatments, the absolute number of cultured cells was globally parallel to the proportion of proliferative response, with the exception of the CNTF and the CNTF plus ethanol treatments, the values of which were maintained similar to the control condition (Fig. 7, right axis, black columns).

4. Discussion

The present data were obtained by working on the second of two temporal windows in which cranial NCCs seem particularly vulnerable to ethanol exposure, the start of the migratory stage from the closing neural tube (Cartwright and Smith, 1995b), using the embryo culture in an in vivo experiment (Rovasio and Battiato, 1995), and cephalic NCC cultures for in vitro experiments (Rovasio and Battiato, 2002).

4.1. NCC apoptosis in normal embryos or embryos exposed to ethanol and trophic factors

The high plasticity of NCCs allows them to spread throughout the embryo body and produce many different derivatives because of their high competence in responding to micro-environmental factors (Le Douarin and Kalcheim, 1999). A trophic modulation is expressed as the high proliferative capacity of NCCs (Paglini and Rovasio, 1994a; Paglini and Rovasio, 1994b; Paglini and Rovasio, 1999; Salvarezza and Rovasio, 1997), as well as the physiologic apoptosis of this cell population during early embryo development (Cartwright and Smith, 1995a; Cartwright and Smith, 1995b; Cartwright et al., 1998; Graham et al., 1993; Hirata and Hall, 2000). In parallel with, or as a consequence of, these and other general characteristics, the complex morphogenesis of NCCs makes them very vulnerable to potential toxic exposure throughout embryo life. Ethanol, among other agents, is frequently taken during pregnancy, inducing embryo–fetal cell death and apoptosis of mammalian neurons (Ikonomidou et al., 2000; McAlhany et al., 2000; McGough et al., 2008; Sulik, 2005) and NCCs (Wentzel and Eriksson, 2009). However, the conviction that ethanol exposure alters embryogenesis and produces Fetal Alcohol Syndrome (FAS) (Jones and Smith, 1973) is not accompanied by adequate knowledge of the mechanism(s) of action of this toxin on the embryo system (Goodlett and Horn, 2001; Kumada et al., 2007; Sulik, 2005; Wentzel and Eriksson, 2009).

An interesting problem, as yet unresolved, is associated with the closure of the neural tube and with the expression of apoptosis in presumptive and early migratory NCCs. Although classic embryology reports refer to the "phagocytic capacity" of NCCs (apoptosis?) to fulfill the function of a "cleaner" of cell debris in such an active region of the closing neural tube, or to eliminate cell overproduction, or as involved in the selective determination of cell lineages (Lawson and England, 1998; Sanders and Wride, 1995), there are as yet no convincing explanations on this subject, and those old proposals still await confirmation. In the present work, the ethanol exposure of in ovo embryos or in a shell-less culture was followed by increased apoptosis expression in the presumptive NCC regions, concomitantly with failures

