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Maternal dietary loads of α-tocopherol depress protein kinase C signaling and synaptic plasticity in rat postnatal developing hippocampus and promote permanent deficits in adult offspring☆

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Abstract

Vitamin E (α-tocopherol) supplementation has been tested as prophylaxis against gestational disorders associated with oxidative damage. However, recent evidence showing that high maternal α-tocopherol intake can adversely affect offspring development raises concerns on the safety of vitamin E extradosages during pregnancy. Besides acting as an antioxidant, α-tocopherol depresses cell proliferation and modulates cell signaling through inhibiting protein kinase C (PKC), a kinase that is deeply involved in neural maturation and plasticity. Possible effects of α-tocopherol loads in the maturing brain, where PKC dysregulation is associated to developmental dysfunctions, are poorly known. Here, supranutritional doses of α-tocopherol were fed to pregnant and lactating dams to evaluate the effects on PKC signaling and morphofunctional maturation in offspring hippocampus. Results showed that maternal supplementation potentiates hippocampal α-tocopherol incorporation in offspring and leads to marked decrease of PKC phosphorylation throughout postnatal maturation, accompanied by reduced phosphorylation of growth-associated protein-43 and myristoylated alanine-rich C kinase substrate, two PKC substrates involved in neural development and plasticity. Although processes of neuronal maturation, synapse formation and targeting appeared unaffected, offspring of supplemented mothers displayed a marked reduction of long-term synaptic plasticity in juvenile hippocampus. Interestingly, this impairment persisted in adulthood, when a deficit in hippocampus-dependent, long-lasting spatial memory was also revealed. In conclusion, maternal supplementation with elevated doses of α-tocopherol can influence cell signaling and synaptic plasticity in developing hippocampus and promotes permanent adverse effects in adult offspring. The present results emphasize the need to evaluate the safety of supranutritional maternal intake of α-tocopherol in humans.

Keywords: Vitamin E; Brain development; Cell signaling; Long-term potentiation; Morris water maze

1. Introduction

Vitamin E (VitE) is a natural antioxidant acting as peroxyl radical scavenger and chain breaker of lipid peroxidation, including two groups of fat-soluble compounds, tocopherols and tocotrienols, each with four distinct analogs (α, β, γ and δ). The term *VitE* is often synonymously used with α-tocopherol (α-T), mostly because of its highest bioavailability and retention in the human body [1].

α-T is regarded as an important molecule during fetal and early postnatal life, playing fundamental role in protecting the developing organism against oxidative stress. In fact, free radicals have been proposed as causative agents of pregnancy-related disorders, such as preeclampsia and maternal diabetes, that induce serious complications in mother and fetus [2,3]. In keeping, blood α-T concentrations are reduced in abnormal pregnancies [4], thus suggesting that VitE requirements may increase during gestation. On this rationale, clinical and preclinical studies have tested the potential benefit of antioxidant prophylaxis in reducing the incidence of pregnancy complications [5–14]. In addition, the growing interest on the antioxidant properties of VitE, together with its easy accessibility, has generally encouraged the spontaneous attitude to increase maternal tocopherol intake in the population [13].

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Exposure to high doses of α-T during fetal and early postnatal ages is expected to occur in the case of an extensive VitE supplementation during pregnancy and lactation. Although the toxicity of VitE is considered low, some recent findings have pointed out that high maternal α-T consumptions may have adverse developmental effects on offspring. Studies in humans, though yielding controversial results, showed that maternal levels of α-T can influence fetal growth. While prospective studies suggested a positive association between maternal VitE concentrations and birth weight and length [15,16], randomized trials have instead reported that VitE supplementations correlate to an increased rate of low birth weight both in normal [17] and in pathological [10] pregnancies. Teratogenic potential of VitE extradosages seems minor in both rodents and humans [8,17,18]. However, a case-control study has recently revealed an association between high maternal VitE intake by diet or supplements with an increased risk of congenital heart defects in offspring [19]. Moreover, concerns on the safety of high maternal α-T intake in pregnancy should conceivably be extended to the lactating period, when α-T transfer is much more efficient and more readily increased via higher intake of VitE by the mother [20].

In this context, it is worth noting that, besides its antioxidant properties, VitE was recently proposed to have alternative, non-antioxidant functions [21]. Studies in different cellular and in vivo models have clearly demonstrated that α-T can depress cell proliferation and regulate the expression of several groups of genes and signaling molecules [22,23]. Most of these effects appear to be dependent on the control of protein kinase C (PKC), which has been tentatively suggested to represent the main and possibly unifying target of VitE in eukaryotic cells [24]. Distinct PKC isozymes have been identified as differing in biochemical properties and expression patterns [25]. PKC isozymes are all variably expressed in neural tissues, where they act as signal transducers involved in synaptic plasticity, as well as in a variety of developmental processes occurring at different stages of CNS maturation, including embryogenesis, cell proliferation and differentiation, dendritic growth, synapse formation and pruning [26–32]. Alterations of PKC isozymes activity and/or expression are thought to play a role in brain developmental dysfunctions induced by gestational and perinatal exposure to neurotoxicants [33,34].

α-T can affect neural plasticity and PKC signaling in adult rat brain in vivo, as evidenced by reports showing that α-T supplementation regulates hippocampal neuronal remodeling and provokes massive PKC inhibition [35]. Moreover, α-T was reported to affect neural precursor proliferation and newborn neuron survival in adult hippocampal neurogenesis, a widely accepted model of neural development in adult brain [36–38], thus suggesting that α-T loads can interfere with some key biological aspects of brain maturation. Whether high doses of α-T may affect PKC signaling also in immature brain, thereby possibly influencing CNS development, remains unknown.

To assess possible neurodevelopmental effects of maternal supranutritional intake of VitE, dams were fed during pregnancy and lactation with supplemental doses of α-T similar to those reported to improve fetal outcome in rat experimental pregnancies [5-8, 11, 14; see Discussion], and the effects on PKC activity and morphofunctional maturation were investigated in offspring's developing hippocampus. In particular, phosphorylation levels of PKC and PKC substrates involved in neural development and plasticity [growth-associated protein (GAP-43) and myristoylated alanine-rich C kinase substrate, MARCKS], and the expression of pre- and postsynaptic markers (synaptophysin and spinophilin), were evaluated throughout postnatal maturation; moreover, synaptic transmission and plasticity were studied in juvenile hippocampus. Finally, biochemical and functional features which were found affected in developing hippocampus, were reinvestigated in adult offspring, where hippocampus-dependent spatial learning ability was also tested by the Morris water maze test.

2. Materials and methods

2.1. Animals and supplementation protocol

Sprague–Dawley albino rats (Charles River, Italy) were used in these studies. Animal care and experimentations were done in accordance with the Policies on the Use of Animals and Humans in Neuroscience.

Virgin female rats weighing 200–250 g were housed individually with free access to food and water and maintained at an ambient temperature of 22±1°C with a 12-h light and 12-h dark cycle (lights on at 6 a.m. and off at 6 p.m.). Animals were randomly divided into two groups: a control group (CTRL; $n=10$), fed with a commercial standard diet, and a supplemented group (TREAT; $n=10$), fed with a diet highly enriched in the natural RRR-α-T form (15 g/kg pellet; Laboratori Dottori Piccioni, Italy). This α-T-supplemented diet was administered constantly from 2 weeks before mating, throughout pregnancy and lactation. Pups were weighed weekly throughout the experimental period. After weaning, offspring were separated from mothers and fed ad libitum with standard diet.

