

Rat Embryo Exposure to All-*Trans* Retinoic Acid Results in Postnatal Oxidative Damage of Respiratory Complex I in the Cerebellum

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ABSTRACT

The results of the present work show that the exposure of pregnant rats to low doses of all-*trans*-retinoic acid (ATRA) (2.5 mg/kg body weight) results in postnatal dysfunction of complex I of the respiratory chain in the cerebellum of the offspring. ATRA had no effect on the postnatal expression of complex I and did not exert any direct inhibitory effect on the enzymatic activity of the complex. The ATRA embryonic exposure resulted, however, in a marked increase in the level of carbony-

lated proteins in the mitochondrial fraction of the cerebellum, in particular of complex I subunits. The postnatal increase of the carbonylated proteins correlated directly with the inhibition of the activity of complex I. ATRA had, on the other hand, no effect on oxygen free-radical scavengers. It is proposed that embryonic exposure to ATRA results in impairment of protein surveillance in the cerebellum, which persists after birth and results in accumulation of oxidatively damaged complex I.

Introduction

The retinoids are a class of more than 4000 natural and synthetic molecules structurally and/or functionally related to all-*trans*-retinoic acid (ATRA), a metabolite of vitamin A. Retinoids control the expression of genes of transcription factors, enzymes, structural proteins, cell-surface receptors, neurotransmitters, neuropeptide hormones, and growth factors (Balmer and Blomhoff, 2002). Retinoic acid (RA) is essential for embryonic and adult growth, activating gene transcription via specific nuclear receptors. During embryo development, RA regulates axial and regional patterning, organogenesis, limb formation, and neurogenesis (Maden,

2007). During adulthood, RA is involved in organ homeostasis, regeneration of tissues (Maden and Hind, 2003; Maden, 2007), prevention of neoplastic growth (De Luca, 1991; Altucci and Gronemeyer, 2001), and neurodegenerative diseases (Corcoran et al., 2004).

The pathways on which RA acts require balanced concentrations of this retinoid, and deviation of RA levels from normal results in abnormal growth and development (McCaffery et al., 2003). The RA action depends on multiple processes, including synthesis and degradation of RA, phosphorylation and degradation of RA receptors, and recruitment of chromatin remodelers and processes involved in the transport, diffusion, and cellular uptake of retinoids (Bastien and Rochette-Egly, 2004). Altogether, these complex interactions determine the amount of ligand available for receptor occupancy in target tissues and set up a prereceptor system that directs timing and tissue-specificity of RA signaling. Despite protective mechanisms that exist to maintain homeostasis, RA is more teratogenic than vitamin A, being the transcriptional active retinoid that binds specific nuclear receptors (Giguere et al., 1987; Petkovich et al., 1987).

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ABBREVIATIONS: ATRA, all-*trans*-retinoic acid; ADU, arbitrary densitometric unit; RA, retinoic acid; DNPH, dinitrophenyl-hydrazine; ECL, enhanced chemiluminescence; GD, gestational day; NHEK, normal human epidermal keratinocyte; KB, human mouth epidermoid cells carcinoma; PBS, phosphate-buffered saline; PND, postnatal day; POD, horseradish peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TTBS, Tris-buffered saline/Tween 20; GSH, glutathione; G + M, glutamate plus malate; PAGE, polyacrylamide gel electrophoresis; DCFH, dichlorofluorescein; TCA, trichloroacetic acid; GSSG, oxidized glutathione.

Because the release of 13-*cis* RA as an effective oral treatment for chronic cystic acne (Peck et al., 1979), the actual or potential use of retinoids (all-*trans* RA, 9-*cis*-RA, 13-*cis* RA) in the treatment of clinical disorders has progressed significantly. Experimental and/or clinical studies have shown benefit of retinoids in malignancy (Altucci et al., 2005; Freemantle et al., 2006; Dragnev et al., 2007), immune (Van et al., 2009; Wasserfall and Atkinson, 2009; Kinoshita et al., 2010), and neurodegenerative disorders (Corcoran et al., 2004; Dheen et al., 2005; Husson et al., 2006; Kaur et al., 2006).

Because of their teratogenicity, the use of retinoids during pregnancy is of great concern, presenting a high risk for the fetus. Embryonic exposure to 13-*cis* RA, which is presumed to be isomerized to all-*trans* RA (Tsukada et al., 2000), induces malformations in hindbrain structures, most commonly in the cerebellum, and forebrain abnormalities (Holson et al., 1997). Interestingly, children embryonically exposed to 13-*cis* RA, who do not present major malformations, nevertheless often exhibit cognitive impairment (Adams et al., 2001).

Studies on the effects induced by rodent embryonic exposure to retinoids have shown that during specific stages of embryonic development, the doses of retinoids that produce malformations are higher than those that impair behavior (Adams and Holson, 1998). Previous investigations have shown that ATRA administration to pregnant rats between embryonic days 11 to 13 at a dose of 2.5 mg/kg induces postnatal neurofunctional deficits associated with transient changes in the cerebellar phenotype with subsequent slow recovery (Coluccia et al., 2008).

A direct correlation between increased number of mitochondria and increased number of spines and synapses has been demonstrated in primary neuronal culture (Liu and Shio, 2008), implicating that mitochondrial proliferation is a biological process facing energy demand during neuronal growth. In the fetus, the respiratory chain complexes are assembled and functional at early stages of development in the brain as in many other organs, and oxidative phosphorylation deficiency may have deleterious consequences (Minai et al., 2008). There is evidence that complex I of the mitochondrial respiratory chain is particularly sensitive to RA. In cultured keratinocytes, RA decreases the activity of complex I and induces cell growth arrest (Papa et al., 2007). In human breast cancer cells, RA reduces the activities of antioxidant enzymes such as catalase and glutathione peroxidase (Hong and Lee-Kim, 2009). Complex I is particularly sensitive to oxidative insults, resulting in nitrosylation and/or carbonylation of its subunits (Folbergrová et al., 2010). In HeLa cells combination of retinoic acids and interferon induces overproduction of reactive oxygen species (ROS), with loss of mitochondrial function and cell death (Huang et al., 2007).

The present study was aimed at verifying whether rat embryo exposure after maternal administration of low doses ATRA (2.5 mg/kg body weight) during pregnancy results in postnatal deleterious effect on mitochondrial energy metabolism of the cerebellum. The results show that ATRA exposure results in postnatal oxidative damage of the functional activity of respiratory chain complex I in the cerebellum.

