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Effects of gestational thiamine-deprivation and/or exposure to ethanol on crucial offspring rat brain enzyme activities

Vasileios Stolakis[a,b], Charis Liapi[a], Hussam Al-Humadi[a,c], Konstantinos Kalafatakis[b,d,e], Vasiliki Gkanti[b], Alexios Bimpis[a], Nikolina Skandali[a,b], Smaragda Tsela[a,b], Stamatios Theocharis[f], Apostolos Zarros[b,c,g] and Stylianos Tsakiris[b]

[a]Laboratory of Pharmacology, Medical School, National and Kapodistrian University of Athens, Athens, Greece; [b]Laboratory of Physiology, Medical School, National and Kapodistrian University of Athens, Athens, Greece; [c]College of Pharmacy, University of Babylon, Hillah, Iraq; [d]Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, School of Clinical Sciences, Faculty of Medicine and Dentistry, University of Bristol, Bristol, UK; [e]Department of Informatics & Telecommunications, School of Informatics & Telecommunications, University of Ioannina, Ioannina, Greece; [f]First Department of Pathology, Medical School, National and Kapodistrian University of Athens, Athens, Greece; [g]Institute of Cancer Sciences, University of Glasgow, Glasgow, Scotland, UK

ABSTRACT

Objective: The fetal alcohol spectrum disorder (FASD) is a group of clinical conditions associated with the *in utero* exposure to ethanol (EtOH). We have recently examined the effects of a moderate maternal exposure to EtOH on crucial brain enzyme activities in offspring rats, and discussed the translational challenges arising when attempting to simulate any of the clinical conditions associated with FASD.

Materials and methods: In this current study, we: (i) address the need for a more consistent and reliable *in vivo* experimental platform that could simulate milder cases of FASD complicated by simultaneous thiamine-deprivation during gestation and (ii) explore the effects of such a moderate maternal exposure pattern to EtOH and a thiamine-deficient diet (TDD) on crucial enzyme activities in the offspring rat brains.

Results: We demonstrate a significant decrease in the newborn and 21-day-old offspring body and brain weight due to maternal dietary thiamine-deprivation, as well as evidence of crucial brain enzyme activity alterations that in some cases are present in the offspring rat brains long after birth (and the end of the maternal exposure to both EtOH and TDD).

Conclusions: Our findings provide a preliminary characterization of important neurochemical effects due to maternal exposure to EtOH and TDD during gestation that might affect the offspring rat neurodevelopment, and that characterization should be further explored in a brain region-specific manner level as well as through the parallel examination of changes in the offspring rat brain lipid composition.

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Acetylcholinesterase (ATPase); ethanol; fetal alcohol syndrome; rat brain; thiamine-deprivation

Introduction

The fetal alcohol spectrum disorder (FASD) is a group of clinical conditions associated with the *in utero* exposure to ethanol (EtOH) [1–3]. Patients diagnosed with some form of FASD might present with severe, mild, or even late-onset symptomatology, depending on the length, extent and timing of the maternal exposure to EtOH during pregnancy [1,3–6], as well on a number of other environmental and genetic factors [2,3]. As EtOH can easily penetrate both the blood–placenta barrier (BPB) and the blood–brain barrier (BBB), its toxic effects during neurodevelopment can be guaranteed to be both structural and functional [2,7–10]: EtOH has been reported to affect optimal neurodevelopment, cause apoptotic neuronal death, disrupt neuronal migration, and deregulate glial function during critical periods for neurogenesis, neuronal proliferation, and myelination [2,4], and as such, it has been linked to the development of neuroanatomical and cognitive deficits that vary both in severity and onset [1,2].

