Ann Ist Super Sanità 2013 | Vol. 49, No. 4: 383-390
DOI 10.4415/ANN_13_04_11

NGF and BDNF long-term variations in the thyroid, testis and adrenal glands of a mouse model of fetal alcohol spectrum disorders

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Abstract

Objectives. Fetal Alcohol Spectrum Disorders (FASD) due to prenatal ethanol consumption may induce long-lasting changes to the newborns affecting also the endocrine system and the nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) signaling. Thus the aim of this study was to investigate in the thyroid, testis and adrenal glands of a FASD mouse model the long-lasting effects of ethanol exposure during pregnancy and lactation on NGF and BDNF and their main receptors, TrkA and TrkB, including their phosphorylated patterns.

Methods. We used aged male CD-1 mice early exposed to ethanol solution or red wine at same ethanol concentration (11% vol).

Results. We found elevations in NGF and BDNF in the thyroid of aged mice exposed to ethanol solution only but not in the red wine group. In the testis NGF resulted to be increased only in the ethanol solution group. In the adrenal glands data showed an elevation in NGF in both the ethanol solution group and red wine. No changes in TrkA, TrkB, phospho-TrkA and phospho-TrkB were revealed in all tissues examined.

Conclusions. Early administration of ethanol may induce long-lasting changes in the mouse thyroid, testis and adrenal glands at NGF and BDNF levels.

Key words
- nerve growth factor
- brain derived neurotrophic factor
- fetal alcohol spectrum disorders
- FASD

INTRODUCTION

Ethanol intake has been shown to elicit changes in the endocrine system [1-4]. During pregnancy, the endocrine systems of the mother and fetus are intricately interconnected to ensure normal fetal development. Accordingly, maternal alcohol consumption during pregnancy can interfere with fetal developmental programming, not only directly, through adverse effects exerted by alcohol that crosses the placenta and enters the fetal bloodstream, but also indirectly, by disturbing the functions and interactions of maternal and fetal hormones [5]. Some of the effects of maternal alcohol consumption on fetal endocrine systems may contribute to the adverse effects observed in children with Fetal Alcohol Spectrum Disorders (FASD) and related disorders [6]. FASD is the term used to describe the range of effects in the fetus caused by the mother drinking alcohol during pregnancy. These effects may include physical, mental, behavioral and/or learning disabilities with possible lifelong implications. Since ethanol consumption during pregnancy represents a major public health concern (it is estimated that the incidence of FASD in western countries is about 4% of live births [7]), early recognition of at-risk children is important for initiating interventional strategies [8-10]. Unfortunately, many crucial aspects about the pathological mechanisms triggered by ethanol exposure during pregnancy are still unknown. Ethanol consumption during pregnancy causes neuronal cell death in the offspring [11] by affecting neu-

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rotrophins including Nerve Growth Factor (NGF) and Brain Derived Neurotrophic Factor (BDNF) [12-15] and their receptors, TrkA and TrkB respectively [16-18]. These neurotrophic factors play also a role in the endocrine system development, maintenance and activity [19-25]. We have previously shown that in a FASD mouse model, trophic factors are severely affected by early ethanol exposure in the fetus both in the central nervous system and in target organs of ethanol intoxication [13, 26, 27]. Quite surprisingly changes in neurotrophic factor levels were also observed in the aged mouse brain [27] exposed in fetus to ethanol. However, only limited information is available on the effects of prenatal ethanol administration on peripheral glands as the thyroid, the testis and the adrenal glands also during aging [5]. Thus the rationale of the present study was to investigate whether or not early ethanol exposure can interfere with the endocrine system by studying NGF and BDNF and their main receptors, TrkA and TrkB, including their phosphorylated patterns in the thyroid, testis and adrenal glands of aged mice exposed in fetus to ethanol or red wine at same ethanol concentration. These receptors belong to a family of tyrosine kinases regulating synaptic strength and plasticity in the nervous system as well as various immune and endocrine functions. Trk receptors affect neuronal survival and differentiation through several signal cascades. However, these receptors have also significant effects on functional properties of neuronal and non-neuronal cells [28, 29]. We compared the effects of ethanol if administered as ethanol solution only or red wine at same ethanol concentration because red wine consumption during pregnancy and lactation is still observed in western countries and as other alcoholic beverages may have a role in the onset of FASD. A control group of mice was exposed to an isocaloric sucrose solution.