Materials and Methods

Animals, Animal Husbandry, and Dosing

Pregnant rats at the gestational day (GD) 7 ($n = 46$) were purchased from Harlan (San Pietro Al Natisone, Italy). On the day of arrival, they were housed individually, allowed free access to food and water, kept under controlled environmental conditions (ambient temperature 24–25°C, humidity 50–60%, 12-h light/dark cycle, light on at 6:00 AM), and randomly assigned to two experimental groups: 1) 2.5 mg/kg body weight ATRA, (Sigma-Aldrich, Milan, Italy) in sesame oil ($n = 26$, initial body weight 251.08 ± 2.82 g); or 2) sesame oil (control, $n = 20$, initial body weight 262.65 ± 4.84 g). Both groups were gavaged once daily for 3 consecutive days between GD 11 and GD 13. Dose volume was 1 ml of oil solution per kg of body weight. The ATRA suspensions were prepared fresh daily under dim illumination and kept in amber bottles to prevent photodegradation. Body weight gain of control and ATRA-treated pregnant rats was monitored every day. On the day of birth [designated postnatal day (PND) 1], all pups were weighed, checked for any external malformations, examined for gender, and then randomly culled to eight pups per litter. Because biochemical assays were performed during the lactation period between PND 3 and PND 21, control and ATRA litters were randomly assigned to PND 3 (control, $n = 7$; ATRA, $n = 7$); PND 8 (control, $n = 5$; ATRA, $n = 6$); PND 15 (control, $n = 4$; ATRA, $n = 7$); and PND 21 (control, $n = 4$; ATRA, $n = 6$). Control and ATRA-treated male pups were killed by decapitation, and the cerebellum was rapidly removed, rinsed free of blood, and placed in ice-cold buffer (215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin, 20 mM HEPES, 1 mM EGTA, and 0.25 mM phenylmethylsulfonyl fluoride, pH 7.2). For each determination at different postnatal days, a pool of a given number of cerebellum was used as described in Table 1. Each biochemical assay was performed in duplicate.

Experiments were carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. All efforts were made to minimize the number of animals used and their suffering.

Cerebellum Homogenate Preparation and Mitochondria Isolation

All steps were carried out at 0 to 4°C. The pool of rat cerebellum was homogenized in 10 volumes (w/v) of 215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin, 20 mM HEPES, 1 mM EGTA, and 0.25 mM phenylmethylsulfonyl fluoride, pH 7.2 (buffer A) by six strokes at 500 rpm with a Potter homogenizer (homogenate). The homogenized suspension was filtered and centrifuged at 1300g for 3 min, and the resulting supernatant was centrifuged at 13,000g for 10 min to obtain mitochondrial pellet and the cytosolic fraction. The mitochondrial pellet was resuspended in the buffer A, manually homogenized, and centrifuged at 10,000g for 10 min. The final pellet was resuspended in 200 μ l of buffer A (Brown et al., 2004).

TABLE 1

Total number of cerebella used for preparation of pooled samples at different postnatal days

Postnatal Day	CTRL		ATRA	
	Total Number of Cerebella	Pooled Samples	Total Number of Cerebella	Pooled Samples
3	33	3	32	3
8	21	3	28	4
15	17	4	31	7
21	18	9	22	11

Protein Measurement

Homogenate and mitochondrial protein concentrations were determined according to the Bradford methods using bovine albumin as standard (Bradford, 1976).

Determination of Enzymatic Activities

For all enzyme activities determination, samples of homogenates or isolated mitochondria were sonicated to allow complete accessibility of substrates to the enzymes. The NADH-UQ-oxidoreductase, cytochrome *c* oxidase, glutathione peroxidase, and lactate dehydrogenase activities were measured spectrophotometrically with a Beckman DU 7400 equipped with a rapid-mixing apparatus.

NADH-UQ-Oxidoreductase (complex I) Activity in the Cerebellum Mitochondria. Complex I activity was performed in the 40 mM potassium phosphate buffer, pH 7.4, 5 mM MgCl_2 , in the presence of 3 mM KCN, 1 $\mu\text{g}/\text{ml}$ antimycin, 100 μM decylubiquinone, by following the rotenone-sensitive initial rate of NADH oxidation at 340 to 425 nm ($\Delta\epsilon = 6.81 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). The activity was 98 to 100% inhibited by rotenone (1 $\mu\text{g}/\text{ml}$).

Cytochrome *c* Oxidase (complex IV) Activity in the Cerebellum Mitochondria. Complex IV activity was measured in 25 mM phosphate buffer and 5 mM MgCl_2 , pH 7.4, by following the oxidation of ferrocytochrome *c* at 550 to 540 nm ($\Delta\epsilon = 19.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

Oligomycin-Sensitive ATP-Hydrolase (complex V) Activity in the Cerebellum Mitochondria. Complex V activity was determined by a coupled pyruvate kinase-lactate dehydrogenase assay in which ADP production was linked to NADH oxidation measured spectrophotometrically (Boffoli et al., 1994).

Citrate Synthase Activity in the Cerebellum Mitochondria. Citrate synthase activity was measured as described in Boffoli et al. (1994).

Glutathione Peroxidase Activity in the Cerebellum Homogenate. For glutathione peroxidase activity, 300 μg of proteins were incubated at 25°C in 5 mM Tris-HCl, pH 8, 0.5 mM potassium-EDTA, pH 8, 2.1 mM GSH (Sigma-Aldrich), 0.5 U/ml glutathione reductase (Sigma-Aldrich), and 0.17 mM NADPH (Sigma-Aldrich) for 3 min. The reaction was started by addition of 300 μM *tert*-butyl hydroperoxide (Sigma-Aldrich). The enzymatic activity was measured after the oxidation of NADPH at 340 nm.

Lactate Dehydrogenase Activity in the Cerebellum Homogenate. Lactate dehydrogenase activity was measured by incubating for 5 min at 37°C 100 μg of protein in 25 mM Tris, pH 7.5, and 0.1 $\mu\text{g}/\text{ml}$ rotenone. The reaction was started adding 100 μM NADH (Sigma-Aldrich). The enzymatic activity was measured after the oxidation of NADH at 340 nm.

Superoxide Dismutase Activity in the Cerebellum Homogenate. To test the superoxide dismutase activity the proteins of homogenate (23 μg) were separated on 8% gel electrophoresis under native conditions. When electrophoresis was completed, the gels were incubated at room temperature in the dark for 5 min with a gel

staining solution (0.05 M potassium phosphate buffer, pH 7.8, 1 mM EDTA, 0.25 mM nitro tetrazolium salt, and 0.5 mM riboflavin). After incubation, gels were exposed to light for 10 or 15 min. When the desired color development was achieved, gels were scanned by VersaDoc imaging system (Bio-Rad Laboratories, Milan, Italy).

Mitochondrial Respiratory Rates

The respiratory activity of freshly prepared cerebellum mitochondria was measured polarographically with a Clark-type oxygen electrode in a water-jacketed chamber (Hansatech Instruments, Norfolk, England) at 25°C. Mitochondria were suspended at a final concentration of 0.4 g/l in a medium containing 300 mM mannitol, 0.2 mM EDTA, 10 mM potassium-phosphate buffer, pH 7.4, 10 mM KCl, and 10 mM Tris-HCl. Respiration was started by adding 10 mM glutamate plus 5 mM malate (G + M) or 10 mM succinate (in the presence of 1 mg/l rotenone) (state IV). State III respiration was induced by the addition of 1 mM ADP.