In a recent study of ours [11], we examined the effects of a moderate maternal exposure to EtOH on crucial brain enzyme activities in offspring rats, and discussed the translational challenges arising when attempting to simulate any of the clinical conditions associated with the, admittedly, broad FASD. Among the latter, severe forms of FASD such as the fetal alcohol syndrome (FAS) are probably the easiest to simulate at the experimental level, while milder FASD conditions are

CONTACT Stylianos Tsakiris stsakir@med.uoa.gr, stsakir@gmail.com Laboratory of Physiology, Medical School, National and Kapodistrian University of Athens, 75 Mikras Asias Street, Athens, Greece

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more rarely studied despite their higher clinical occurrence [12]. Our recently employed approaches aimed at: (i) addressing the need for a more consistent and reliable *in vivo* experimental platform that could simulate these milder cases of FASD and (ii) exploring the effects of such moderate maternal exposure patterns to EtOH on important neurochemical parameters of the offspring rats, in a brain region-specific manner [11]. The first goal was achieved by implementing an experimental setting that allowed for the comparative assessment of the developmental neurotoxicity of EtOH when administered in the drinking water (at a 10% v/v concentration) during gestation alone or throughout both gestation and lactation; an experimental setting designed to simulate two significantly different clinical situations in terms of developmental neurotoxicity: that of a moderate maternal exposure to EtOH during the first trimester and halfway through the second trimester, and that of a moderate maternal exposure to EtOH throughout a 9-month pregnancy and beyond (lactation during early postnatal life), respectively [11]. The second goal was achieved by studying the activities of acetylcholinesterase (AChE) and two important adenosine triphosphatases (Na^+,K^+-ATPase and Mg^{2+}-ATPase) in the homogenates of important brain regions of the 21-day-old offspring of rats exposed to EtOH, following the two aforementioned schemes of exposure [11].

In this study, the first experimental scheme is used as a basis for the assessment of the effects of a moderate maternal exposure to EtOH during the early stages of the offspring neurodevelopment, and this scheme is examined in the presence of a further complication: that of a dietary-induced thiamine-deprivation [13]. Thiamine is a crucial cell membrane component [14,15], and its metabolism and availability *in utero* are closely linked to maternal EtOH consumption [13,16]. In view of this important fact as well as in view of previous reports suggesting a major role for thiamine in neurodevelopment [15,17–23], our study aimed at examining the effects of gestational thiamine-deprivation (through the maternal feeding of a thiamine-deficient diet; TDD) and/or exposure to EtOH on the activities of AChE, Na^+,K^+-ATPase, and Mg^{2+}-ATPase in the offspring rat brains.

Materials and methods

Animals and experimental procedure followed

Twenty-four adult female albino Wistar rats (2-month-old) were purchased by the National Center of Scientific Research "Demokritos" (Agia Paraskevi, Athens, Greece) and were housed two in a cage, at a constant room temperature (22 ± 1 °C) under a 12 h light:12 h dark (light 08:00–20:00 h) cycle. Food and water were provided *ad libitum*. Animals were cared for in accordance with the principles for the care, use and protection of experimental animals as set by the EEC Council Directive 86/609/EEC and aligned according to the Recommendation 2007/526/EU. Permission for the conduction of the herein described experiments was granted by the local authorities (K/243; 22-01–2010).

Twelve adult male albino Wistar rats were used for mating purposes only; each male was placed with two females in each cage, in order for mating to be achieved. Following that (as assessed through the examination for the presence of an ejaculatory plug in the vagina), males were removed and female rats were divided into four groups (Figure 1(a)): (i) control (receiving normal diet and tap water during both gestation and lactation, $n = 6$), (ii) EtOH (receiving normal diet and 10% v/v of EtOH in the drinking water during gestation, $n = 6$) [11], (iii) TDD (receiving TDD[1] and tap water during gestation, $n = 6$), and (iv) EtOH + TDD (receiving TDD and 10% v/v EtOH in the drinking water during gestation, $n = 6$). Upon delivery, five newborn rats ($n = 5$) from each group were weighted and sacrificed by decapitation and their brains were rapidly removed, providing brain tissue samples for the control, EtOH, TDD, and EtOH + TDD groups (Figure 1(a)). The rest of the newborn rats were allowed to grow, while food and water were switched back to normal (as in Control) in all groups. At the end of the lactation period, the 21-day-old rat offspring ($n = 5$–6 per group) were also weighted, sacrificed by decapitation and their brains were rapidly removed, providing brain tissue samples for their respective groups (Figure 1(a)). It should be noted that all offspring groups consisted of rats of both sexes, as previous data of ours have shown that no significant sex-dependent differences exist among newborn or 21-day-old rats with regard to their herein studied brain homogenate AChE and ATPase activities [24]. Exposure to EtOH and/or TDD did not cause any statistically significant effects on litter size. However, offspring body and brain weight differences due to the followed treatments have been observed and are presented in Figure 1.