MATERIALS AND METHODS

General description of the FASD Mouse Model

CD-1 outbred female mice were housed singularly in Plexiglas cages (33x13x14 cm) under standardized conditions with pellet food (enriched standard diet purchased from Mucedola, Settimo Milanese, Italy). A 12L:12D lighting regime was used. To fully mimic chronic ethanol exposure the liquid administration started 60 days before pregnancy as previously shown [13]. Animals were exposed to ethanol as ethanol solution only or as red wine at the same ethanol concentration. Indeed, dams were divided in 3 groups: ethanol, red wine and sucrose (n = 6 for each group). Animals of the ethanol group received *ad libitum*, as only source of liquid, ethanol (11% vol) dissolved in water [30, 31]. Ethanol used for the preparation of the drinking solutions was obtained from Merck (Darmstadt, Germany) and was of analytical grade. Red wine *ad libitum* (11% vol, red wine from different grapes of different regions of center Italy, Vignetta rosso, Caldirola, www.caldirola.it) was the only source of liquid in the red wine group. We used 11% concentration of ethanol since this value is comparable to the amount of ethanol present in the red wine commonly consumed by humans. The sucrose group (Controls) received sucrose dissolved in water at equivalent caloric intake of the ethanol group and was used as control group. This treatment schedule ended at the time of pups weaning (postnatal day 28). This method of liquid administration was chosen to limit the stress due to handling to pregnant mice. Fluid intake was measured regularly and the amounts consumed were calculated [13]. All groups received pellet food *ad libitum* as above. Mating took place over a period of 2 days. The day of plug detection was designated as gestational day 0. At birth each litter was reduced to 4 males and 4 females when possible to maintain a uniform sex ratio as previously shown [32]. Pups remained with their own mother. After weaning cages contained 4 males or 4 females. 18-month old male mice exposed in uterus to ethanol solution, red wine or sucrose were used in the present study (one animal per litter, n=6 per group). We have defined aged our experimental animals following indications previously released [33]. These animals were littermates of subjects used in previous experiments on FASD, aging and brain neurotrophins [13, 27]. Previously published data on this FASD mouse model revealed no differences between groups in pregnancy duration, neither in pups delivery, pups mortality and sex ratio [13]. Ethanol and red wine groups consumed equivalent amounts of liquid corresponding to the same caloric intake [13]. The body weight of dams [13] was comparable between groups before and immediately after delivery: however 7 days after the delivery the sucrose group had highest values which returned similar to the other groups 28 days after delivery. Dams blood ethanol levels [13] collected during the light cycle were comparable between the red wine and the ethanol groups measured at gestational day 15 (133.43 + 13.18, ethanol group; 122.917 + 13.72, red wine group; 0, controls as mg/dl). The blood ethanol level collected during the light cycle of 7-day-old offspring was also analogous between the two groups (11.93 + 1.33, ethanol group; 11.11 + 1.19, red wine group; controls 0 as mg/dl). Also animal body weight at the time of animal sacrifice and thyroid/testis/adrenal glands weight during dissection did not differ between experimental groups (data not shown). Animals were killed by decapitation between 10.00 a.m. and 12.00 a.m. and the thyroid, testis and adrenal glands were quickly removed for biochemical and morphological analyses. As for the adrenal glands we analyzed both adrenal cortex and adrenal medulla because the perfect dissection of both sections is extremely difficult to perform in the mouse by the small dimensions. All tissues were immediately frozen with liquid nitrogen and then stored at -80 °C. All efforts were made to minimize and reduce animal suffering and for limiting the number of animals used. All animal experiments were carried out following the procedure described by the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC) and all experiments were authorized by the local ethical committees (Ministero della Salute, Regione Lazio, following the Decreto Legislativo 116/92; no permit number or approval ID are required since we only inform the committees of our experimentations without receiving any reply in case of positive answer).