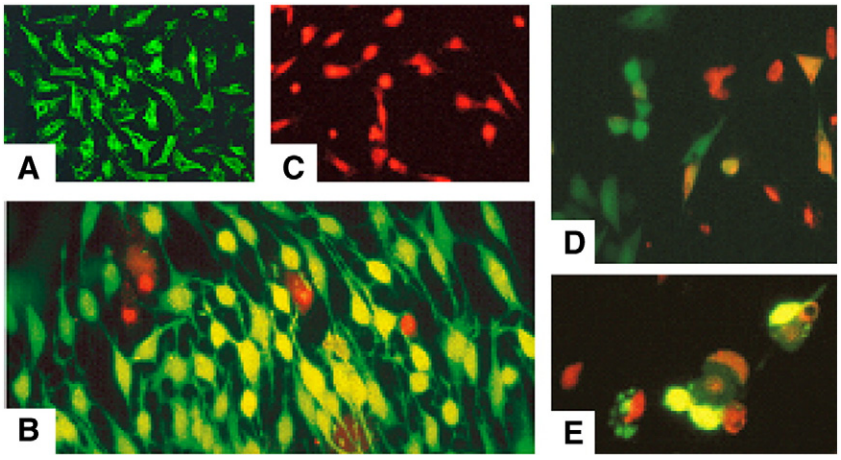

Fig. 4. Primary cultures of mesencephalic NCCs. (A) Immunolabeling with NC-1 antibody without previous permeabilization, showing the typical distribution of the epitope as fine points on the cell surface. The field represents the almost pure population of NCCs resulting from a careful microdissection technique. 20×. (B) Control group after calcein–ethidium technique; almost all cells are alive. 40×. (C) Positive control of cultured NCCs pre-treated with sodium azide; all cells are dead. 20×. (D) NCC culture after exposure for 3 h to ethanol; the proportion of dead cells is higher than in the control. 40×. (E) Apoptosis-like images showing DNA fragmentation and NCCs phagocyting dead cells, in ethanol-exposed NCC culture. 60×.

of neural tube closing. This data supports evidence of ethanol vulnerability in various mice embryo cell populations (including NCC-derivates) in late stages of development (Dunty et al., 2001; Kumada et al., 2007; Sulik, 2005; Wentzel and Eriksson, 2009).

Several lines of evidence suggest that ethanol effects can be modulated by controlling the trophic environment of cells (Kilburn et al., 2006; McGough et al., 2008). It was shown that the administration of exogenous Sonic hedgehog protein to the embryo rescues NCCs from ethanol-induced cell death (Ahlgren et al., 2002). Also, it is known that NCCs express several trophic factor receptors (Le Douarin and Kalcheim, 1999), and that ethanol exposure produces reduction of NT3 secretion (Heaton et al., 2004) and alterations in the concentration of nerve growth factor in the rat forebrain (Arendt et al., 1995) and brain-derived neurotrophic factor in the hippocampus (MacLennan et al., 1995), without necessarily affecting their receptors. On the other hand, an ethanol-induced low number of neonatal Purkinje cells associates with decreased expression of TrkC receptor in this cell population, suggesting that ethanol may induce alteration of NT3/TrkC regulation (Light et al., 2002). Interestingly, rats, prenatally exposed to ethanol and postnatally housed for 6 weeks in environmentally enriched conditions, showed a high level of NT3 on cerebellar vermis (Parks et al., 2008). This data encouraged the use of trophic factors in the potential treatment and/or prevention of ethanol-induced embryotoxicity (Kilburn et al., 2006; McAlhany et al., 2000; McGough et al., 2008).

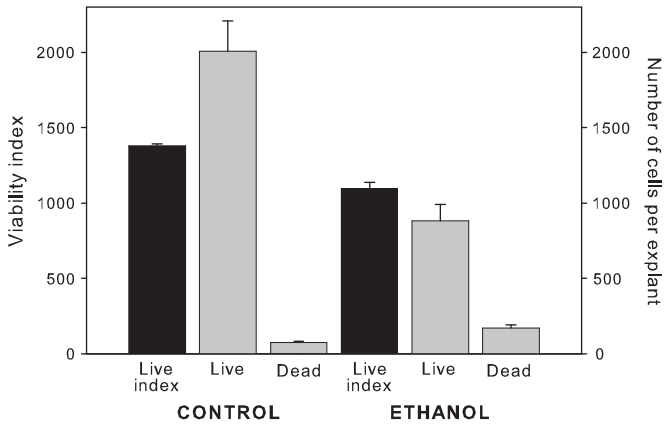

Fig. 5. The cell numbers of live and dead cells per explant, corresponding to cultures of ethanol-exposed and control, are shown (right axis, gray columns). In ethanol-treated NCCs, the number of live and total cells is lower than in the control group ($p<0.0001$). The number of dead cells in ethanol-treated cultures significantly exceeds the corresponding control group ($p<0.0003$). Student t-test. Mean ± SEM. Viability index (Live index) of NCC cultures after 3 h of ethanol exposure (left axis, black columns). Cell viability in ethanol-treated cultures is lower than in controls ($p<0.001$). Values in black columns correspond to the median. Mann-Whitney test. Control group: 41 explants (8,541 cells). Ethanol group: 44 explants (4,623 cells).