Tissue preparation and brain enzymes' activity determination

The obtained rat brain tissue was homogenized in 10 vol. ice cold (0–4 °C) medium containing 50 mM

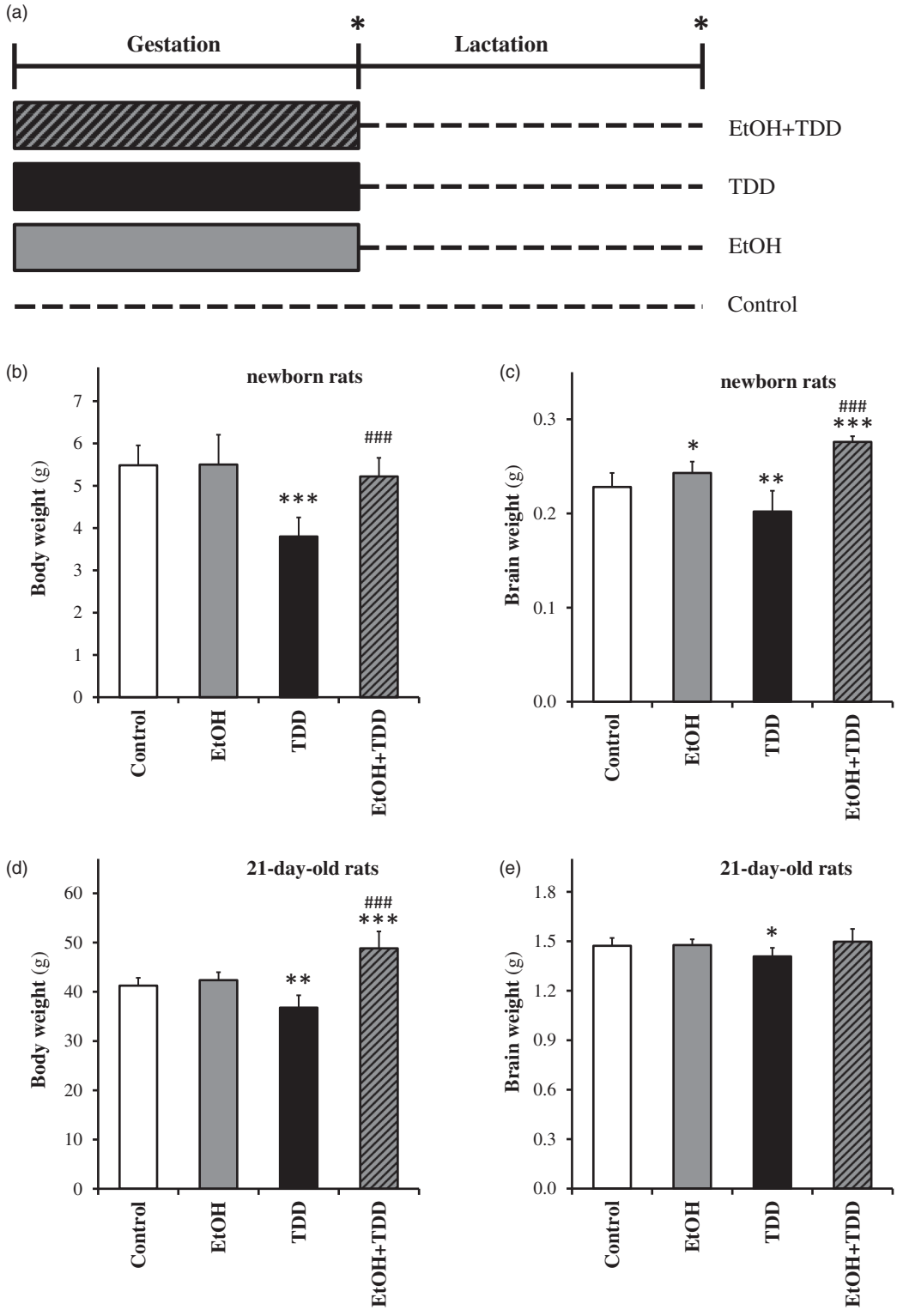

Figure 1. (a) Schematic overview of the experimental protocol followed. Pregnant albino Wistar rats were exposed to ethanol (EtOH; 10% v/v in the drinking water) and/or thiamine-deficient diet (TDD) during gestation, but not during lactation. The studied brain homogenates derived from the newborn and the 21-day-old offspring rats of all four studied groups during postnatal day 1 and at the end of the lactation period, respectively. *Timepoints of animal sacrifice. (b–e) Effects of gestational exposure to ethanol (EtOH; 10% v/v in the drinking water) and/or thiamine-deficient diet (TDD) on the body and brain weight of newborn (b,c) and 21-day-old (d,e) offspring rats. Significance versus the control group: $^{***}p < .001$; $^{**}p < .01$; $^{*}p < .05$. Significance versus the TDD group: $\#\#\#p < .001$.

Tris (hydroxymethyl) aminomethane–HCl (Tris–HCl), pH 7.4 and 300 mM sucrose, using an ice-chilled glass homogenizing vessel at 900 rpm (4–5 strokes). Then, the homogenate was centrifuged at 1000×*g* for 10 min to remove nuclei and debris [25]. In the resulting supernatant, the protein content was determined according to the method of Lowry et al. [26] and then the enzyme activities were measured as previously described in detail [27].