For the present experimental purposes, pups from the CTRL and TREAT litter groups were sacrificed at various developmental ages: postnatal day 0 (P0, day of birth), P7, P14, P21 (weaning) and P60–90 (adulthood).

2.2. Measurement of α-T tissue incorporation

Rats at P0, P21 and P60–90 were used for these experiments. Animals were deeply anesthetized (by hypothermia at P0 and with sodium pentothal, 45 mg/kg, at P21 and P60–90) and perfused transcardially with ice-cold physiological saline, and brains (from which hippocampi were immediately excised) and livers were quickly removed and frozen in liquid N_2. Measurement of α-T in hippocampal and hepatic tissues was performed by HPLC-ECD analysis as previously described [39]. Briefly, 50 mg of tissue was suspended in 1 ml of phosphate-buffered saline (PBS), pH 7.4, and then were homogenized by sonication (50 W×15 s in ice). An aliquot of 200 μl suspended in 1 ml of absolute ethanol was then transferred into a Pyrex tube for lipid extraction by hexane. The organic layer was dried down under a stream of nitrogen at 50°C and the residue was resuspended in 200 μl of HPLC-grade methanol for the injection in the HPLC system. Tocopherols were separated under isocratic conditions using 98% methanol in ultrapure water and a column Gemini C18, 5 μl, 150×4.6 mm (Phenomenex, Milan, Italy). Test compound detection was obtained using a coulometric detector set at the oxidation potential of 750 mV with a 50-nA scale. Compound identification and analysis calibration were based on the use of appropriate external and internal standards (RRR-α-T and tocol, respectively).

2.3. Immunoblotting experiments

2.3.1. Antisera used

Rabbit polyclonal raised against the phosphorylated forms of PKCpan (anti-p-PKCpan; Ser660), PKCalpha (anti-p-PKCα; Thr638) and PKCdelta (anti-p-PKCδ; Thr505) were obtained from New England Biolabs Inc. (Beverly, MA, USA). Rabbit polyclonal anti-GAP-43 and its phosphorylated form (anti-p-GAP-43; Ser41) were purchased from Abcam (Cambridge, UK). Rabbit polyclonal anti-p-MARCKS (Ser152/156) was from PhosphoSolution (Aurora, CO, USA). Mouse monoclonal anti-synaptophysin and rabbit polyclonal anti-spinophilin antisera were obtained from Chemicon International Inc. (Temecula, CA, USA). Antiactin antibody (rabbit polyclonal) was purchased from Sigma (St. Louis, MO, USA).

2.3.2. Electrophoresis and Western blotting procedures

Rats at all developmental ages were used for these studies. Hippocampal tissue homogenization and sample preparation were done as previously described [40]. Blots were incubated with specific primary antisera (anti-p-PKC pan, anti-p-PKCα, anti-p-PKCδ, anti-GAP-43, anti-p-GAP-43, anti-p-MARCKS, anti-spinophilin, anti-synaptophysin; all diluted 1:1000), and subsequently with the appropriate secondary antibodies conjugated with horseradish peroxidase (goat antirabbit and antimouse IgG, all diluted 1/3000; Bio-Rad, Milan, Italy). Immune complexes were visualized using an enhanced chemiluminescence Western blot analysis system (Amersham-Pharmacia, Milan, Italy). Blot images were then digitized (Chemidoc, Bio-Rad), and areas of all labeled bands were quantified using the computerized imaging system software (QuantityOne; Bio-Rad). After visualization of immunolabeled bands, nitrocellulose membranes were stripped for 30 min at 50°C with stripping buffer (62.5 mM Tris–HCl, pH 6.7, containing 10 mM β-mercaptoethanol and 2% SDS) and reprobed with antiactin antibody (1:200). In each series, relative optical densities (arbitrary units) were finally normalized for densitometric values obtained from actin-labeled bands (taken as control).

2.4. Immunohistochemistry

Rats (at P4 and P21) were anesthetized with sodium pentothal (45 mg/kg) and perfused transcardially with physiological saline followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB, 0.1 M; pH 7.4). Brains were postfixed in 4% PFA for 2–12 h. Thirty-micrometer-thick sections were cut on a Vibratome and collected in PBS. Free-floating sections were treated for 30 min in PBS containing 1% H_2O_2, preincubated

for 1 h in 10% nonimmune goat serum in PBS plus 0.2% Triton X-100 and then incubated overnight at 4°C in primary antibodies against spinophilin and synaptophysin (1:500 and 1:1000 in PBS, respectively). The next day, sections were incubated for 1 h in the appropriate biotinylated secondary antibodies (antirabbit and goat antimouse IgG; Vector Lab, Burlingame, CA; 1:200 in PBS), and processed according to the avidin–biotin peroxidase complex procedure (Vector; PK-6100; 30 min). Finally, the reaction product was demonstrated by 3′,3-diaminobenzidine tetrahydrochloryde (DAB; 40 mg/50 ml) with 0.03% hydrogen peroxide.

2.5. Electrophysiological experiments

Male pups from different CTRL and TREAT litters, aged from P14 to P21, were used. On the same experimental day, two aged-matched pups, one born to CTRL and one from TREAT dams, were chosen to obtain slices to perform electrophysiological recordings in CA1 subfield of hippocampus.

Six hundred-micrometer-thick brain slices were prepared as previously described [41]. Field potential and whole-cell patch clamp recordings were carried out. All recordings started following a slice equilibration period in the recording chamber. Electrophysiological data analysis was carried out offline using a WinWCP software (Strathclyde electrophysiology software whole-cell analysis V 3.2.9, John Dempster, University of Strathclyde, UK).

2.5.1. Field potential recordings

To test the effects of maternal α-T supplementation on synaptic plasticity in the Schaffer collateral-CA1 pathway, basal synaptic transmission (input/output curves), paired-pulse facilitation and the ability to elicit LTP in the abovementioned pathway were examined. Recording and bipolar stimulating electrodes were prepared and filled with artificial cerebrospinal fluid (ACSF) as previously described [41]. Recording electrode was placed in the *stratum radiatum* of CA1 subfield and bipolar stimulating electrode was positioned in *stratum radiatum*, approximately 300 μm from the recording electrode.

Recordings were performed on slices giving extracellular field excitatory postsynaptic potentials (fEPSPs) of at least 1 mV in amplitude. Input/output curves were obtained applying to the slice square pulses of current (500 μs in duration) with A385 stimulus isolator (World Precision Instruments, USA). To test for paired-pulse facilitation (PPF), two stimuli were delivered at interpulse interval of 50 ms at the recording stimulation intensity eliciting 50% of the maximum response. PPF ratios were calculated by measuring the peak amplitude of the fEPSP evoked by each stimulus and taking the ratio of the second to the first. Then, baseline responses were elicited using low-frequency test pulses (at 30-s intervals) and recorded at approximately 50% of the maximum response over 20–60 min, a period sufficient to ensure stability.

To investigate the capability to elicit LTP in CA1, after a stable baseline had been obtained, Schaffer collateral was stimulated using stimulus pattern including 10 trains of 100 Hz applied for 0.1 s separated by an interval of 0.2 s; the fEPSP was then recorded for 30 min. The ability to induce and maintain LTP in the same pathway was also assessed in 400-μm-thick brain slices of P60-90 rats from different CTRL and TREAT litters, by using the same stimulus pattern repeated for three times 5 s apart. Slope (between 10% and 80% of max) of the fEPSP was analyzed as measures of synaptic strength; values were normalized to the mean value obtained over the last 20 min of the baseline period and expressed as a percent of this baseline value.