In Vitro ATRA Treatment of Mitoplasts Isolated from Mammalian Cell Cultures

Cell Cultures. Normal human epidermal keratinocyte (NHEK) (Lonza Walkersville, Walkersville, MD) and human mouth epidermoid cells carcinoma (KB) (American Type Culture Collection, Manassas, VA) were grown in high-glucose Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum plus 2 mM glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin (all from Euroclone, Pero, Italy) at 37°C, 10% CO_2 .

Mitoplast Preparation and NADH-UQ Oxidoreductase Activity. Cells were harvested from Petri dishes with 0.05% trypsin and 0.02% EDTA, pelleted by centrifugation at 500g, and then resuspended in phosphate-buffered saline, pH 7.4 (PBS). The cell suspension was exposed for 10 min on ice to 2 mg of digitonin per milligram of cellular proteins. The mitoplast fraction, obtained by digitonin cell disruption, was pelleted at 14,000g and resuspended in PBS. The isolated mitoplasts were exposed to ultrasound energy for 15 s at 0°C. The sonicated mitoplasts were incubated for 1 h at 25°C in the absence or in presence of 20 μM ATRA.

The NADH-UQ oxidoreductase activity was performed in 40 mM potassium phosphate buffer, pH 7.4, 5 mM MgCl_2 , in the presence of 3 mM KCN, 1 $\mu\text{g}/\text{ml}$ antimycin, and 200 μM decylubiquinone using 30 μg of mitoplast proteins by following the oxidation of 1.25 to 25 μM NADH at 360 to 374 nm ($\Delta\epsilon = 2.01 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). The activity was corrected for the residual activity measured in the presence of 1 $\mu\text{g}/\text{ml}$ rotenone. V_{max} values were obtained from Lineweaver-Burk plots.

Electrophoretic Procedures and Western Blotting

The homogenate proteins (10 μg) were separated on 12% SDS-PAGE and transferred on nitrocellulose. The membrane was blocked with 5% nonfat dry milk in 500 mM NaCl, 20 mM Tris

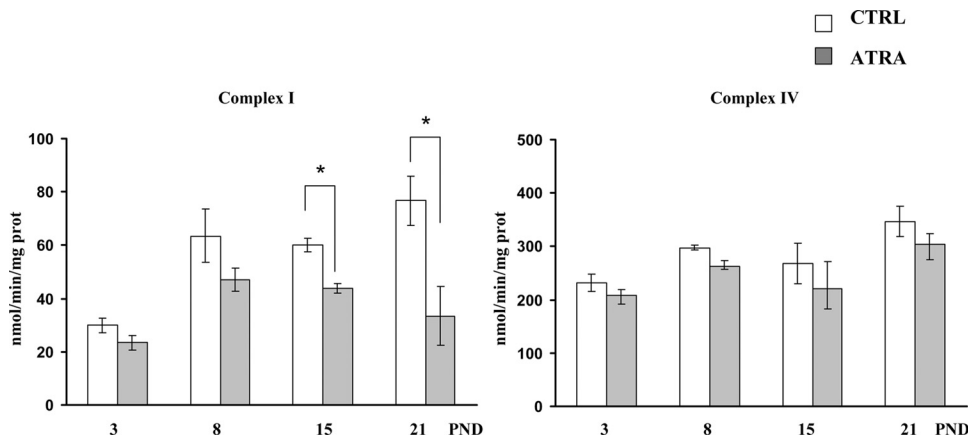


Fig. 1. Effects of ATRA on activities of mitochondrial respiratory chain complexes. Complexes I and IV activities were measured in sonicated mitochondria isolated from the cerebellum of control (CTRL) and ATRA-exposed offspring at PND 3, 8, 15, and 21. The bar graphs represent the mean values \pm S.E.M. *, $p < 0.05$ versus CTRL (Student's *t* test). The number of determinations at each age is reported in Table 1. For other details, see *Materials and Methods*.

0.05%, and Tween 20, pH 7.8 (TTBS) for 3h at 4°C and probed with antibodies against the β -subunit of ATP synthase complex (1:500; Invitrogen, San Giuliano Milanese, Milan, Italy), anti-39 kDa of complex I (1:500; Invitrogen), anti-subunit IV of cytochrome *c* oxidase (1:2000; Invitrogen), and anti- β -actin (1:3000; Sigma-Aldrich). After being washed in TTBS, the membrane was incubated for 60 min with anti IgG peroxidase-conjugated antibody (1:5000). Immunodetection was then performed, after further TTBS washes, with the enhanced chemiluminescence (ECL; Euroclone). Densitometric analysis was performed by VersaDoc imaging system.

Separation of oxidative phosphorylation complexes was performed by two-dimensional blue-native/SDS-PAGE (Schägger and von Jagow, 1991). Mitochondrial proteins (80 μ g) were suspended in 750 mM aminocaproic acid, 50 mM Bis-Tris, 0.5 mM EDTA, and 0.4% (w/v) lauryl maltoside. Proteins were applied on 5 to 12% gradient blue-native gel followed by a 12% two-dimensional SDS-PAGE. After electrophoretic separation, the proteins were transferred on nitrocellulose, and immunodetection was performed as described above.

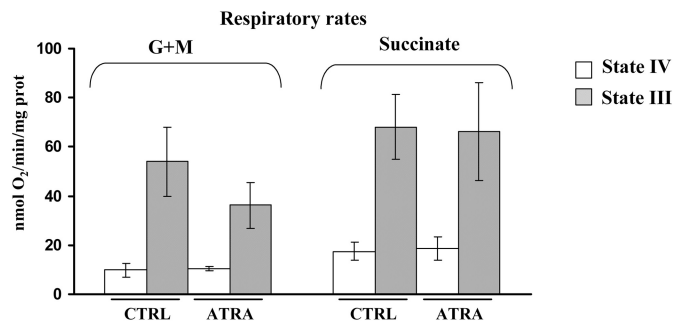


Fig. 2. Effects of ATRA on respiratory rates in mitochondria isolated from cerebellum of offspring at PND 21. Oxygen consumption rates were measured polarographically in freshly isolated mitochondria from the cerebellum of controls (CTRL) and ATRA-exposed offspring at PND 21. Respiration was started by adding 10 mM glutamate plus 5 mM malate (G + M) or 10 mM succinate in the presence of 1 mg/l rotenone (state IV). State III respiration was induced by the addition of 1 mM ADP. The bar graphs represent the mean values \pm S.E.M. For further details, see *Materials and Methods*.