In particular, the activity of AChE was measured by following the hydrolysis of acetylthiocholine according to the method of Ellman et al. [28], as described by Tsakiris [25]. The incubation mixture (1 ml) contained 50 mM Tris–HCl, pH 8, 240 mM sucrose, and 120 mM NaCl. The protein concentration of the incubation mixture was 80–100 μg/ml. The reaction was initiated after addition of 0.03 ml of 5,5′-dithionitrobenzoic acid (DTNB) and 0.05 ml of acetylthiocholine iodide, which was used as substrate. The final concentration of DTNB and substrate was 0.125 and 0.5 mM, respectively. The reaction was followed spectrophotometrically by the increase of absorbance ΔOD at 412 nm.

The activity of Na^+,K^+-ATPase was calculated from the difference between total ATPase activity (Na^+,K^+,Mg^{2+}-dependent ATPase) and that of Mg^{2+}-dependent ATPase. Total ATPase activity was assayed in an incubation medium consisting of 50 mM Tris–HCl, pH 7.4, 120 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 240 mM sucrose, 1 mM ethylenediamine tetraacetic acid K_2-salt (K^+-EDTA), 3 mM disodium ATP, and 80–100-mg protein of the homogenate in a final volume of 1 ml. Ouabain (1 mM) was added in order to determine the activity of Mg^{2+}-ATPase. The reaction was started by adding ATP and stopped after an incubation period of 20 min by addition of 2 ml mixture of 1% lubrol and 1% ammonium molybdate in 0.9 M H_2SO_4 [25,29]. The yellow color which developed was read at 390 nm.

Chemicals and statistical analysis

All chemicals used in this study were of analytical grade and/or of the highest purity available and were purchased from Sigma-Aldrich (St. Louis, MO). The data were analyzed using one-way ANOVA followed by Bonferroni's correction (where applicable). All analyses were performed by SPSS for Windows Software (Chicago, IL). Values of *p* less than .05 were considered statistically significant.