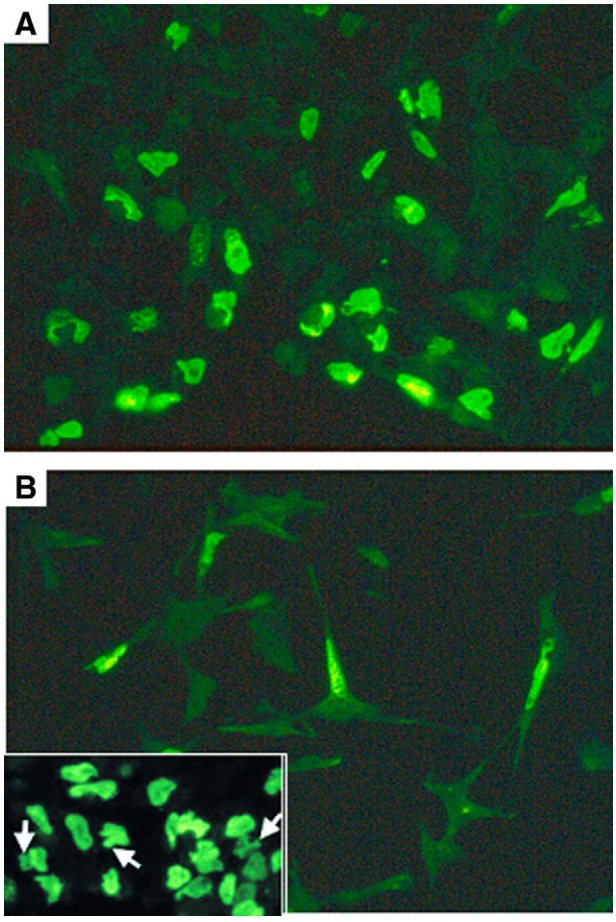

Fig. 6. Primary culture of mesencephalic NCCs after BrdU intake and immunolabeling. (A) Control group. (B) NCCs after 3 h of 100 mM ethanol treatment; a low proportion of proliferative activity is seen compared with the control. Apoptosis-like images are frequently seen (arrows in the inset) 40×.

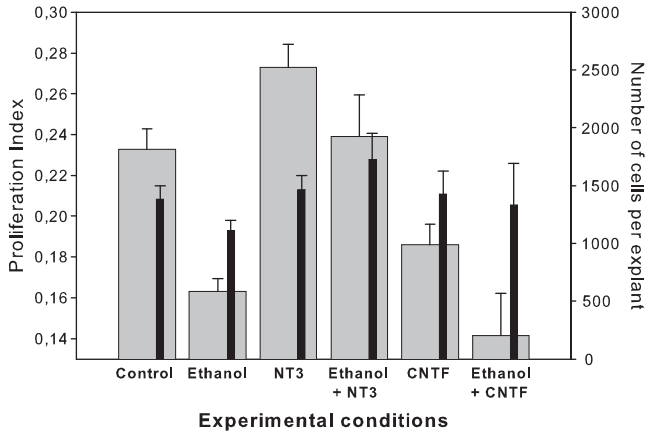

Fig. 7. Proliferation of NCCs after ethanol and trophic factor exposure (left axis, gray columns). Values correspond to arcsine transformation of proliferation index (BrdU-positive/total cells). Ethanol vs. Control: $p<0.001$; NT3 vs. Control: $p<0.015$; CNTF vs. Control: $p<0.005$; Ethanol + CNTF vs. Control: $p<0.001$; NT3 vs. Ethanol: $p<0.001$; Ethanol + NT3 vs. Ethanol: $p<0.004$; CNTF vs. Ethanol + CNTF: $p<0.05$; Ethanol + NT3 vs. Control: NS; Ethanol + NT3 vs. NT3: NS. ANOVA (Tukey test) or Kruskal–Wallis (Dunn test). Mean ± SEM. Number of NCCs per explant (right axis, black columns). Ethanol vs. Control: $p<0.05$; NT3 vs. Control: $p<0.01$; CNTF vs. Control: NS; Ethanol + CNTF vs. Control: NS; NT3 vs. Ethanol: $p<0.001$; Ethanol + NT3 vs. Ethanol: $p<0.006$; CNTF vs. Ethanol + CNTF: NS; Ethanol + NT3 vs. Control: NS; Ethanol + NT3 vs. NT3: NS. Student t-test. Mean ± SEM. The initial size of the explants was equivalent. Control: 19 explants (26,221 cells). Ethanol: 23 explants (25,578 cells). NT3: 19 explants (27,879 cells). Ethanol + NT3: 7 explants (12,080 cells). CNTF: 12 explants (17,111 cells). Ethanol + CNTF: 6 explants (7977 cells). NS: no significant difference.