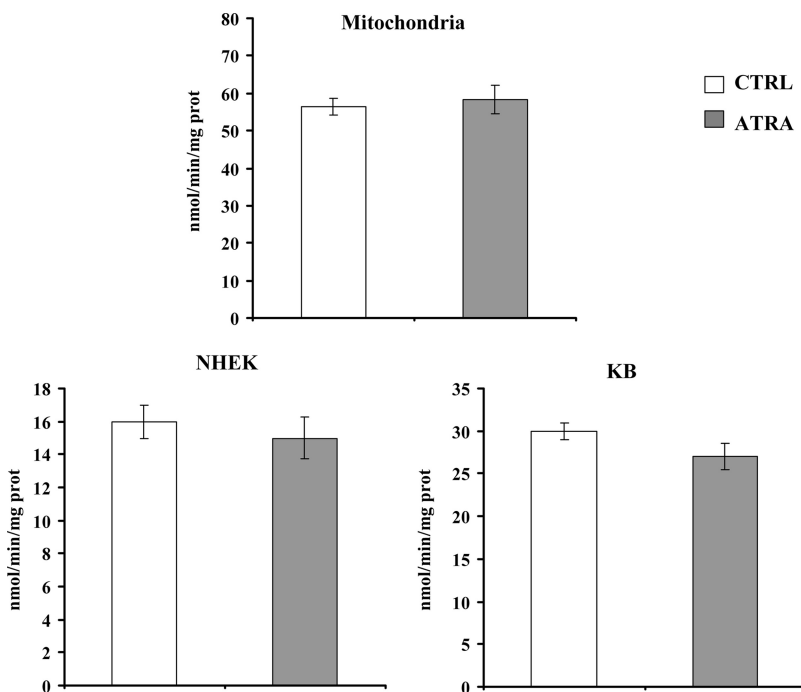


Fig. 3. Effects of ATRA on complex I activity after in vitro incubation of cerebellum mitochondria or mitoplasts isolated from NHEK and KB cells. Mitochondria isolated from the cerebellum of control offspring at PND 21 or mitoplasts, isolated from NHEK and KB cell cultures, were sonicated at 0°C and incubated for 1 h at 25°C in the absence (CTRL) or the presence of 20 μ M ATRA. Complex I activity was measured following the oxidation of NADH at 360 to 374 nm. For further details, see *Materials and Methods*. The bar graphs represent the mean of V_{max} values \pm S.E.M. of three different experiments.

Measurement of Mitochondrial ROS Level

The mitochondrial level of ROS was estimated by measuring the linear fluorescence increase (475 nm excitation, 525 nm emission) caused by the H_2O_2 -dependent oxidation of dichlorofluorescein (DCFH) to the fluorescent compound dichlorofluorescein in the presence of horseradish peroxidase (Black and Brandt, 1974). Immediately before determinations, DCFH was obtained from the stable reagent DCFH-diacetate by alkaline treatment (Rota et al., 1999). Mitochondrial proteins (100 μ g) were resuspended in 1 ml of 75 mM sucrose, 5 mM KP_i , 40 mM KCl, 3m M $MgCl_2$, 0.5 mM potassium-EDTA, and 30 mM Tris/HCl, pH 7.4, in the presence of 0.5 μ M horseradish peroxidase and 1.5 μ M DCFH. The ROS level was determined in the presence of 10 mM glutamate plus 5 mM malate in the absence or presence of 1 mg/l rotenone or with 5 mM succinate.

Determination of Total and Oxidized Glutathione Level in the Cerebellum Homogenate

The homogenate was divided into two aliquots. For the total glutathione (GSH + GSSG) assay, 0.25 ml of homogenate was added to 100 mM potassium phosphate buffer, pH 7.5, containing 17.5 mM EDTA and 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid). For the oxidized glutathione (GSSG) assay, 0.25 ml of homogenate was added to 100 mM potassium phosphate buffer, pH 6.5, containing 17.5 mM EDTA and 10 mM *N*-ethylmaleimide. The samples were centrifuged at 2000g for 5 min, and the supernatants were used for the spectrophotometric assay of total or oxidized glutathione (Calabrese et al., 2008).

Spectrophotometric Assay of Carbonylated Proteins in the Cerebellum Homogenate

The homogenate proteins (0.4 mg) were incubated for 1 h at 25°C in 0.2% (w/v) of dinitrophenyl-hydrazine (DNPH) in 2 N HCl, or in 2 N HCl only, as a control blank. The proteins were after precipitated with 10% TCA (w/v) and centrifuged at 3000g for 5 min at 4°C. The precipitated proteins were washed with 10% TCA and 1:1 (v/v) ethanol/ethyl acetate. The final precipitate was dissolved in 6 M guanidine, pH 2.5, and the spectrum of the hydrazone derivatives versus HCl controls was followed spectrophotometrically. The concentration of carbonyl groups was calculated from the spectrum absorbance,

using $\Delta\epsilon$ 21.5 mM⁻¹ · cm⁻¹ as the extinction coefficient for aliphatic hydrazones (Levine et al., 1990).

Carbonylation and Nitrosylation of Mitochondrial Proteins

Carbonylated Proteins Determination. For carbonyl groups determination, mitochondria samples (0.1 mg) were incubated for 1 h at 25°C in 5 mM DNPH in 2 N HCl. The proteins were after precipitated with 10% TCA (w/v) and centrifuged at 10,000g for 5 min. The proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane.

The membrane was blocked with 5% nonfat dry milk in TTBS for 2h at 4°C and probed with antibody against the anti-DNPH (Invitrogen) (1:3000). After being washed in TTBS, the membrane was incubated for 60 min with anti IgG peroxidase-conjugated antibody (1:5000). Immunodetection was then performed, after further TTBS washes, with ECL (Euroclone). The same nitrocellulose was immunoblotted with antibody against subunit IV of cytochrome *c* oxidase (1:2000).

Nitrosylated Proteins Determination. The nitrotyrosine groups detection was performed on 30 µg of mitochondrial proteins separated on 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked for 1 h in blocking buffer (3% bovine serum albumin in PBS/Tween 20) and then incubated with anti-nitrotyrosine antibody (Invitrogen) (1:1000) in PBS/Tween 20, for 1.5 h. After incubation, the membrane was washed in PBS/Tween 20 for 10 min three times and then incubated for 1 h with a goat anti-mouse IgG secondary antibody in PBS/Tween 20 (1:3000). The membrane was washed three times in PBS/Tween 20 for 10 min, and the immunodetection was performed with the ECL. The same nitrocellulose was treated for immunodetection of subunit 39 kDa on complex I of respiratory chain. Densitometric analysis was performed by VERSADOC imaging system (Bio-Rad).

Carbonylation of Complex I Subunits

Complex I was separated from cerebellum mitochondria with blue-native electrophoresis. The band corresponding to complex I was excised by gel, and electroelution was performed. The electroeluted subunits of complex I were treated with DNPH, and carbonyl groups were detected by electrophoresis and immunoblotting, as described above.

Results

The study of the asset of mitochondrial respiratory enzymes in the cerebellum was performed on the offspring of pregnant rats treated with ATRA (2.5 mg/kg body weight) or ATRA vehicle (control). The enzymatic activity of NADH-UQ oxidoreductase (complex I) and cytochrome *c* oxidase (complex IV) was determined in the cerebellum mitochondrial fraction at PND 3, 8, 15, and 21 (Fig. 1). In the control groups, the activities of both enzymes showed an age-dependent increase during the postnatal development of the cerebellum, which was particularly marked in the case of complex I at PND 21 (256% with respect to PND 3). The ATRA treatment depressed markedly the postnatal increase of the activity of complex I. A slight depressing effect was also exerted by ATRA treatment on complex IV activity (Fig. 1). Direct enzymatic assays showed that the ATRA treatment of pregnant rats had no effect on the ATP hydrolase activity of complex V (FoF1ATP synthase) and citrate synthase in the cerebellum mitochondria of the offspring at PND 21 (data not shown).