2.5.2. Patch clamp recordings

To test the effects of maternal supplementation on the electrophysiological properties of CA1 pyramidal cells, patch clamp recordings in whole-cell configuration were carried out under visual guidance as previously described [41]. Patch clamp electrodes were filled with internal solution containing (in millimolars) 126 potassium gluconate, 8 NaCl, 0.2 EGTA, 10 HEPES, 3 Mg_2ATP, 0.3 GTP; pH=7.2; 290 mOsm) and biocytin (Sigma; 0.2%), for subsequent determination of cell morphology. Electrode resistance in the bath ranged from 3 to 5 MΩ. No correction was made for junction potential between internal and external solutions.

Somata were selected to be recorded based on their pyramidal shape. Resting membrane potential (RMP), input resistance (IR) and capacitance (C), and cell excitability were determined as previously described [41]. The amplitude of the first elicited spike and the number of spikes obtained applying a current step leading cell membrane potential at approximately −35 mV along with stimulus duration (300 ms) were evaluated.

A bipolar stimulating electrode filled with ACSF solution was placed in *stratum radiatum* approximately 300 μm from the recording cell. Postsynaptic currents (PSCs) were evoked as previously described [41]. Spontaneous PSCs were also recorded for approximately 5 min and mean frequency was calculated. During PSC recordings, cells were held at a membrane potential of −70 mV, if not otherwise specified.

Recordings were rejected if the initial series resistance was >30 MΩ, if the series resistance measured at the end of the experiment had changed (±5 MΩ) or if DC offset exceeded 5 mV after withdrawal from the cell.

2.6. Biocytin detection and morphological analysis

Patch clamp recorded slices were fixed with PFA (4% in PBS) overnight at room temperature, and biocytin, injected by patch recording electrode, was revealed as previously described [41]. Morphological reconstruction of each stained pyramidal cell was performed using a Leica TCS-SL confocal microscope equipped with Argon and He/Ne laser sources. Morphological analysis was carried out on a subset of reconstructed neurons showing no clear dendritic cutting at the slice surface. The total length of pyramidal cell dendrites was evaluated using the image analysis software NeuronJ. Scholl concentric ring analysis was adopted in order to estimate dendrite arborization; concentric rings were superimposed to reconstructed cells and dendritic crossings were counted along both the basal (negative number) and apical (positive number) dendrites.

Table 1
Comparison of postnatal ponderal growth in offspring from α-T-supplemented and unsupplemented mothers

	Offspring body weight (g)			
	P0	P7	P14	P21
CTRL	7.8±0.8	12.9±2.1	32.8±7.3	45.6±8.3
TREAT	6.1±0.7	14.8±3.2	37.2±6.8	48.8±6.9

Rats born to α-T-supplemented mothers did not show significant differences in their ponderal growth as compared to offspring from normally fed dams.

2.7. Morris water maze

P60-90 CTRL and TREAT male rats were tested for spatial learning using the paradigm of Morris water maze. The apparatus consisted of a black circular swimming pool 1.65 m wide and 60 cm high, half-filled with water at temperature of 21±1°C, in which an escape black platform 10 cm in diameter was placed, submerged 1.5 cm below the water surface and kept in the same position at 15 cm from pool wall. To guide rat escape behavior, three different extramaze cues were used. A pretraining session without platform was carried out to accustom the rat to water. Then, the training protocol was applied consisting of two sessions per day (8 a.m. and 2 p.m.) of four trials each (60 s with intertrial time of 60 s), over two consecutive days. The time taken to reach the platform (escape latency) and the path length covered by the rats to get to the platform were recorded (Video tracking system; Smart-BS, 2biological Instruments, Italy) and considered as parameters to evaluate learning. Moreover, in order to obtain a more reliable measure of learning, the day after the end of training, rats were tested for probe task by removing the platform from the pool and tracking the swim path for 1 min. To evaluate the result of this test, the maze was virtually divided in four quadrants, one of which was centered on the position formerly occupied by the platform (goal quadrant); the time spent in the goal quadrant as compared to that in the opposite one was calculated.

2.8. Statistical analyses

Student's *t* test, mixed-factor ANOVA test or one-way ANOVA test was appropriately applied to analyze the results obtained in TREAT vs. CTRL rats; $P=.05$ was considered the significance threshold for each statistical test.

3. Results

Based on the average amount of pelleted food consumed by supplemented rats (20 g/day), the estimated daily intake of α-T was around 300 mg (1000 mg/kg/day). α-T-supplemented dams did not show differences in ponderal growth curve as compared to unsupplemented rats. Survival rate, weight of the pups and litter size were also unaffected by maternal α-T load, and no obvious teratogenic effects were observed in TREAT newborn. Lactating pups showed no significant intergroup differences in ponderal growth (Table 1), as well as in the timing of major maturational

Table 2
α-T accumulation in offspring hippocampus and liver

[α-T] ng/mg protein

	Hippocampus		Liver	
	CTRL	TREAT	CTRL	TREAT
P0	14.6±1.3	29.4±2.6 *	8.2±0.6	69.1±2.4 *
P21	17.1±0.9	36.4±1.4 *	331.1±57.7	4314.4±185.3 *
P60	23.3±0.4	27.6±0.5	396.7±62.1	589.2±93.4

Changes in hippocampal and hepatic α-T content in offspring (at P0, P21 and P60–90; at each time point, for each group, $n=8$ pups were sampled from four different litters) in response to maternal supplementation with α-T. Student's *t* test: *$P<.05$.

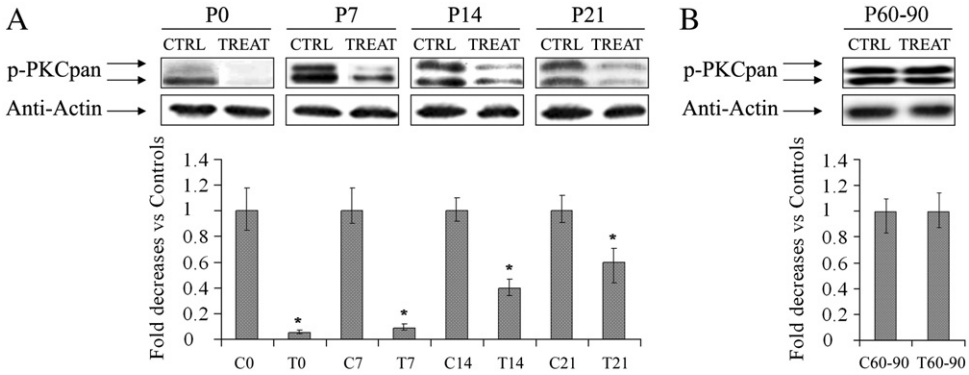

Fig. 1. Effect of α-T maternal supplementation on PKCpan phosphorylation in the hippocampus of developing and adult offspring. Hippocampal protein extracts taken from CTRL and TREAT developing and adult offspring (at each time point, for each group, $n=8$ animals from four different litters) were subjected to SDS/PAGE (12% polyacrylamide) followed by Western blotting using polyclonal phosphospecific antibody directed to PKCpan. Antiactin blot is shown as loading control. Representative blots of PKCpan phosphorylation during postnatal development (A) and in adult progeny (B) are displayed. In A and B, histograms represent densitometric analyses of blots from three independent experiments (means±S.D.). Relative decreases in band absorbance values (arbitrary units) were normalized for the control band in each series. Student's *t* test: *$P<.05$.

steps, that is, hair coat development, eye opening and onset of movement control; neither obvious abnormalities were observed in older pups.