In the present work, we found that the administration of exogenous NT3 attenuated in vivo NCC death and the associated defects of neural tube closure induced after 10 h of embryo treatment with 100 mM ethanol. This ethanol dose is equivalent to the blood alcohol concentration inducing FAS in mice (Webster and Ritchie, 1991), and is also similarly associated with aberrant neuronal migration (Kumada et al., 2006; Kumada et al., 2007), as well as apoptosis produced in fetal neurons (McAlhany et al., 2000) and cells of early gastrulating embryos (Kilburn et al., 2006).

These results lead us to think that NT3 is involved in some NCC rescue mechanism. The NT3 protection effect, not previously reported, is seen as a distribution and density of apoptosis similar to that of control groups, concomitant with the normality of the neural tube closure. Even if the regulation of NCC apoptotic phenomena is only partially known, the present results support the proposed general mechanism of an interaction of ethanol with apoptosis-inhibitor trophic factors (Cui et al., 1997). It is also well known that premigratory and migratory NCCs express the Trk-C receptor responding trophically to NT3 (Le Douarin and Kalcheim, 1999), and recent evidence indicates that subpopulations of cephalic NCCs respond with chemotactically guided Trk-C-dependent migratory behavior towards a concentration gradient of NT3 [Rovasio et al., unpublished results].

Our study on whole-mount embryos also allows us to confirm that NCCs of all segments of the neural tube are involved in ethanol-induced apoptosis, corroborating our preliminary and other partial descriptions utilizing selective markers and strict morphological criteria in optical and electron microscopy (see Rovasio and Battiato, 2002; Sanders and Wride, 1995; Wyllie et al., 1980).

The idea of a direct action of ethanol in a short time window matches reports on an ethanol-induced rapid increase of intracellular calcium followed by high G protein/phospholipase C activity and apoptosis in in vivo presumptive NCC regions (Debelak-Kragtorp et al., 2003; Garic-Stankovic et al., 2005; Garic-Stankovic et al., 2006). The short time window was also shown as ethanol-induced apoptosis and survival alterations of rat neonatal cerebellum, most occurring within the first 2 h of ethanol exposure, described as the "critical survival/death period" (Heaton et al., 2003). This point of view is supported by our present results showing that in vitro treatment of NCCs with a teratogenic concentration of ethanol during a lapse as short as 3 h induced a significant lowering of cell viability.

4.2. Viability and proliferative activity of in vitro NCCs exposed to ethanol and trophic factors

Several classic studies showed an ethanol-induced low proliferation of brain and cerebellum neuronal precursors, whereas other studies have reported increased proliferation of glia and neurons in in vivo and in vitro conditions (Bonthius and West, 1991; Goodlett and Horn, 2001; Miller, 1995; NIAAA, 2000). Our present results are the first report about a direct determination of the action of ethanol in NCCs producing low viability consequent to an increase of apoptotic/dead cells, and also a significant diminution of proliferative capacity, after only 3 h of in vitro ethanol treatment, and support evidence of similar occurrences in the neural tube later in development (Giles et al., 2008). These results seem to conflict with another report claiming that ethanol exposure did not significantly alter cell proliferation within neural crest-populated regions (Cartwright et al., 1998). However, this conclusion arose from experiments of the incorporation of thymidine analogs by homogenates of the entire cranial region that are not specific for NCCs and are consequently not comparable with our present results.