Results

Offspring rats exposed to a TDD during gestation recorded a significantly lower body and brain weight on both day 1 and day 21 of their postnatal life (Figure 1(b–e)). The body weight of the newborn rats of the TDD group was 31% lower ($p < .001$; Figure 1(b)), while their brain weight was 11% lower ($p < .01$; Figure 1(c)) than that of the age-matched control group. Similarly, but to a milder extent, the 21-day-old rats of the TDD group presented with a lower body (−11%, $p < .01$; Figure 1(d)) and brain (−4%, $p < .05$; Figure 1(e)) weight as compared to the respective Control group. The offspring rats exposed to EtOH during gestation did not present with differences in terms of the recorded body weight (Figure 1(b,d)), while their brain weight was only found to be significantly altered on day 1 of their postnatal life (+7%, $p < .05$; Figure 1(c)) as compared to the Control group. Interestingly, offspring rats that were exposed to both EtOH and TDD during gestation (EtOH + TDD group) presented only with an increase in their brain weight on day 1 (+21%, $p < .001$; Figure 1(c)) and an increase in their body weight on day 21 (+18%, $p < .001$; Figure 1(d)) of their postnatal life, as compared to the Control group.

The measurement of the newborn rat brain AChE activity in the herein studied groups revealed only a statistically significant increase in the TDD group (+17%, $p < .05$; Figure 2(a)) as compared to that of the Control group. This increase was not evident in the brain homogenates of the 21-day-old rats of the same group, but instead, statistically significant changes in the activity of this crucial cholinergic enzyme were observed in the EtOH (+10%, $p < .05$) and the EtOH + TDD (−13%, $p < .01$) groups as compared to that of the Control (Figure 2(b)).

Newborn rats demonstrated significant changes in their brain Na^+,K^+-ATPase activity after a gestational exposure to EtOH and/or TDD (Table 1): the enzyme was found to be significantly inhibited in the TDD group (−32%, $p < .05$), while both the EtOH and the EtOH + TDD groups' brain Na^+,K^+-ATPase activities were found to be significantly increased (+51%, $p < .05$ and +37%, $p < .05$, respectively), as compared to Control. Following a TDD- and EtOH-free lactation, all these changes in the offspring brain Na^+,K^+-ATPase activity were found to be completely or partially restored (Table 1).

Finally, none of the examined groups of offspring rats demonstrated any statistically significant changes with regards to their brain Mg^{2+}-ATPase activity, at

Table 1. Effects of gestational exposure to ethanol (EtOH) and/or thiamine-deficient diet (TDD) on newborn and 21-day-old offspring rat brain Na^+,K^+- and Mg^{2+}-ATPase activities.

		Na^+,K^+-ATPase activity (μmol Pi/h × mg protein)		
	Group	Mean ± SD	Versus Control	Versus TDD
Newborn rats	Control ($n = 5$)	0.76 ± 0.16	–	
	EtOH ($n = 5$)	1.15 ± 0.24	* (+51%)	
	TDD ($n = 5$)	0.52 ± 0.09	* (−32%)	
	EtOH + TDD ($n = 5$)	1.04 ± 0.15	* (+37%)	*** (+100%)
21-day-old rats	Control ($n = 6$)	2.84 ± 0.27	–	
	EtOH ($n = 6$)	2.90 ± 0.21	NS (+2%)	
	TDD ($n = 5$)	3.17 ± 0.29	NS (+12%)	
	EtOH + TDD ($n = 6$)	3.21 ± 0.26	* (+13%)	NS (+1%)
		Mg^{2+}-ATPase activity (μmol Pi/h × mg protein)		
Newborn rats	Control ($n = 5$)	4.76 ± 0.50	–	
	EtOH ($n = 5$)	4.86 ± 0.65	NS (+2%)	
	TDD ($n = 5$)	4.62 ± 0.39	NS (−3%)	
	EtOH + TDD ($n = 5$)	4.97 ± 1.05	NS (+4%)	NS (+8%)
21-day-old rats	Control ($n = 6$)	6.30 ± 1.06	–	
	EtOH ($n = 6$)	6.63 ± 0.40	NS (+5%)	
	TDD ($n = 5$)	6.48 ± 0.24	NS (+3%)	
	EtOH + TDD ($n = 6$)	6.37 ± 0.53	NS (+1%)	NS (−2%)