NGF and BDNF determination

Growth factors were analyzed in the thyroid, testis and adrenal glands of 18-month-old mice with ELISA kits

following the indication provided by the manufacturer by a researcher who was unaware of the tissue group assignment. NGF/BDNF evaluation (n = 6 for group, one animal per litter) was carried out with ELISA kits "NGF Emaxtm ImmunoAssay System number G7631" and "BDNF Emaxtm ImmunoAssay System number G7611" by Promega (Madison, WI, USA) following the instructions provided by the manufacturer. The tissues were homogenized with ultrasonication in extraction buffer (Tris– acetate 20 mM, pH 7.5, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, sodium-pyrophosphate 2.5 mM, ortovanadate 1 mM, b-glycerol-phosphate 1 mM, NaF 100 mM, PMSF 1 mM, leupeptin 1 mg/ml) and centrifuged at 4 °C for 10 min, 13000 rpm and supernatants were recovered (EDTA, ethylenediamine-tetraacetic acid; EGTA, ethyleneglycol-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride). Briefly, 96-well immunoplates were coated with 100 µl per well of polyclonal anti-NGF antibody. After an overnight incubation at 4 °C, the plates were washed one time with washing buffer (Tris–HCl 20 mM, pH 7.6, NaCl 150 mM, 0.05% Tween 20) and then blocked for 1 h with block and sample 1 buffer provided by manufacturer (200 µl for well). After washing the samples were incubated in the coated wells (100 µl each) for 6 h at room temperature with shaking. After an additional five washes the immobilized antigen was incubated with an anti-NGF monoclonal antibody overnight at 4 °C. The plates were washed again with wash buffer, and then incubated with an anti-rat IgG HRP (horseradish peroxidase) conjugate (100 µl for well) for 2.5 h at room temperature. After washing the plates were incubated with a TMB/peroxidase substrate solution for 15 min (100 µl/well) (TMB, tetramethyl benzidine) provided by the manufacturer. Reaction was then stopped with 100 µl/well 1N HCl. The colorimetric reaction product was measured at 450 nm using a microplate reader (Dynatech MR 5000, PBI International, USA). NGF/BDNF concentrations were determined, from the regression line for the NGF/BDNF standard (ranging from 7.8 to 500 pg/ml purified NGF or BDNF) incubated under similar conditions in each assay. Under these conditions, the recovery of NGF or BDNF in our assay ranged from 80 to 90%. The NGF sensitivity of the assay was about 3 pg/g of wet tissue and cross-reactivity with other related neurotrophic factor (BDNF, neurotrophin-3 and neurotrophin-4) was less than 3%. The BDNF sensitivity of the assay was about 15 pg/ml of wet tissue and cross-reactivity with other related neurotrophic factor (NGF, neurotrophin-3 and neurotrophin-4) was less than 3%. Data from samples are represented as pg/mg total proteins and all assays were performed in duplicate [34, 35].

Western blotting analyses for TrkA, TrkB, phospho-TrkA and phospho TrkB

The mouse tissues were homogenized in sample buffer (0.01 M TRIS-HCl buffer pH 7.4, containing 0.1 M NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 10% Glycerol, 0.1% SDS) and centrifuged at 10,000 *g* for 15 min at 4 °C. The supernatants were then used for western blotting. Samples (5-30 µg total protein) were dissolved with loading buffer (50 mM TRIS-HCl buffer pH 6.8, 140 mM b-mercaptoethanol, 1% SDS, 10% glycerol, and 0.05% bromophenol blue), boiled for 5 min thus separated by 8% SDS-PAGE, and electrophoretically transferred to polyvinylidene fluoride membrane (PVDF) in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membranes were incubated for 1 hour at room temperature with blocking buffer (5% BSA, bovine serum albumine, or 5% non-fat dry milk in Tris-buffered saline and Tween (TBS-T) (10 mM TRIS pH 7.4, 100 mM NaCl, 0.1% Tween 20). Membranes were washed three times for 10 min each at room temperature in TBS-T and then incubated for 1 hour at room temperature with anti-TrkA 1:500 (Cell Signaling, USA, catalog number 25055), anti-TrkB 1:1000 (BD Biosciences Pharmingen, San Jose, CA, USA, catalog number 610101), anti-phospho-TrkA (Cell Signaling Technology, USA, catalog number 9141), anti-phospho-TrkB (Santa Cruz Biotechnology, CA, USA catalog number sc-135645) or anti-GAPDH 1:1000 (Santa Cruz Biotechnology, CA, USA). As shown in the instructions TrkA antibody does not react with TrkB or TrkC. Membranes were washed three times for 10 min each at room temperature in TBS-T and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG for TrkA and for GADPH 1:4000 or anti-mouse IgG for TrkB 1:4000 (Cell Signalling, Beverly, Mass., USA) as secondary antibodies. The blots were developed with enhanced chemiluminescent (ECL) assay (Millipore) as chromophore. Similar results were obtained in 6 independent Western blot runs. The public domain Image J software (http://rsb.info.nih.gov/ij/) was used for gel densitometry and protein quantification. The OD of GAPDH (Glyceraldehyde 3 phosphate dehydrogenase) bands was used as a normalizing factor. The optical density of GAPDH bands was used as an internal control for difference in sample loading. For each blotting, normalized values were expressed as percentage of relative normalized controls and used for the statistical analyses [35]. Each blotting for sample was repeated at least 2 times.