The respiratory rate measurements, presented in Fig. 2, show a depression of state III respiration with NAD-linked substrates in the cerebellum mitochondria of the offspring of

ATRA-treated rats, compared with controls, but no effect on state IV respiration rate was observed. The inhibition of state III respiration reflected the inhibition of complex I. Mitochondrial respiration with the flavin adenine nucleotide-linked substrate succinate in state III and IV and the index of respiratory control ratio (state III/ state IV) were, in this case, unaffected in the ATRA groups compared with controls. These data show that the mitochondrial capacity to conserve

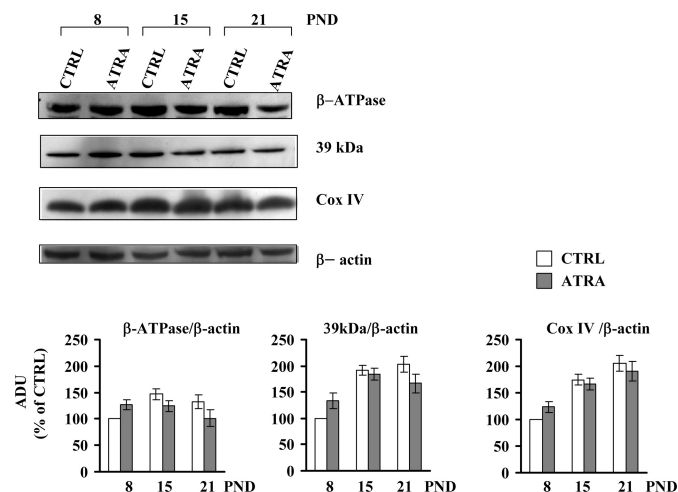


Fig. 4. Analysis of protein expression of mitochondrial respiratory chain complexes. The proteins of homogenates from the cerebellum of control (CTRL) and ATRA-exposed offspring at PND 8, 15, and 21 were loaded on 12% SDS-PAGE. Proteins were transferred on nitrocellulose and immunoblotted with antibodies specified in the figure. Arbitrary densitometry units (ADUs) of immunorevealed proteins with antibody against 39 kDa, Cox IV, and β-ATPase were normalized to ADU of β-actin. All data obtained were normalized to the control value at PND 8. The bar graphs represent the mean values ± S.E.M. The number of determinations at each age is reported in Table 1.

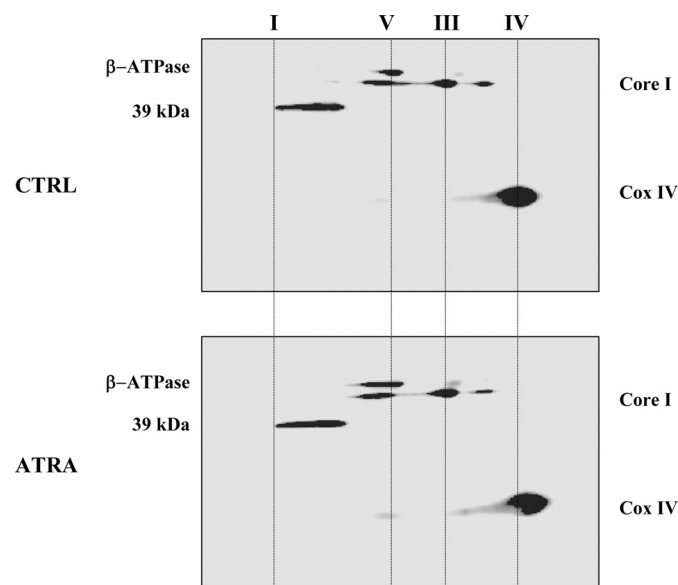


Fig. 5. Assembly of oxidative phosphorylation complexes in control (CTRL) and ATRA-exposed offspring at PND 21. The proteins of mitochondria isolated from cerebellum of CTRL and ATRA-exposed offspring at 21 PND were separated by two-dimensional blue-native/SDS-PAGE electrophoresis and transferred for immunodetection with antibodies against complex I (39 kDa), complex III (core I), complex IV (cox IV), and complex V (β-ATPase) of mitochondrial respiratory chain. For other details, see *Materials and Methods*.