3.1. α-T accumulation in offspring tissues

α-T content in hippocampus and liver of pups at P0 (day of birth) and P21 (end of lactation) was measured by HPLC analysis. Results (Table 2) showed that in TREAT newborn, hippocampal α-T concentration doubled that of CTRL pups; such increase remained substantially unchanged at P21. More remarkable differences in α-T incorporation were observed in liver, where α-T concentration in TREAT pups was approximately eightfold higher than in CTRL at P0 and 13-fold higher at P21. These differences in α-T uptake in both cerebral and hepatic tissues were missed in adult offspring (Table 2).

3.2. PKC activity is reduced in developing hippocampus of tocopherol-exposed pups

At all postnatal time points, immunoblots using antisera to p-PKCpan (that detect all kinase isozymes) revealed the presence of two separate bands migrating at approximately 78 and 85 kDa (Fig. 1A), suggesting that diverse PKC isozymes are activated at various phases of hippocampal maturation. Densitometric analyses revealed that the average intensity of p-PKCpan bands in TREAT pups was 5% of CTRL at P0, increased to approximately 10% at P7, raised to 40% at P14 and reached 60% at P21 (Fig. 1A). Differences in p-PKCpan intensity between TREAT and CTRL disappeared in adult offspring (Fig. 1B).

In the same hippocampal homogenates, immunoblot analyses were performed to visualize the phosphorylated forms of PKCα (p-PKCα; a conventional, Ca^{2+}-dependent isozymes) and PKCδ (p-PKCδ; a novel, Ca^{2+}-independent isoform). For both isozymes and at all time points, immunoblots revealed the presence of a single band with apparent molecular weight of 80 and 78 kDa for α and δ isoforms, respectively (Fig. 2A and B). Densitometric analyses revealed that changes of p-PKCα bands followed a developmental pattern similar to p-PKCpan: in TREAT pups, signal was around 10% of CTRL at P0, 17% at P7, 40% at P14 and 55% at P21 (Fig. 2A). For p-PKCδ, differences between TREAT and CTRL were even more remarkable in the first postnatal week, when signal was virtually absent in TREAT pups (P0 and P7 in Fig. 2B), and became much narrower at P14 (80% in TREAT respect to CTRL) and P21 (72%) (Fig. 2B).

3.3. Phosphorylation of PKC substrates is reduced in developing hippocampus of tocopherol-exposed pups

Immunoblotting analyses were performed to detect phosphorylated forms of GAP-43 (p-GAP-43) and MARCKS (p-MARCKS). At all time points, immunoblots for the two antigens (Fig. 3A and B)

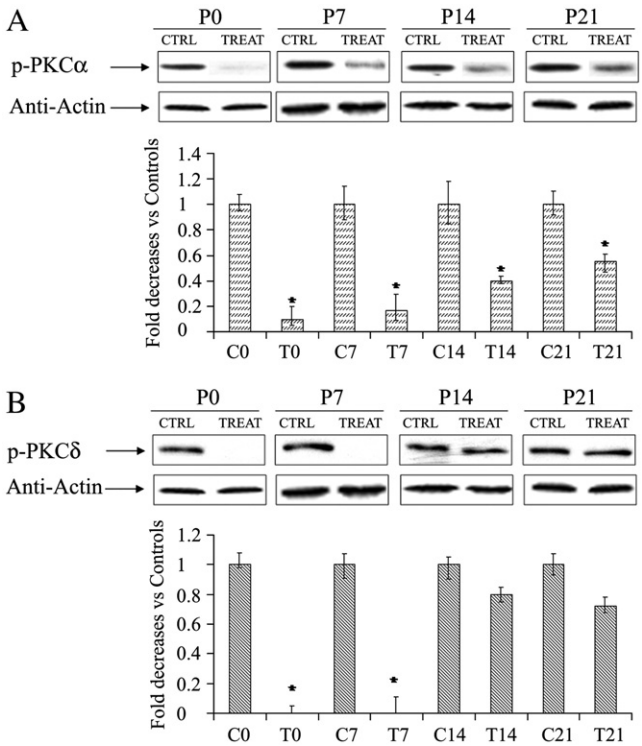

Fig. 2. Effect of α-T maternal supplementation on PKCα and PKCδ phosphorylation in offspring's developing hippocampus. Protein extracts from developing hippocampi of CTRL and TREAT pups (at each time point, for each group, $n=8$ pups from four different litters) were subjected to SDS/PAGE (12% polyacrylamide) followed by Western blotting using polyclonal phosphospecific antibodies directed to PKCα and PKCδ isozymes. Antiactin blots are shown as loading control. Representative blots of PKCα and PKCδ phosphorylation are displayed in A and B, respectively. In A and B, histograms represent densitometric analyses of blots from three independent experiments (means ±S.D.). Relative decreases in band absorbance values (arbitrary units) were normalized for the control band in each series. Student's *t* test: *$P<.05$.

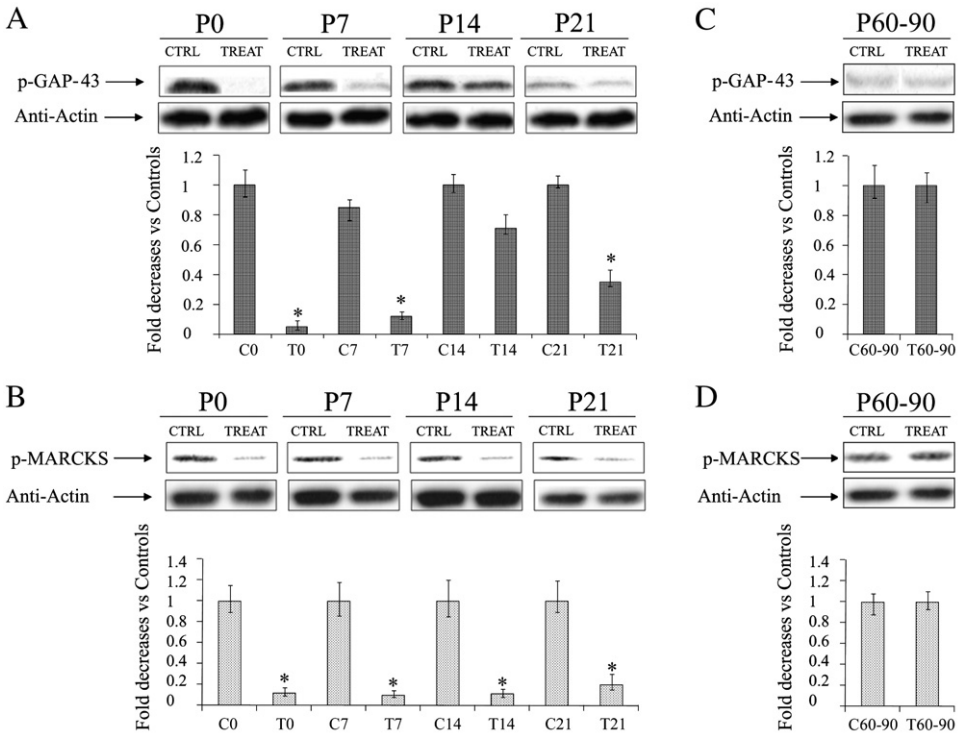

Fig. 3. Effect of α-T maternal supplementation on phosphorylation of PKC substrates GAP-43 and MARCKS in the hippocampus of developing and adult offspring. Hippocampal protein extracts taken from CTRL and TREAT developing and adult offspring (at each time point, for each group, n=8 animals from four different litters) were subjected to SDS/PAGE (12% polyacrylamide for GAP-43 and 7% polyacrylamide for MARCKS) followed by Western blotting using polyclonal phosphospecific antibodies directed to GAP-43 and MARCKS. Antiactin blots are shown as loading control. Representative blots of hippocampal GAP-43 phosphorylation are displayed in developing (A) and adult (C) progeny; immunoblots of MARCKS phosphorylation through postnatal development and in adulthood are shown in B and D, respectively. Histograms in A–D represent densitometric analyses of blots from three independent experiments (means±S.D.). Relative decreases in band absorbance values (arbitrary units) were normalized for the control band in each series. Student's t test: *P<.05.

revealed the presence of single bands with apparent molecular weight of 87 and 50 kDa for p-GAP-43 and p-MARCKS, respectively. For p-GAP-43, signal intensity was very low in TREAT rats at P0–P7 (5–12% of CTRL) and then raised steeply at P14 (reaching 71% of CTRL), to decrease again at P21 (35%) (Fig. 3A). For p-MARCKS, phosphorylation levels were severely reduced in TREAT pups throughout hippocampal development, with intensity of labeled bands ranging between 10% and 20% of CTRL (Fig. 3B). For both p-GAP-43 and p-MARCKS, differences between the two groups vanished in adulthood (Fig. 3C and D).