Statistical analysis

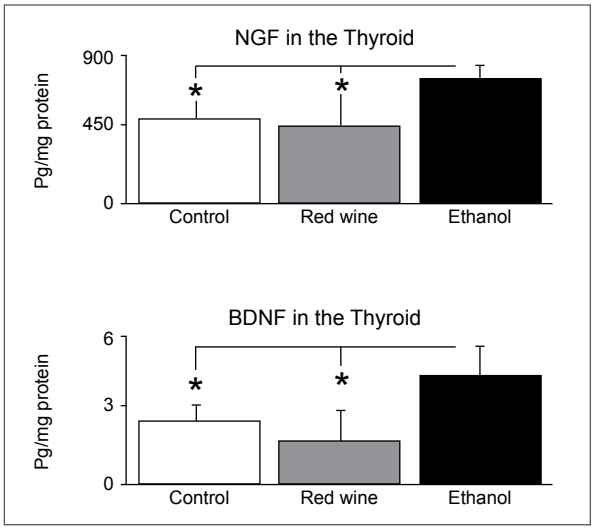
Data were analyzed by using StatView for the Mac considering ANOVA with the solution type as main factors. Post-hoc comparisons were performed using the Tukey's HSD test. The vertical lines in the figures indicate pooled standard error means (SEM) derived from appropriate error mean square in the ANOVA. Asterisks indicate significant differences between groups (* = $p < 0.05$).

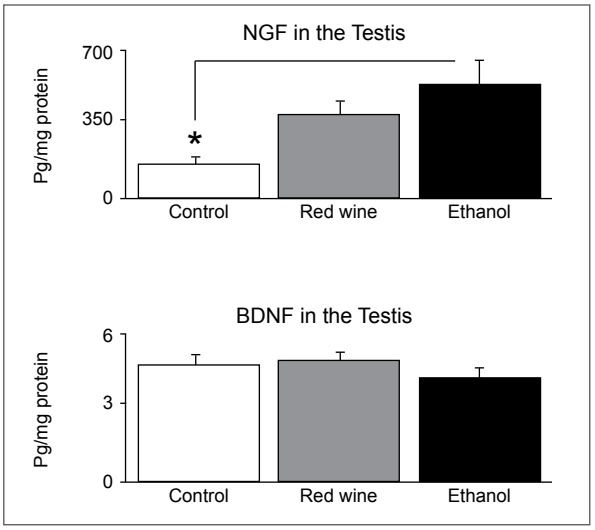
RESULTS

NGF and BDNF determination

Data on NGF and BDNF levels in the thyroid expressed as pg/mg total proteins of aged male mice exposed to ethanol during fetal and suckling period to ethanol, red wine and sucrose as controls are shown in *Figure 1*. ANOVA revealed that both neurotrophins resulted to be elevated in animals exposed *in uterus* and during lactation to ethanol solution [$F(2,15) = 5.11, 8.35$, $p < 0.05$ in the ANOVA, respectively, $p < 0.05$ in post-hoc comparison, Ethanol *vs* Controls, Ethanol *vs* Red Wine]. Conversely, animals exposed to red wine had NGF and BDNF thyroid values comparable to Controls.

Figure 2 shows the NGF and BDNF levels in the testis. ANOVA revealed that the ethanol solution group had