NS: nonstatistically significant.
Each value indicates the mean ± SD of five or six independent experiments (five or six rats per group). The average value of each experiment was obtained from three evaluations in the homogenated rat whole brain.
*$p < .05$.
***$p < .001$.

any of the timepoints of the herein studied experimental scheme (Table 1).

Discussion

In his well-designed study on Wistar rats, one of the leading figures in the field of perinatal thiamine-deprivation-associated neurotoxicity, Abdoulaye Bâ, has shown that the maternal consumption of TDD during gestation could result to a significant decrease of the offspring rat body weight as measured on postnatal day 1, but not on postnatal days 10, 15, 20, 25, 30, or 45 [30]. The same author has suggested that perinatal and postnatal thiamine-deprivation could affect the offspring early growth in a severe manner, in contrast to the maternal consumption of TDD during gestation alone that seems to be easily corrected by thiamine-administration right after delivery [30]. Moreover, in a similar earlier study, Bâ et al. [20] have also suggested that the offspring rat brain weight can only be affected by perinatal, and not by prenatal or postnatal dietary thiamine-deprivation. Our findings suggest that the maternal consumption of a TDD during gestation alone, could on its own have a significant effect on the offspring rat body and brain growth (Figure 1(b–e)). Interestingly, these are not the only findings of ours that are not in agreement with those of Bâ and his colleagues [20,30]: we did neither observe abortions [13], nor significantly lower litter sizes at birth due to maternal consumption of TDD [13]. As Bâ admits, the clinical association of gestational thiamine-deprivation to lower birth weight is complicated, and so is the experimental one [30]; we suspect that variations should be expected due to the different diet formulas used, the susceptibility of the rats to TDD and/or the litter sizes achieved. The latter could certainly affect the offspring postnatal weight gain, while an important finding of ours is also the fact that EtOH does not exacerbate this offspring rat growth retardation caused by maternal exposure to a TDD during gestation (Figure 1(b–e)).

The maternal exposure to a TDD during gestation was found to stimulate the newborn rat brain AChE (Figure 2(a)); an effect that is silenced by a maternal coexposure to EtOH, as well as by a normal diet-supplemented lactation (Figure 2(b)). We have previously shown that thiamine can inhibit *in vitro* the pure (eel *E. electricus*-derived) AChE enzyme, but not the adult rat brain homogenate AChE one [31]. The ability of thiamine to inhibit AChE is well-characterized [32], and thus, its decreased availability could to an extent account for the observed increased AChE activity in the newborn offspring rat brains. However, the latter finding is of particular importance, as very few are the studies exploring the interaction between these two factors in neurodevelopment; in fact, the most important experimental study linking thiamine-deficiency to the cholinergic system during neurodevelopment is that of Kulkarni and Gaitonde [33], and it suggests a decrease of the offspring (21- and 28-day-old) rat