Here, we also demonstrated that NT3 is mitogenic on early cranial NCC cultures, also increasing the cell number by a survival effect, and exerting a protective action when administered together with ethanol treatment. Although present data do not allow us to make inferences about the protection mechanism of NT3, it is important to note that our results emerged after a brief 3 h treatment, which is significant for future experiments focused on their signal pathways background, including protein synthesis involvement.

In relation to CNTF, here their mitogenic role on in vitro early cranial NCCs was not verified, but its effect was rather associated with a lowering of proliferative cell behavior. Also, the treatment with ethanol plus CNTF induced an additional significant diminution of NCC proliferation, curiously maintaining a high value of the absolute number of cultured NCCs. This result, difficult to interpret, may be the consequence of a trophic CNTF action, not on NCC proliferative behavior but lengthening the viability of live cells. It is known that CNTF is a survival factor that exerts multiple effects later in development, aiding several NCC-derived cell lineages, such as motor, sensory, sympathetic and parasympathetic neurons, to recover from cell death (Hapner et al., 2006; Le Douarin and Kalcheim, 1999). On the other hand, it was suggested that CNTF could not have an essential role in the survival of early neuroblasts, since mice with null mutation of the CNTF gene did not reveal abnormalities during their embryo stages (Masu et al., 1993). Even if CNTF does not seem to have a trophic function on NCCs at early developmental stages, recent results from our laboratory suggest that they may have a chemokinetic action on the directional migration of early cephalic NCCs [Rovasio et al., unpublished results].

Concerning the concentration of the growth factors used in the present work, we considered the values of ED-50 of biological activity applied in other reports, as well as the general data about the NT3-CNTF responses of NCC-derived cells (Hapner et al., 2006), resulting in a selection of a "pharmacological dose" within the range of 10–50 ng/ml.

Taken as a whole, the present results show that the direct action of ethanol induces trophic perturbations after a short treatment of 3 h using a dose sufficient to induce FAS, thus supporting the idea of a primary effect on the near cell surface environment. It is known that the cell adhesion molecule L1 is a target sensitive to ethanol at a concentration as low as 1 mM, much lower than the blood alcohol concentration after a single glass of beer (Charness et al., 1994; Ramanathan et al., 1996). Moreover, the loss of the L1 expression gene induces brain anomalies and a clinical phenotype similar to FAS (Fransen et al., 1996; Wong et al., 1995). Our previous results showed irreversible

perturbations of cell morphology, cytoskeleton and motility patterns of in vitro cranial and trunk NCCs from the start of ethanol exposure (Rovasio and Battiato, 2002). These and present data suggest that a sensitive site of the cell surface milieu may be "touched" by ethanol, triggering deep changes in morphology and migratory regulation (Kumada et al., 2006; Kumada et al., 2007), as well as in proliferative and trophic cell behavior (Giles et al., 2008; Kilburn et al., 2006; McAlhany et al., 2000). They also give support to the importance of a brief exposure to ethanol as a factor leading to embryo cell/molecular damage (Dunty et al., 2001; Dunty et al., 2002; Kumada et al., 2006; Kumada et al., 2007; Wentzel and Eriksson, 2009), and agree with the idea of a critical survival/death period for ethanol-induced embryonic disturbance (Heaton et al., 2003; Webster and Ritchie, 1991).

5. Conclusions

The present results from in vivo and in vitro experiments, in line with the conditions inducing FAS, allow us to conclude that: (1) The early development of embryos exposed for 10 h to 100 mM ethanol exhibited anomalies of neural tube closure and increase of apoptosis in the NCC region along the neural axis, which were prevented by simultaneous treatment with 40 ng/ml NT3. (2) Cephalic NCC cultures exposed for 3 h to 100 mM ethanol presented high cell death and diminution of proliferative capacity, which were avoided after adding 40 ng/ml NT3 (but not CNTF) to the ethanol treatment.

These results support the current consensus on the potential risk of alcohol-exposure (even for a short time), during any stage of pregnancy and lactation, and encourage the perspectives for future work aimed at investigating the underlying molecular mechanism(s) of ethanol-exposure, as well as the use of trophic factors in the treatment and/or prevention of ethanol-induced embryopathologies.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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