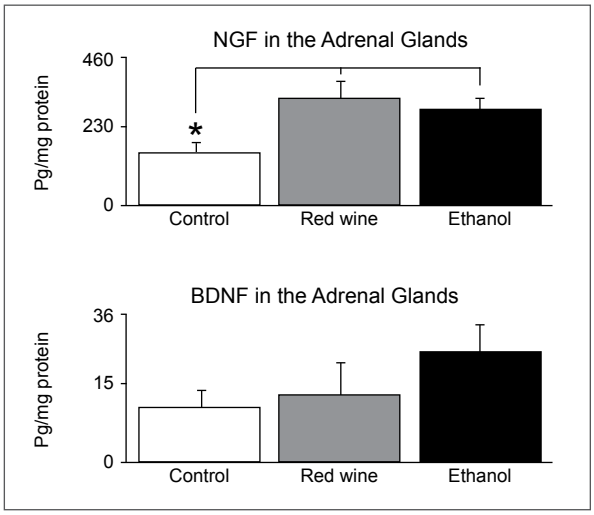


Figure 1 (top left)
NGF and BDNF levels in the thyroid expressed as pg/mg total proteins of aged male mice exposed during fetal and suckling period (n = 6 per group) to ethanol, red wine and sucrose as controls. Asterisks indicate significant differences between groups (* = $p < 0.05 \pm$ standard deviation).

Figure 2 (top right)
NGF and BDNF levels in the testis expressed as pg/mg total proteins of aged male mice exposed during fetal and suckling period (n = 6 per group) to ethanol, red wine and sucrose as controls. Asterisks indicate significant differences between groups (* = $p < 0.05 \pm$ standard deviation).

Figure 3 (bottom left)
NGF and BDNF levels in the adrenal glands expressed as pg/mg total proteins of aged male mice exposed during fetal and suckling period (n = 6 per group) to ethanol, red wine and sucrose as controls. Asterisks indicate significant differences between groups (* = $p < 0.05 \pm$ standard deviation).

NGF values significantly higher compared to controls [$F(2,15) = 6.23$, $p < 0.05$ in the ANOVA, $p < 0.05$ in post-hoc comparison, Ethanol *vs* Controls] but no NGF changes were found for the red wine group. As for BDNF no differences between groups were found in the testis.

Data on the NGF and BDNF levels in the adrenal glands are shown in *Figure 3*. Statistical analysis demonstrated that prenatal ethanol elicited a significant increase in NGF in both ethanol solution and red wine groups [$F(2,15) = 4.98, 5.32$, $p < 0.05$ in the ANOVA, respectively, $p < 0.05$ in post-hoc comparison, Ethanol *vs* Controls, Red Wine *vs* Controls]. No differences in BDNF levels between groups were found in the adrenal glands.

Western Blottings for TrkA, TrkB, phospho-TrkA and phospho-TrkB

To assess whether or not early ethanol exposure would influence the NGF and BDNF-target cells in aged mice the thyroid, the testis and the adrenal glands were used for testing TrkA, TrkB, phospho-TrkA and phospho-TrkB expression.

TrkA Western Blotting analyses are reported in *Figure 4*. Indeed, we have shown that in the thyroid TrkA stained with 2 bands at 110 and 140 kDa. In the testis the signal was weak at both 110 and 140 kDA as also shown in the rat [36] and in the adult human testis [37, 38]. In the adrenal glands we found a very weak TrkA signal only at 110 kDa findings in line with previous data showing that in the rat TrkA signal was weak and present only in the adrenal medulla [38]. In all examined tissues densitometric analyses of the mature band (140 kDa; 110 for the adrenal glands) did not reveal significant differences between groups.

TrkB Western Blotting analyses are reported in *Figure 5*. In the thyroid we found that under our experimental conditions TrkB stained with 3 different separate bands at 70, 116 and 145 kDa. However, in the testis the staining was absent at 70 kDa [38] while, as also stated by the TrkB antibody manufacturer, in the adrenal glands a further band was observed at 95 kDA mostly in both groups exposed to ethanol. Again in all examined tissues densitometric analyses of the mature band (145 kDa) did not reveal significant differences between groups.

The signal of phospho-TrkA and phospho-TrkB in all examined tissues was not discernible indicating no receptor's phosphorylation (data not shown).