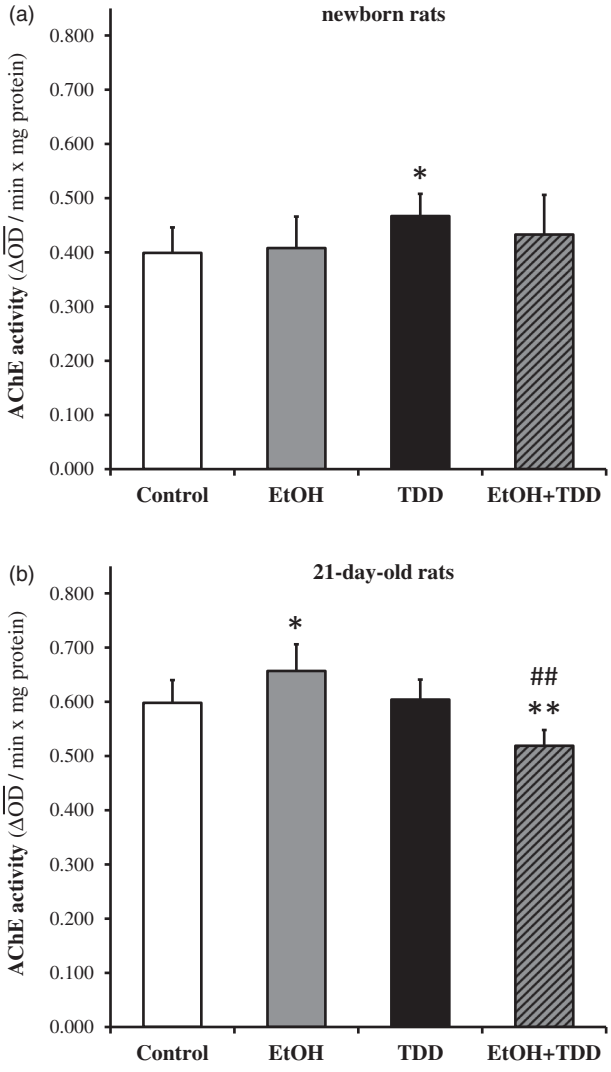

Figure 2. Effects of gestational exposure to ethanol (EtOH; 10% v/v in the drinking water) and/or thiamine-deficient diet (TDD) on the acetylcholinesterase (AChE) activity of the newborn (a) and 21-day-old (b) offspring rat brain. Each value indicates the mean ± SD of five or six independent experiments. The average value of each experiment was obtained from three evaluations in the homogenized rat brain. Significance versus the Control group: $^{**}p < .01$; $^{*}p < .05$. Significance versus the TDD group: $\#\#p < .01$.

brain acetylcholine levels as a result of thiamine-deprivation during both gestation and lactation that, interestingly, is not accompanied by any significant changes in the AChE activity of these same brains as compared to their respective controls. Due to its unique experimental design with regards to this matter, our study also provides evidence of the development of an interesting late-onset (if one might suggest) effect of the maternal thiamine-deprivation and EtOH-exposure during gestation: that of the decreased AChE activity in the 21-day-old offspring rats (Figure 2(b)). The latter inhibition cannot possibly be attributed to low thiamine availability, but to a more complex mechanism that might involve neuronal excitability (as evident by the changes observed with regards to Na^+,K^+-ATPase activity at the same time-point; Table 1) and/or complex dynamic and adaptive neuromodulatory events aiming at "recovering" the developing neural tissue from the increased brain weight observed in the EtOH + TDD group upon birth. In either case, the pattern of the enzymatic behavior of AChE in the offspring rat brains of the combined exposure group (EtOH + TDD) does not seem to mimic any of the other two groups (TDD or EtOH; Figure 2).

We have previously provided an in-depth discussion about the role of Na^+,K^+-ATPase in the neurodevelopmental toxicity of EtOH [11]; our current findings are in agreement with those of Stolakis et al. [11] as far as 21-day-old rat whole brain Na^+,K^+-ATPase activity is concerned, but also add up to our knowledge on the behavior of this crucial neurochemical parameter in the case of newborn rats exposed to EtOH throughout gestation (Table 1). The observed stimulation of newborn rat brain Na^+,K^+-ATPase (+51%, $p < .05$; Table 1) might be a precursor of the 21-day-old offspring rat frontal cortex Na^+,K^+-ATPase stimulation (+98%) reported by Stolakis et al. [11] following a maternal exposure to EtOH throughout gestation. This finding is of unique interest, as the literature has provided – though a variety of *in vivo* experimental approaches to FASD utilizing different EtOH doses and/or exposure timeframes, that are in no case identical to those employed in our current study – evidence of an EtOH-induced inhibition of the offspring rat brain Na^+,K^+-ATPase following a maternal exposure [34–36]. In their very important study, Marques and Guerri [37] have provided evidence of a stimulation of rat synaptosomal Na^+,K^+-ATPase activity by EtOH concentrations that are lower than 100 mM, and of an inhibition of this same enzyme by higher EtOH concentrations (>300 mM). As the same authors [37] also report an abolishment of the concentration-dependent effects of EtOH on synaptosomal Na^+,K^+-ATPase activity following a delipidization of the synaptic membranes, one could suggest that apart from the EtOH concentration present, the lipid microenvironment of the enzyme might play a critical role in defining the interaction between this xenobiotic and the enzyme. In view of this finding, one should not oversee the fact that both EtOH [38] and thiamine-deprivation [9] are known to modify the rat brain lipid composition. A modified brain lipid composition could also alter the availability of EtOH and the response of Na^+,K^+-ATPase to it, and might be the reason behind the significant variation