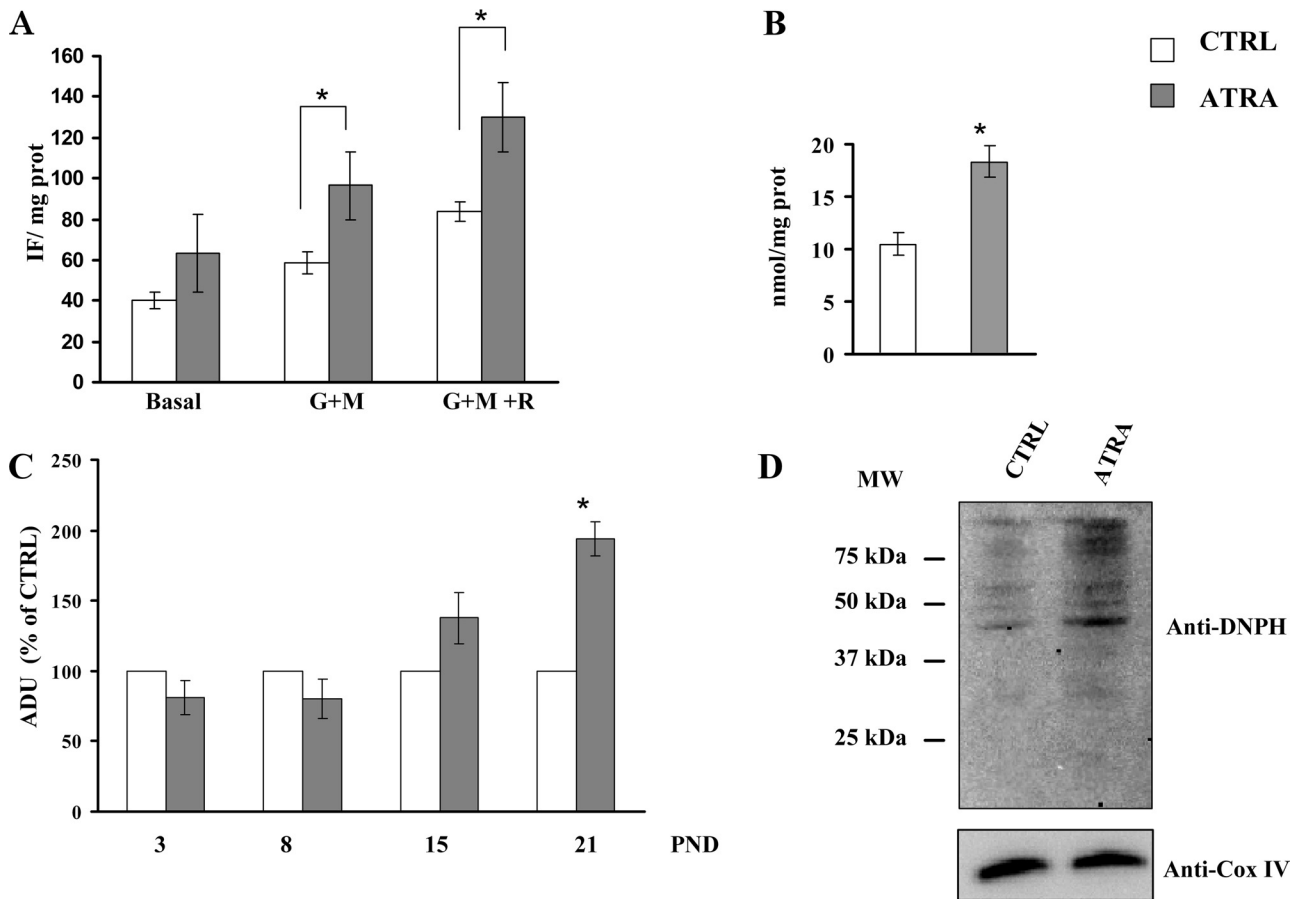


Fig. 6. ROS production and protein carbonylation levels in the cerebellum homogenate and isolated mitochondria. A, ROS level was assayed in freshly isolated mitochondria from cerebellum, after the oxidation of DCFH. ROS production was estimated in the absence of substrates (basal) and in the presence of 10 mM glutamate plus 5 mM malate (G + M) or G + M plus 1 mg/l rotenone (R). *, $p < 0.05$ versus CTRL (Student's t test). For further details, see *Materials and Methods*. B, carbonylated proteins were measured spectrophotometrically in homogenate samples obtained from the cerebellum of control (CTRL) and ATRA-exposed offspring at PND 21. For further details, see *Materials and Methods*. C, mitochondria were isolated from the cerebellum of CTRL and ATRA-exposed offspring at PND 3, 8, 15 and 21. Proteins were treated with DNPH, as reported under *Materials and Methods*, loaded onto 12% SDS-PAGE, transferred on nitrocellulose, and immunoblotted with anti-DNPH and anti-Cox IV subunit. The ADUs of anti-DNPH were normalized to ADU of anti-Cox IV, and the data obtained were normalized to the respective control value. The bar graphs represent the mean values \pm S.E.M. The number of determinations at each age is reported in Table 1. *, $p < 0.05$ versus CTRL (Student's t test). D, representative immunoblotting of mitochondrial proteins with anti-DNPH and anti-Cox IV at PND 21.

the energy-linked electrochemical proton gradient, generated by respiration, was unaffected in the ATRA group mitochondria.

To verify whether the decrease of complex I activity, detected in the mitochondria from the offspring of ATRA-treated rats, was due to the direct effect of ATRA on the enzyme, sonicated mitochondria of the cerebellum from PND 21 control rats and mitoplasts, isolated from two different cell lines (NHEK and KB), were incubated with 20 μ M ATRA. Under these conditions, no significant effect was exerted by ATRA on the activity of complex I (Fig. 3).

To evaluate whether the decreased activity of the complexes was associated with changes in their content, the specific levels of protein subunits of complexes I, IV, and V (FoF1ATP synthase) were estimated by Western blotting with specific antibodies in cerebellum homogenates of rats at PND 8, 15, and 21. The results of this analysis show that the levels of the subunits of the three complexes increased during the postnatal development of the cerebellum (Fig. 4). ATRA treatment did not exert, however, a significant effect on the postnatal increase in the level of the subunits of the three complexes (Fig. 4).

Two-dimensional blue native/SDS-PAGE separation and

immunoblot with subunit-specific antibodies showed that at PND 21, there was no change in the assembly pattern of respiratory complexes I, III, and IV and ATP synthase (complex V) in the cerebellum mitochondria of the offspring of ATRA-treated pregnant rats compared with controls (Fig. 5).

Complex I is a main site of oxygen-free radical production,

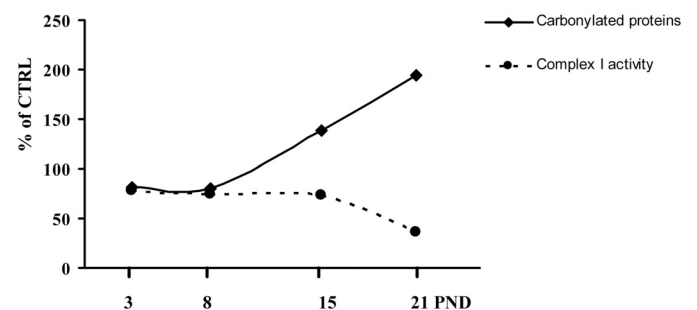


Fig. 7. Relationship between carbonylated proteins and complex I activity in the cerebellum mitochondria of ATRA-exposed offspring. Ordinate, percentage increase in the level of mitochondrial carbonylated proteins and percentage decrease of complex I activity with respect to control; abscissa, PND.

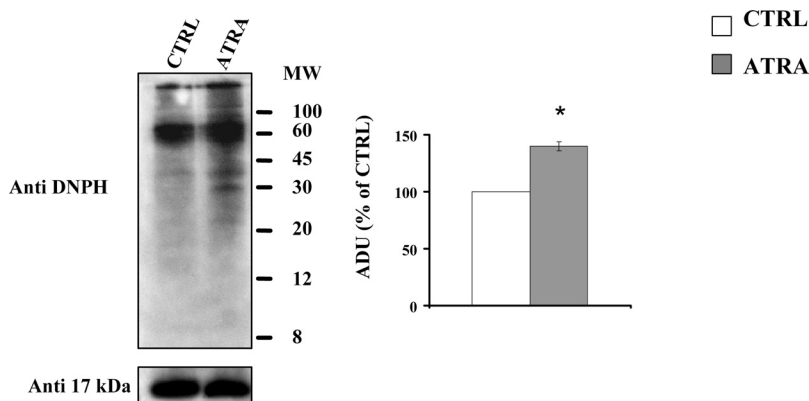


Fig. 8. Carbonylated proteins in complex I subunits. The NADH-UQ oxidoreductase complex was isolated by blue-native gel, from the cerebellum mitochondria of control (CTRL) and ATRA-exposed offspring at PND 21. The complex was excised by gel, electroeluted, and treated with DNPH (see *Materials and Methods*). Proteins were loaded on 12% SDS-PAGE and transferred on nitrocellulose for immunoblotting with antibody against DNPH groups and anti-17 kDa subunit of complex I. The ADU of all bands immunorevealed with antibody anti-DNPH were normalized to ADU of anti-17 kDa. The bar graphs represent the mean values \pm S.E.M. *, $p < 0.05$ versus CTRL (Student's *t* test).

which can become significant under particular pathophysiological conditions (Koopman et al., 2010).