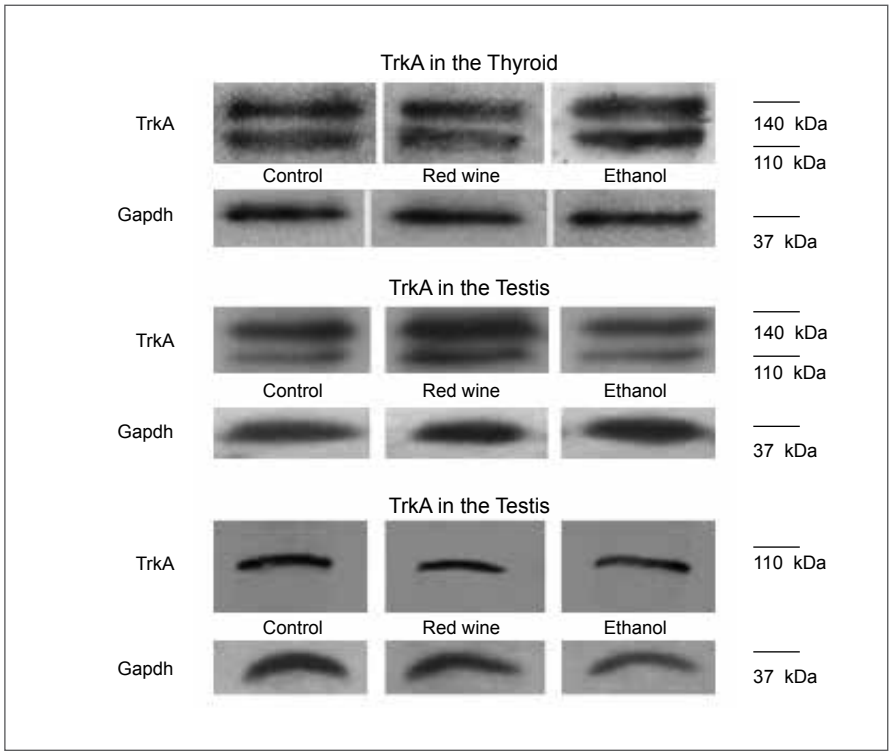


Figure 4
Typical western blot TrkA gel expression in the thyroid, testis and adrenal glands of aged male mice exposed during fetal and suckling period (n = 6 per group) to ethanol, red wine and sucrose. Gel expression of TrkA indicates that this receptor has two close bands at 140 and 110 kDa. However in the adrenal glands we stained only the band at 110 kDa. As shown in the Results section no significant differences between groups were found.