3.4. Hippocampal synaptic plasticity is impaired in tocopherol-exposed pups

3.4.1. Field synaptic responses

Basal synaptic transmission in Schaffer collateral-CA1 pathway was not different in TREAT pups as compared to CTRL. The dendritic field potential responses to increasing intensity stimuli of the Schaffer collateral were essentially identical in the two groups; the relationship between the fiber volley amplitude and the stimulus intensity showed that a given intensity stimulus activated approximately the same number of fibers in both groups (Fig. 4A). The fEPSP slope vs. stimulus intensity (Fig. 4B) also displayed no significant intergroup difference. Finally, PPF ratios measured from the Schaffer collateral-CA1 pathway were not altered in TREAT pups compared to CTRL (PPF ratio calculated as percentage of second-to-first peak ratio; TREAT: 174.4±14.1, n=7 pups from different litters, seven slices; CTRL: 170.2±2.8, n=6 pups from different litters, 6 slices).

On the contrary, α-T supplementation affected LTP; in fact, the fEPSP slope post-high frequency stimulation was significantly reduced in TREAT pups in comparison to CTRL (Fig. 5A). The acquired potentiation was maintained unchanged for 30 min in both groups, thus indicating that α-T did not influence LTP maintenance. The ability to elicit LTP was evaluated also in adult offspring: interestingly,

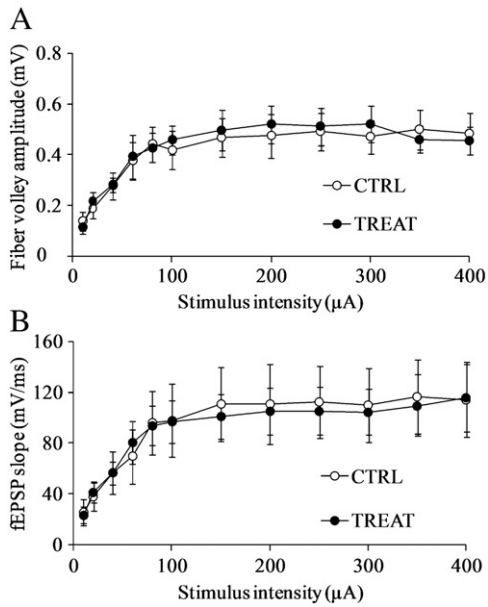

Fig. 4. Dendritic field potential responses at the Schaffer collateral to CA1 stratum radiatum. CTRL (n=5 pups from different litters, five slices) vs. TREAT (n=5 pups from different litters, five slices) animals: (A) relationship between the fiber volley amplitude and the stimulus intensity; (B) fEPSP slope vs. stimulus intensity relationship. Values are expressed as mean±S.E.M.

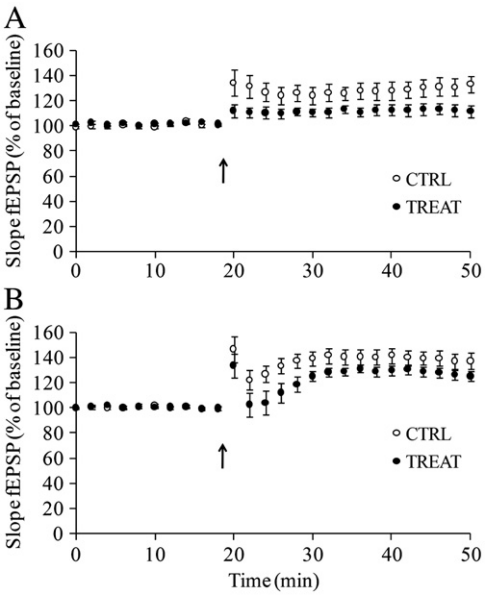

Fig. 5. α-T supplementation is associated to LTP impairment in both developing (A) and adult (B) offspring. Arrows indicate the stimulus application (see text). Slope (between 10% and 80% of max) of the fEPSP was analyzed as measures of synaptic strength; values were normalized to the mean value obtained over the last 20 min of the baseline period and expressed as a percentage of this baseline value. Mixed-factor ANOVA test: in A, P=.043 CTRL (n=6 pups from different litters, six slices) vs. TREAT (n=7 pups from different litters, seven slices); in B, P=.041 CTRL (n=10 rats from different litters, 10 slices) vs. TREAT (n=10 rats from different litters, 10 slices).

LTP remained significantly affected (Fig. 5B) in early exposed rats compared to CTRL, despite the time elapsed from the suspension of α-T supplementation.

3.4.2. Whole-cell analysis

Electrophysiological characteristics of CA1 pyramidal neurons did not significantly differ in the two groups (Table 3); in fact, RMP and membrane passive properties (IR and C) of CA1 pyramidal cells did not result altered by α-T supplementation, and neither did neuron excitability nor spontaneous and evoked synaptic activity (Table 3).

3.5. Maternal tocopherol ingestion did not modify CA1 pyramidal cells morphology

The lack of electrophysiological differences of CA1 pyramidal neurons between TREAT and CTRL pups was paralleled by the absence of significant variations in cell morphology, as revealed by morphometrical analyses carried out on a subset of CA1 pyramidal neurons filled with biocytin during whole-cell recordings (Fig. 6A). Measurements of total dendritic length showed no significant difference between the two groups (5319.6±653.2 and 5308.9±516.6 μm for TREAT and CTRL; n=10 and n=9 neurons, respectively). Moreover, Scholl analysis revealed that the numbers of basal (negative numbers in Fig. 6B) and apical (positive numbers in Fig. 6B) dendritic crossings along the Scholl rings were highly similar in CA1 pyramidal cells of the two groups (Fig. 6B), thus indicating that maternal α-T supplementation did not influence the global complexity of dendritic arborization of hippocampal neurons.

Table 3
Analysis of the electrophysiological features of CA1 pyramidal neurons in juvenile offspring from α-T-supplemented and unsupplemented mothers

	CTRL (n=28)	TREAT (n=27)
RMP (mV)	−40.3±2.3	−43.7±2.0
IR (MΩ)	384.7±45.5	331.4±28.0
C (pF)	101.7±5.8	111.1±6.0
Threshold (mV)	−44.9±1.6	−43.7±1.3
Action potential amplitude (mV)	106.7±3.6	112.3±2.3
Number of action potentials	4.7±0.6	4.9±0.5
PSC amplitude (pA)	−104.1±19.5	−97.2±18.7
sPSC frequency (Hz)	3.1±0.3	2.1±0.4

Basic electrophysiological characteristics were unchanged in offspring from α-T-exposed mothers as compared to controls. Number of action potentials=number of spikes obtained applying a 300-ms current step leading cell membrane potential at approximately −35 mV from about −70 mV. CTRL=control; TREAT=supplemented. n=number of recorded CA1 pyramidal neurons in five pups from each group.