observed among the *in vivo* and *in vitro* studies of the effects of EtOH on Na^+,K^+-ATPase activity [11,39–41].

Na^+,K^+-ATPase is an enzyme implicated in neuronal excitability, metabolic energy production, as well as the uptake and release of a number of neurotransmitters [27,42]. One could argue that the herein reported EtOH-induced stimulation of the newborn offspring rat brain Na^+,K^+-ATPase (Table 1) could be related to the diminished cortical dendritic branching observed under conditions involving maternal exposure to EtOH [12], and the attempt of the neurons to enhance neuronal excitability over a smaller number of axons and dendrites. In such a case, catecholaminergic neurotransmitters (particularly, norepinephrine) could play some role in regulating the response of Na^+,K^+-ATPase to EtOH [42–44], while thiamine-deprivation ought to have further enhanced this stimulation [31,45]. Interestingly, in our current study, gestational thiamine-deprivation caused an inhibition of the newborn rat brain Na^+,K^+-ATPase (Table 1), and when combined with a maternal exposure to EtOH, resulted to an increase in the offspring rat brain Na^+,K^+-ATPase activity at both examined time-points (postnatal days 1 and 21; Table 1). The decrease in Na^+,K^+-ATPase activity due to thiamine-deprivation could be a result of a lower presence of high-affinity ouabain binding sites [46], altered lipid composition [9] and/or even the result of an interaction with the cholinergic system, based on the suggested by Meyer and Cooper [47] inverse relationship between the activities of Na^+,K^+-ATPase and AChE. However, irrespectively of the mechanism(s) involved, one should keep in mind that an enhanced or decreased Na^+,K^+-ATPase activity might not necessarily reflect an actual increased or decreased enzyme efficiency, respectively [48].

Finally, the unaltered Mg^{2+}-ATPase activity in all brain samples is indicative of the absence of serious metabolic consequences as a result of the herein examined experimental conditions. Our previous study exploring the effects of gestational or gestational and lactational maternal exposure to EtOH has also failed to reveal any significant changes in the offspring rat brain Mg^{2+}-ATPase activity, but it did reveal a significant stimulation of this crucial enzyme in the 21-day-old offspring rat hippocampus [11].

In conclusion, this study was undertaken in order to: (i) address the need for a more consistent and reliable *in vivo* experimental platform that could simulate milder cases of FASD complicated by simultaneous thiamine-deprivation during gestation and (ii) explore the effects of such moderate maternal exposure patterns to EtOH and TDD on crucial enzyme activities in the offspring rat brains. Our findings provide a preliminary characterization of important neurochemical effects due to maternal exposure to EtOH and TDD during gestation that might affect offspring neurodevelopment, and that characterization should be further explored in a brain region-specific manner level as well as through the parallel examination of changes in the offspring rat brain lipid composition.

Note

1. TDD was purchased from Mucedola (Italy) and contained: corn starch, casein vitamin free, sucrose, corn oil, cellulose powder, dicalcium phosphate, potassium citrate, calcium carbonate, D,L-methionine, ethoxyquin, vitamin A, vitamin D_3, vitamin E, copper, and selenium.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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