Determination of H_2O_2 production in the cerebellum mitochondria (Fig. 6A) showed that in ATRA groups, there was at PND 21 a significant increase in the ROS level in the presence of NAD-linked substrates compared with controls. The ROS level increased in the presence of the complex I inhibitor rotenone. No difference in ROS production was observed when succinate was used as respiratory substrate (data not shown). Complex I is particularly susceptible to oxidative stress (Taylor et al., 2003; Choksi et al., 2004), and functional defects of the complex have been associated with nitrosylation and/or carbonylation of its subunits (Choksi et al., 2008; Folbergrová et al., 2010).

A direct determination was performed of the nitrosylation and carbonylation of proteins in postnatal cerebellum mitochondria of the offspring of control pregnant rats and rats treated with ATRA. A marked increase, at PND 21, of carbonylated proteins in the cerebellum homogenate and mitochondria of the ATRA-treated offspring with respect to controls was found (Fig. 6, B and C). SDS-PAGE of the cerebellum mitochondria showed increase of carbonylated proteins of various apparent molecular weights at PND 15

and 21 (Fig. 6D). Figure 7 shows that there was a direct relationship in the cerebellum mitochondria between the postnatal increase of carbonylated proteins and the decrease of the enzymatic activity of complex I in the cerebellum of ATRA-exposed offspring.

Two-dimensional blue-native SDS-PAGE electrophoresis of complex I subunits separated from cerebellum mitochondria revealed that at the PND 21, when the level of carbonylated proteins and the decrease of complex I activity was the largest, a significant increase in the level of carbonylated subunits of complex I of apparent molecular weights between 75 and 20 kDa was detectable (Fig. 8). The densitometric analysis of immunodetected nitrosylated proteins showed no significant difference between control and ATRA treated offspring at PND 8, 15, and 21 (Fig. 9).

To verify whether the treatment with ATRA was associated with a decreased capacity of free radical scavengers, the GSH and GSSG levels, glutathione peroxidase activity, and superoxide dismutase (copper/zinc-SOD and manganese-SOD) activities were directly determined. As shown in Fig. 10, no alteration in the levels of GSH and GSSG or in the activity of the scavenger enzymes was found.

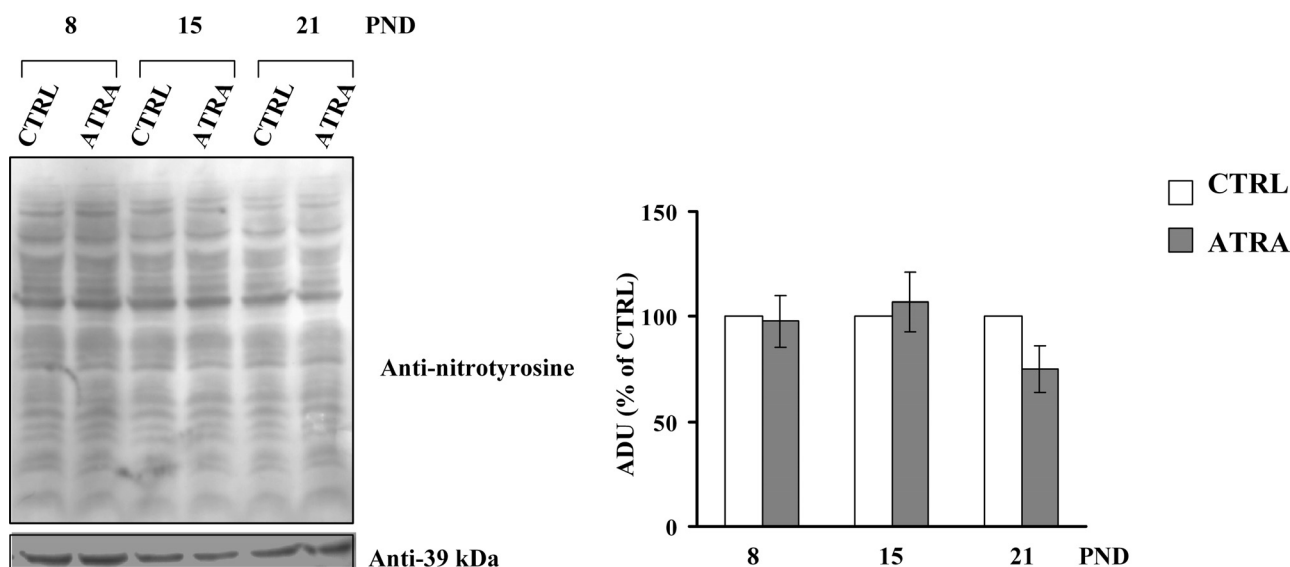


Fig. 9. Nitrosylation levels of mitochondrial proteins. Mitochondrial proteins (30 μ g) isolated from the cerebellum of control (CTRL) and ATRA-exposed offspring at PND 8, 15, and 21 were loaded on SDS-PAGE, transferred on polyvinylidene difluoride membrane, and treated with antibody against anti-nitrotyrosine. ADUs of immunorevealed proteins with anti-nitrotyrosine antibody were normalized to the ADU of antibody against 39-kDa subunit of complex I. Data were normalized to the respective control value. Bar graphs represent the mean values \pm S.E.M. The number of determinations at each age is reported in Table 1.