3.6. Developmental expression of synaptic and growth-related proteins is unaffected by maternal tocopherol supplementation

Immunoblotting studies were performed to detect developmental changes of synaptophysin and spinophilin (pre- and postsynaptic marker proteins, respectively), and total GAP-43, including both the phosphorylated and unphosphorylated form of the protein. At all time points, immunoblots showed single-labeled bands with apparent molecular weights of 38, 140 and 50 kDa for synaptophysin, spinophilin and GAP-43, respectively (Fig. 7). For all proteins, densitometric analyses revealed that intensity levels of immunolabeled bands were not significantly different between TREAT and CTRL pups (Fig. 7).

Immunohistochemical studies were performed at P14–P21 to investigate whether hippocampal LTP impairment was associated to concomitant variations of topographical distribution and cellular expression of synaptic markers. In line with immunoblots, intensity of synaptophysin- and spinophilin immunoreactivity

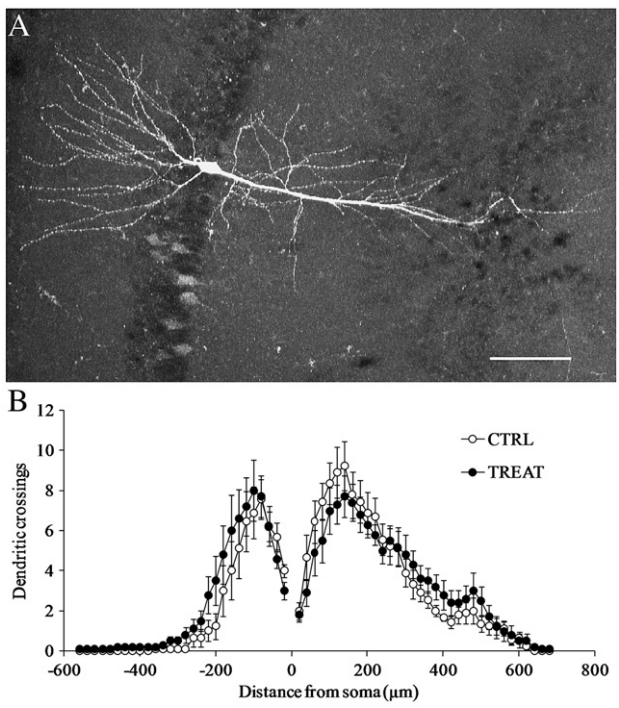

Fig. 6. α-T maternal supplementation does not affect neuronal morphology. (A) An example of CA1 pyramidal neuron processed for biocytin detection and reconstructed by confocal microscope, on which was carried out the morphological analysis; (B) Scholl analysis plot showing numbers of dendritic crossings along the Scholl rings as a function of distance from soma. Distributions of both basal and apical dendritic crossings do not result in significant differences between the two groups at mixed-factor ANOVA (basal: P=.41; apical: P=.96). CTRL: n=9 neurons; TREAT: n=10 neurons). Bar: 100 μm.

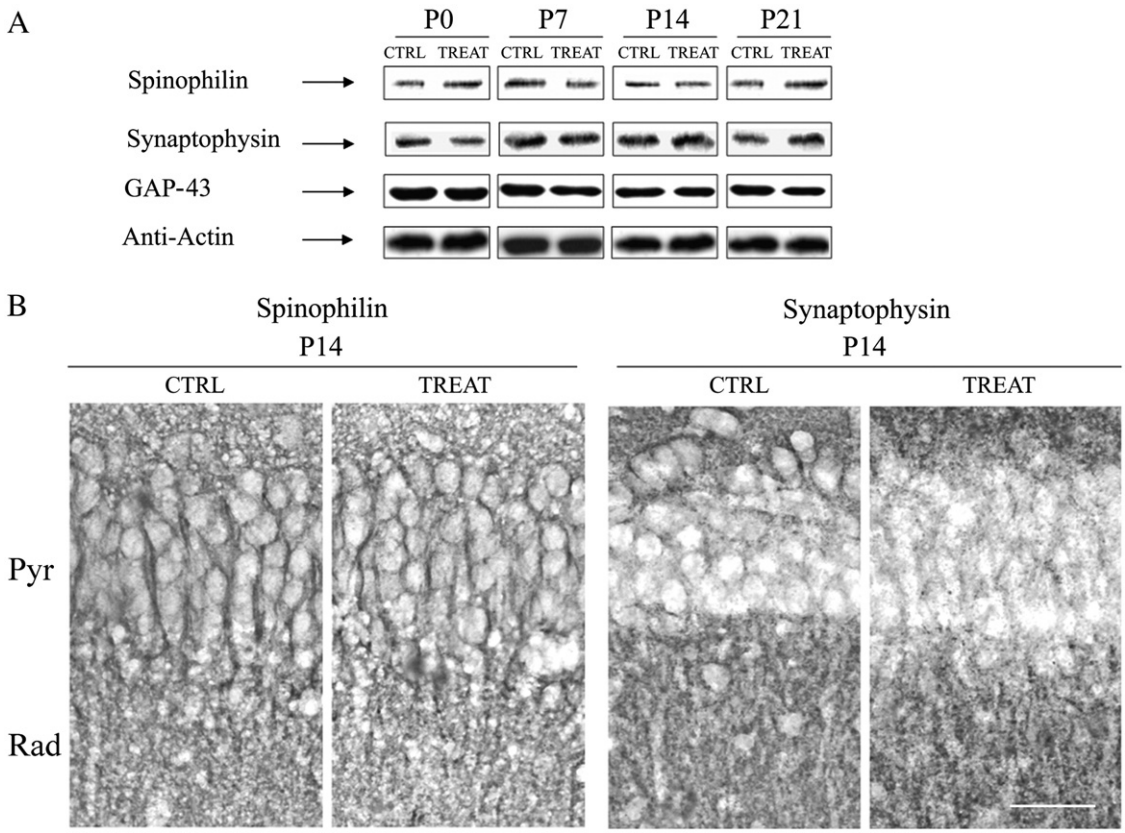

Fig. 7. Developmental expression of synaptic and growth-related proteins is unaffected by maternal tocopherol supplementation. Protein extracts from CTRL and TREAT offspring hippocampi (at each time point, for each group, $n=8$ pups from four different litters) were subjected to SDS/PAGE (12% polyacrylamide for synaptophysin and GAP-43, 7% polyacrylamide for spinophilin) followed by Western blotting using specific antibodies directed to spinophilin, synaptophysin and GAP-43. Antiactin blot is shown as loading control. (A) Representative blots of spinophilin, synaptophysin and GAP-43 at all postnatal points (from P0 to P21). (B) Spinophilin and synaptophysin immunoreactivity in CA1 sections taken from control and supplemented offspring at P14. Distribution and morphological features of immunolabeling show no differences between the two experimental groups (for each group, $n=2$ pups from each litter at P14 and P21): spinophilin immunoreactivity is associated to sparsely distributed puncta and to neuronal cell bodies and proximal dendrites; synaptophysin immunoreactivity stains punctate structures scattered in the neuropil and densely aggregated around somata and proximal dendrites of unlabeled neuronal profiles. *pyr*, pyramidal cell layer; *rad*, stratum radiatum. Bar: 50 μm.

failed to show obvious differences between TREAT and CTRL pups (Fig. 7). For both antigens, no remarkable difference was found in topographical and laminar distribution pattern and morphological features (Fig. 7).