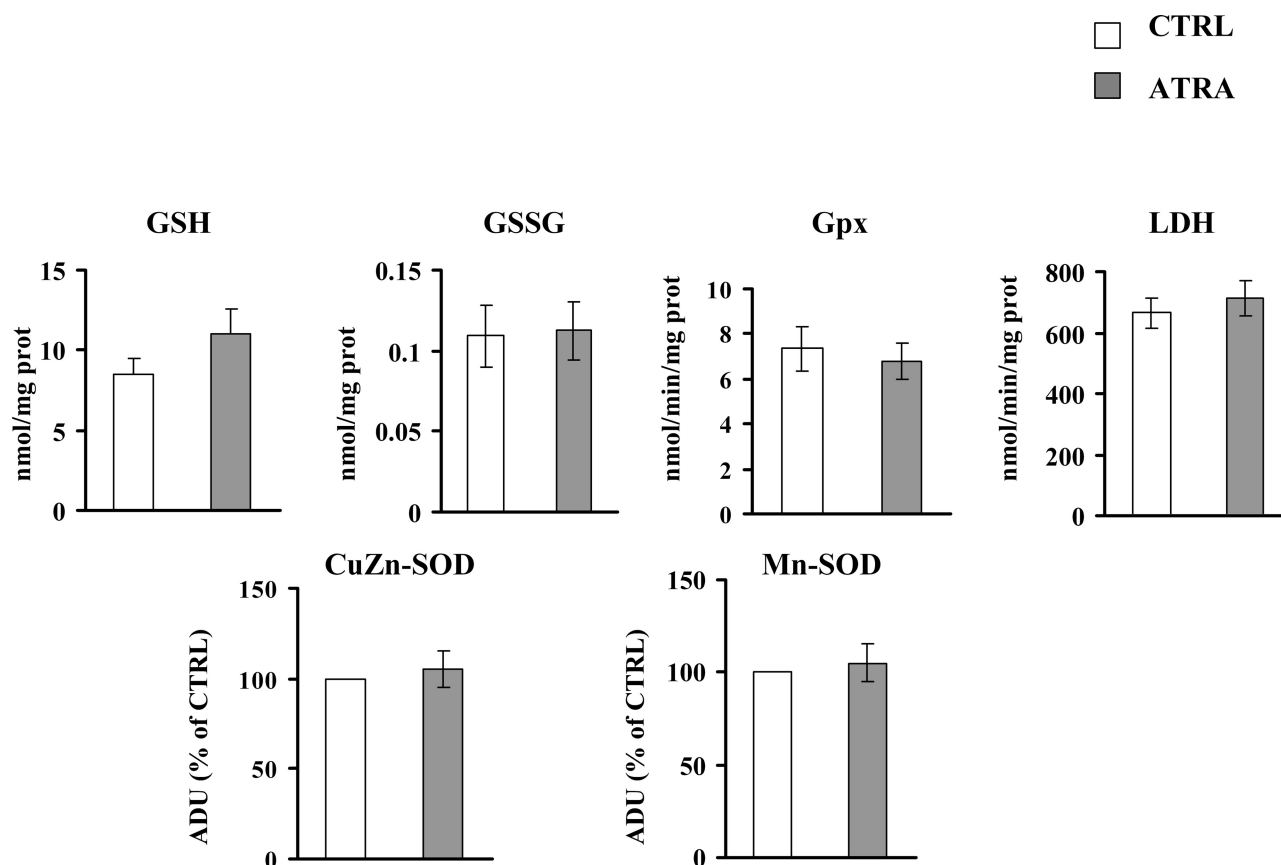


Fig. 10. Scavenger systems in the cerebellum homogenate at PND 21. Homogenate samples were obtained from the cerebellum of control (CTRL) and ATRA-exposed offspring at PND 21. The levels of total and oxidized glutathione (GSH and GSSG) were determined spectrophotometrically. The glutathione peroxidase activity (Gpx) was assayed in homogenate after the oxidation of NADPH at 340 nm. For lactate dehydrogenase activity (LDH), 100 μ g of the homogenate proteins were resuspended in 25 mM Tris, pH 7.5, in the presence of rotenone and pyruvate, and reaction was started with NADH. The manganese and copper/zinc superoxide dismutase (Mn-SOD and CuZn-SOD) were determined by in gel activity. For further details see *Materials and Methods*. The bar graphs represent the mean values \pm S.E.M. ADU, arbitrary densitometric units.

Discussion

The present study shows that the postnatal development of rat cerebellum is associated with increased expression of mitochondrial respiratory chain complexes. This results in an increased functional capacity of respiratory chain complexes, in particular complex I, which, under various conditions, seems to be the pacemaker of the mitochondrial respiratory activity (Papa et al., 2008; De Rasmo et al., 2011). These observations thus provide further direct evidence that mitochondrial energy supply is critical for the development of brain regions and their functionality.

Rat embryo exposure to low doses of ATRA (2.5 mg/kg body weight), which have been found to result in a postnatal transient neurofunctional deficit (Coluccia et al., 2008), is shown here to prevent the postnatal increase of the functional activity of complex I in the cerebellum compared with that observed in control rats. It can be noted that the impairment of complex I activity in the offspring of the ATRA-treated rats resulted in inhibition of the overall state III respiratory rate with NAD-linked substrates, but there was no damage of the capacity of mitochondria to store the membrane electrochemical proton gradient, as shown by the lack of any effect of ATRA on coupled state IV respiration.

Impairment of the postnatal increase in the functional activity of complex I was not due to inhibition of the postnatal expression burst of complex I, because ATRA treatment did

not cause significant depression of the postnatal increase in the level of subunits or in the assembly pattern of the complex. Neither ATRA exerted any direct inhibitory effect on the enzymatic activity of complex I. Evidently embryo exposure to ATRA induces some events in the embryonic cells that persist after birth and result in a postnatal detrimental effect on the functional capacity of complex I. It is conceivable that direct exposure of neonatal rats to inhaled oxygen could, in addition to stimulating the expression of respiratory complexes, result in some increase in the level of oxygen free radicals. There are in the cells systems that prevent and/or counteract the deleterious effect of free radicals, such as oxygen free radical scavengers (Finkel and Holbrook, 2000; Andreyev et al., 2005) or surveillance systems that remove from the cells oxidatively damaged nucleic acid (Dizdaroglu, 2005), lipids, and proteins (Friguet et al., 2008; Grimm et al., 2011). The ATRA treatment did, in fact, result in postnatal days, in a substantial elevation of mitochondrial ROS production, and in the level of carbonylated proteins in the cerebellum homogenate and in the mitochondrial fraction.

In particular, carbonylation of complex I subunits in the range of 20 to 75 kDa was detected, and the extent of inhibition of complex I activity seemed to be directly correlated with the extent of protein carbonylation (Taylor et al., 2003; Keeney et al., 2006; Choksi et al., 2008; Folbergrová et al., 2010). Carbonylation of several subunits of complex I, such

as NDUFS1, NDUFS2, NDUFV1, NDUF9, ND4, and NDUF54, has been reported previously (Wen and Garg, 2004; Keeney et al., 2006; Choksi et al., 2008). On the other hand, no differences were observed in the nitrosylation of proteins, a modification which involves, in addition to oxygen superoxide, nitric oxide and peroxyxynitrite formation (Stadtman and Levine, 2000).

The increased oxidative damage of complex I caused by ATRA treatment was not due to impairment of scavenger systems, which were unaffected. It is conceivable that embryonic exposure to ATRA induces in embryo cells events resulting in impairment of cellular surveillance systems (Hernebring et al., 2006; Grimm et al., 2011), which normally clean off oxidatively damaged subunits of complex I (Friguet et al., 2008; Stanyer et al., 2008), detrimental for the activity of the enzyme. This impairment apparently persists after birth and results in a critical postnatal accumulation of oxidatively damaged complex I in the cerebellum, when the offspring is directly exposed to inhaled oxygen. Evidence is available that there are, in cells, systems that can ensure an exchange of damaged subunits in the assembled complex I with newly synthesized subunits (Lazarou et al., 2007; De Rasmio et al., 2008; Papa et al., 2010). Further work is required to verify the type of the surveillance pathways eventually affected by ATRA.

The present work provides further evidence, at a molecular level, of postnatal detrimental effects that ATRA treatment of pregnant mammals, even at low doses, can have on the offspring, thus raising warning for better control of the therapeutic use of retinoids in pregnancy.

Authorship Contributions

Participated in research design: Signorile, Sardaro, De Rasmio, Scacco, F. Papa, Borraci, Carratù, and S. Papa.

Conducted experiments: Signorile, Sardaro, De Rasmio, and Borraci.

Performed data analysis: Signorile and De Rasmio.

Wrote or contributed to the writing of the manuscript: Signorile, Carratù, and S. Papa.

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