3.7. Spatial learning is impaired in adult rats perinatally exposed to α-T

Figure 8 displays the learning curves for escape latency (A) and for path length (B) over the course of the 2-day training period, and performance on the probe test on Day 3 (C). In both groups of rats, the escape latency and path length became progressively shorter, showing an asymptotic trend; moreover, probe test revealed a clear preference for the quadrant in which platform was previously located, indicating that both TREAT and CTRL learned to navigate to the hidden platform. Nonetheless, TREAT rats performed significantly worse than CTRL in this task: in fact, evaluating performances in the first trial of every session, as an index of long-lasting memory, significant intergroup differences appeared on the second session of the training regarding both escape latency and path length (Fig. 8A and B). These differences in long-lasting memory were better evaluated by probe testing (Fig. 8C) showing a significantly lesser swimming time spent in the goal quadrant by TREAT rats in comparison to controls. These findings indicate that TREAT rats needed longer time to develop a precise spatial preference for the goal.

4. Discussion

The present work provides the first in vivo evidence that maternal exposure to elevated doses of α-T during pregnancy and lactation can affect PKC-mediated cellular signaling and synaptic plasticity in offspring's developing brain and exert permanent adverse effects on neurophysiological and cognitive functions in adult progeny.

Previous reports in experimental pregnancy have shown that supplemental doses of maternal VitE intake needed to induce protective effects on offspring development are elevated: in rats, the efficacy of tocopherol supplementation in reducing embryo malformations in gestational diabetes is dose-dependent, with 400–500 mg/day [5,7] and 2–15% VitE in diet [6,8] being the most effective; more recently, protective effects were described in diabetic embryopathy with maternal tocopherol intake of 1000–1200 mg/kg per day [11], and similar levels of vitamin intake through pregnancy and lactation were proved effective in reducing brain atrophy and DNA damage in postnatal offspring of ethanol-exposed mothers [14]. In our supplementation protocol, rats were given food enriched with 1.5% α-T, thus consuming a daily tocopherol amount of about 300 mg (1000 mg/kg/day), that is, a tocopherol intake similar to that proposed in prevention studies. Supplementing dams with such elevated doses of α-T may thus represent a valid model to ascertain whether supranutritional loads of maternal VitE consumption, which reportedly normalize offspring development

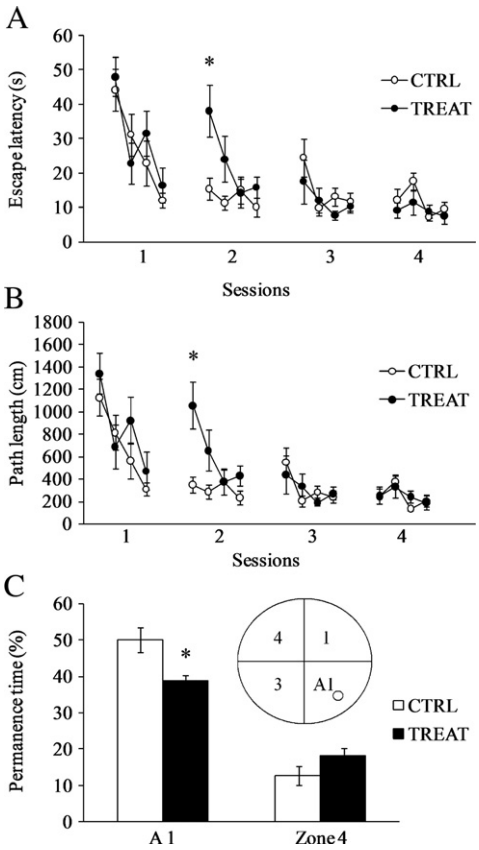

Fig. 8. Spatial learning is permanently affected by maternal α-T supplementation. Evaluation of spatial learning in CTRL (n=10 from different litters) and TREAT (n=10 from different litters) rats. (A) Escape latency calculated for each trial of every session; each point represents the mean value (±S.E.M.) of all animals used. One-way ANOVA test: for CTRL, P<.001; for TREAT, P<.001. Student's t test: *P=.011 vs. the corresponding point obtained in controls. (B) Path length measured for each trial of every session; each point represents the mean value (±S.E.M.) of all animals used. One-way ANOVA test: for CTRL, P<.001; for TREAT, P<.001. Student's t test: *P=.005 vs. the corresponding point obtained in controls. (C) Water maze probe test performed the day after the end of training assessed as permanence time of the animal in the goal quadrant (A1) relative to the opposite one (Zone 4); Student's t test: *P=.007 vs. the corresponding controls.

in complicated pregnancy, may have adverse effects on offspring from normal pregnancy.

Concerns on the fetal safety of maternal exposure to elevated doses of α-T have emerged from both human and animal studies. Clinical trials, besides proving the inefficacy of VitE supplementation as a prevention strategy in gestational disorders [10,12], mostly found no clear association between gestational VitE supplementation and the rate of fetal malformations, preterm birth, and neonatal or perinatal death [9,17]; however, an increased rate of babies born with a low birth weight has been consistently observed [10,12,17], and major congenital heart defects have been reported at least in one case-control study [19]. In rodents, the teratogenic role of supranutritional VitE consumption was suggested only for tocopherol intakes 10-fold higher than in our study (15% tocopherol in diet), leading to facial dysmorphogenesis in a limited number of cases [8]. In our model, long-term maternal exposure to elevated doses of α-T did not produce any obvious teratogenic effects: litter size, survival rate and weight of the pups were not modified by maternal supplementation, and no malformations were observed in newborn pups from α-T-exposed mothers. These findings seem to indicate that, in rats, high maternal VitE intake is relatively harmless for the fetus and are in good agreement with previous evidence showing that maternal doses of α-T up to 2250 mg/kg per day do not affect birth weight and do not increase the rate of fetal malformations [5,18].

Here we show that offspring of supplemented mothers display a remarkable increment of α-T tissular incorporation already at birth: in newborn rats, tocopherol accumulation in hippocampus and liver was increased by two- and eight-fold, respectively, compared to that detected in control pups. These findings suggest that levels of maternal α-T dietary intake positively correlate with the rate of tocopherol placental transfer from mother to fetus. Present observations are only partially in line with previous reports, which provide a rather conflicting scenario. Studies in different mammalian species, including humans, have proposed that net placental transfer is low and barely influenced by maternal VitE intake [42,43]; on the contrary, but in better agreement with present results, it has been shown that VitE levels in amniotic fluid at delivery closely correlate with maternal plasma vitamin levels in supplemented women [44], and that fetal and maternal concentrations of α-T in rodents are positively correlated and vary as a function of maternal intake [45,46]. Such discrepancies may conceivably reflect the heterogeneity of experimental models and supplementation protocols used in various reports. The range of supplemental doses used in the literature is considerably wide, thus possibly leading to important differences in tocopherol fetal transfer. Here, dams were fed with relatively high α-T doses: similar levels of tocopherol intake have been reported to cause massive tocopherol increase in maternal plasma [5,18] and an increment of α-T concentrations in embryonic tissues comparable to that presently observed in neonatal brain [5]. In addition, it is worth noting that the natural form of α-T, RRR-α-T, showing the highest biological activity in terms of both bioavailability and biopotency [47], is considered as the isomer preferentially transferred through the placenta [43,46]. Therefore, the choice of supplementing dams with elevated doses of RRR-α-T might have strengthened α-T transfer to fetal tissues in our experimental model.

At the end of lactation, hepatic levels of α-T incorporation appear dramatically increased in offspring from both supplemented and nonsupplemented mothers, with differences between the two groups larger than at birth; in contrast, brain α-T concentrations show only a slight increment after lactation, with intergroup differences at P21 similar to those observed at P0. By revealing a high hepatic-to-cerebral ratio of α-T incorporation in developing rats, such findings are in line with recent evidence in adult rats showing that infusions of pharmacological doses of α-T lead to much larger tocopherol accumulation in liver with respect to extrahepatic tissues [48].

α-T inhibitory effect on PKC activity has been documented in different cellular models in vitro, including neural cells [21–24,40], and confirmed in vivo in the hippocampus of α-T-supplemented adult rats [35]. The present results show that supranutritional α-T intake in pregnant and lactating rats strongly reduces PKC phosphorylation in offspring's developing hippocampus. Interestingly, while hippocampal α-T concentrations do not change relevantly after birth, levels of PKC inhibition gradually decrease through development, thus revealing that PKC susceptibility to α-T follows a decremental pattern during brain maturation, being highest at early postnatal phases and then lowering with time. Since α-T supplementation in adult rats virtually abolishes hippocampal PKC phosphorylation [35], thus showing that α-T inhibition of PKC activity resumes to its highest levels in mature brain, our findings point to major differences between adult and immature brain in responding to α-T and call for using appropriate, age-related models to assess biological effects of exogenous compounds in developing CNS.

Consistent with PKC inhibition, phosphorylation levels of MARCKS and GAP-43, two PKC substrates coupling PKC signaling to plastic changes in cellular structure and motility [49,50], were reduced throughout postnatal development in α-T-exposed pups. Interestingly, developmental variations of MARCKS and GAP-43

phosphorylation levels follow temporal profiles similar to those of PKCα and PKCδ activity, respectively. A certain degree of isozyme-related specificity may thus occur in the activation of the two PKC-substrates in vivo, with a prominent role for calcium-dependent PKC isozymes in phosphorylating MARCKS and for calcium-independent kinases in regulating GAP-43 activity. Although evidence from molecular and cellular studies have suggested that both MARCKS and GAP43 can be phosphorylated by different classes of PKC isozymes [51,52], present data are in accordance with studies in vitro showing a strong correlation between MARCKS translocation and PKCα activation in human neuroblastoma cells [53] and proposing PKCδ as prominently involved in GAP-43 phosphorylation [54].

PKC and its substrates play important roles in neuronal development and plasticity. PKC activity controls neural cell proliferation, differentiation, and migration induced by a number of neuromodulators and growth factors [29,31] and regulates activity-related developmental circuit refinement through neuronal plasticity processes, such as neuritogenesis, synaptogenesis, dendritic branching and spine formation [26–28,30–32,55–57]. PKC-dependent GAP-43 phosphorylation is involved in cell growth, neurite outgrowth, synaptic remodeling and axonal guidance [49,58], and GAP-43 knockout mice show disrupted cortical maps [59]. MARCKS expression is developmentally regulated in association with neuronal migration, cortical lamination, process outgrowth and synaptic maturation [60], while MARCKS-deficient mice exhibit decreased brain size and lamination abnormalities in neocortex and hippocampus [61]. Since morphological, physiological and neurochemical maturation of rodent hippocampus is virtually complete only after P14 [62], developmental deviations possibly induced by PKC activity dysregulation should be fully expressed in the third week. At this time, however, hippocampal processes of neuronal morpho-functional maturation, as well as synapse formation and targeting appear unaffected by maternal α-T supplementation in spite of concomitant, remarkable inhibition of PKC and PKC-substrate activity.

Resolving this apparent inconsistency is not a trivial task. In rat CNS, levels of PKC activity and PKC-substrate phosphorylation are reportedly low at birth and then increase with maturation [63]. Since α-T inhibition of PKC phosphorylation is strongest in the first week of life, when the role of PKC-mediated signaling is supposed to be minor, the lack of PKC activity in early postnatal brain could be readily taken over by alternative, vicarious signaling pathways. Moreover, it has been shown that GAP-43 can control growth-cone motility and extension independently from its phosphorylation [64]. Present finding that maternal α-T ingestion does not affect developmental expression of GAP-43 suggests that plasticity-related functions of unphosphorylated GAP-43 may well be retained in our model. In addition, both MARCKS and GAP-43 can be targeted and activated by different signaling factors other than PKC [50,65]. Since our antisera selectively recognize MARCKS and GAP-43 phosphorylated at the PKC site, alternative PKC-independent activation of these substrates would go undetected.

A key finding in this study is that α-T maternal supplementation reduces the efficiency of long-term synaptic plasticity in offspring juvenile hippocampus. PKC activity has been proposed as a major coordinator for processes underlying activity-dependent synaptic modifications [66,67]. Moreover, accumulating evidence indicates that LTP requires F-actin cytoskeletal assembly–disassembly dynamics, thus calling for an involvement of GAP-43 and MARCKS in synaptic plasticity [68]. LTP disruption observed in α-T-exposed pups may thus be accounted for, at least in part, by the inhibition of PKC and PKC-substrate activity. Concomitant with LTP impairment, in fact, we found a strong reduction in phosphorylation levels of Ca^{2+}-dependent PKCα isoform, whose activation was proposed as an early event in LTP induction in Schaffer collateral-CA1 pathway [69], and MARCKS, whose function is thought to regulate activity-driven plastic changes in dendritic spines [70].

Although underlying mechanisms are poorly understood, early life events producing subtle changes in brain maturation can give rise to persistent behavioral and cognitive deviations [71], and the hippocampus, given its long-spanned period of maturation, may be especially vulnerable to perinatal insults [72,73]. Here we found that adult rats born to α-T-supplemented mothers show reduced performances in Morris water maze, a hippocampus-dependent spatial learning task, as compared to age-matched controls. Although both groups of animals do acquire the task at the end of training, escape latency and path length display significant intergroup differences, and α-T-exposed rats spend lesser swimming time in the goal quadrant after platform removal. These findings clearly indicate that spatial memory performances are impaired in maternally supplemented rats long after suspension of α-T exposure, when hippocampal tocopherol concentration, as well as PKC and PKC-substrate phosphorylation had recovered to control levels. Consistently, we found that maternally exposed adult rats exhibit a marked reduction of hippocampal LTP, thus indicating that the impairment in long-term synaptic plasticity observed in juvenile offspring persists in adulthood. Taken together, present evidence suggests that early loads of α-T, by influencing brain developmental processes, may promote permanent dysfunctions in the hippocampal circuitry, thereby adversely affecting activity-driven neuronal plasticity and hippocampus-dependent, long-lasting learning ability in adult progeny.

In conclusion, the lack of obvious teratogenic effects does not rule out the possibility that massive maternal intake of VitE may promote slight and delayed alterations in offspring development. In this context, the present data show that supranutritional tocopherol intake in pregnant and lactating rats may indeed influence neural cell signaling and affect neurophysiological and cognitive functions in offspring, though in the absence of significant modifications of birth weight and postnatal ponderal growth. Interestingly, it has been recently reported that neonatal cord blood VitE status is positively correlated with higher cognitive and behavioral scores in 2-year-old children [74], thus suggesting the possibility, also in humans, of a potential association between antioxidant vitamin status during pregnancy and subsequent cognitive and behavioral development in offspring. Although extrapolation from rats to humans is always difficult and fraught with errors, present findings emphasize the need to carefully evaluate the safety of developmental exposures to high doses of α-T in humans, with particular focus on possible delayed, long-term effects on neurocognitive and behavioral functions.

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