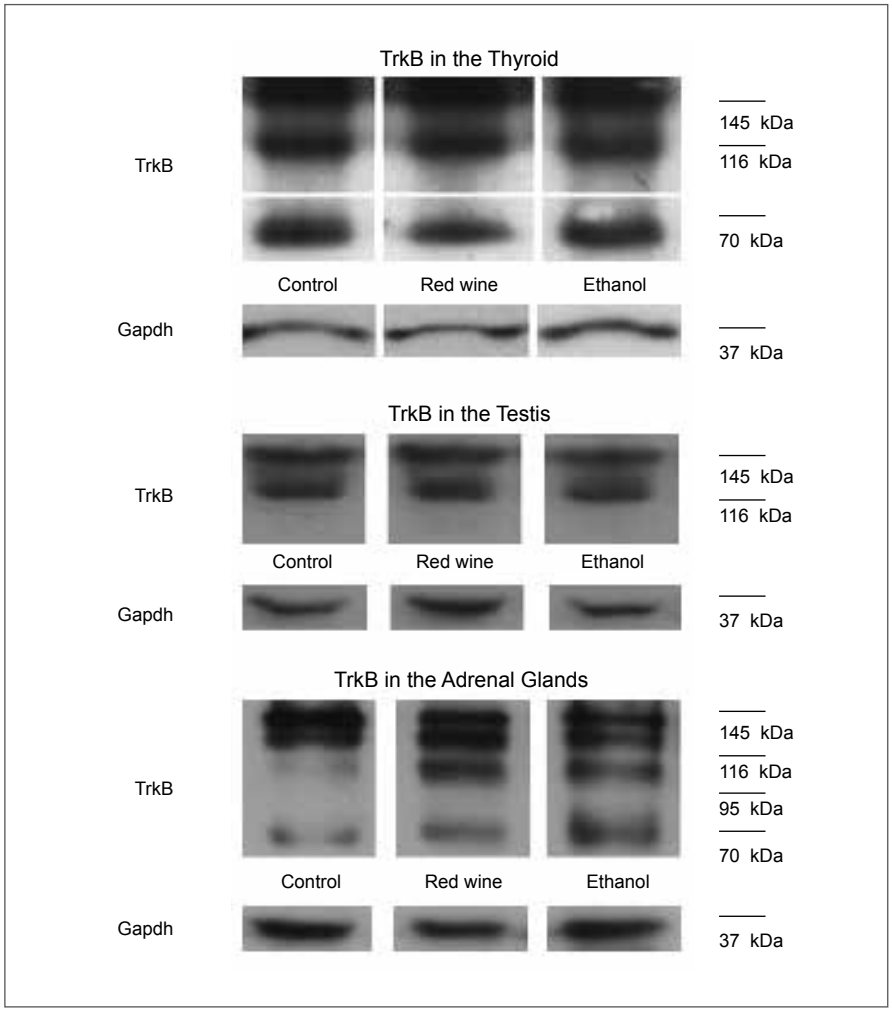


Figure 5
Typical western blot TrkB gel expression in the thyroid, testis and adrenal glands of aged male mice exposed during fetal and suckling period (n = 6 per group) to ethanol, red wine and sucrose. Gel expression of TrkB indicates that this receptor has three close bands at 145 and 116 and 70 kDa. However, in the testis the 70 kDa band was absent and in the adrenal glands we stained a further band at 95 kDa. As shown in the Results section no significant differences between groups were found.

DISCUSSION AND CONCLUSIONS

Since Jones, Clarren and Smith pioneer reports [39, 40] described that FASD is largely regarded as a variety of neurodevelopmental disorders associated with alterations not only in the neuronal architecture of brain areas but also at the endocrine system level several laboratories tried to develop animal models with these characteristics. Indeed, we have previously shown that ethanol administration in the mouse during gestation as ethanol solution only or red wine induces abnormalities at trophic factor levels in target organs of ethanol toxicity as brain, liver, testis, kidney [13, 26, 27]. In particular, we demonstrated behavioral, cellular, biochemical and molecular changes suggesting that one mechanism through which the brain and other target organs of ethanol toxicity are affected involves the dysregulation of the synthesis and secretion of neurotrophic factors such as NGF and BDNF. In this 2-years long study we demonstrate for the first time that exposure in the fetus and during lactation to ethanol may have long lasting effects by potentiating NGF presence in some glands of the aged mouse as the thyroid, the testis and the adrenal glands whereas BDNF elevation was observed only in the thyroid. Quite interestingly we also show that in this FASD mouse early ethanol exposure does not affect TrkA and TrkB expression or the expression of their phosphorylated patterns in these glands. This suggests that TrkA and TrkB receptor signaling/activation pathways may not have a significant role in the elevation of growth factors and some other mechanisms may be involved. However, the functional significance of NGF/BDNF potentiation is not known although increased NGF levels during aging have been prospected as a damage sign leading to precocious brain aging [41, 42].

As for the thyroid we have shown that NGF and BDNF are detectable in this mouse gland [22, 23] although with different concentrations. ELISA data indicate that NGF is highly present in the thyroid if compared to BDNF. In a previous study it has been found that in the rat thyroid tissue BDNF mRNA was undetectable and very low levels of mRNA for truncated TrkB and no catalytic TrkA or TrkB were shown [22]. In another study addressing growth factors and tumors in the thyroid it has been shown that NGF immunoreactivity was found in thyroid tissues and tumors of all types [23] whereas the NGF receptor staining was found in the vascular stroma and immunoreactivity correlated with the degree of vascularization, but no staining was seen in normal or affected thyroid cells. As for the testis we found increased NGF in the ethanol group. In the rodent it has been studied the expression of NGF and TrkA in the testis [36, 43, 44] suggesting an important role for NGF in gonadal function. Analogous results we found for the adrenal glands with an elevation in NGF in both experimental groups following prenatal ethanol. The adrenal gland is composed of two distinct, ontogenetically unrelated organs. The cortex is the prominent site of hormones production, whereas the medulla, of neural crest origin, synthesizes a complex cocktail of peptides including growth factors [45]. Interestingly, in a previous study on developing and adult rats it has been shown that BDNF is highly expressed in the developing and adult adrenal glands compared to NGF [38].

The present finding also supports the hypothesis that alcohol-induced endocrine imbalances may contribute to the etiology of fetal alcohol syndrome. Alcohol-mediated alterations in hypothalamic-pituitary-adrenal/thyroid/testis axis function are supposed mechanisms by which alcohol causes neurodevelopmental injury to the fetus [46-51]. Furthermore, it has been hypothesized that peripheral glands as thyroid, testis and adrenal glands may be directly affected by prenatal ethanol [50, 52, 53] and FASD animal models studies have shown short- and long-term consequences in the hypothalamic-pituitary-adrenal axis [5, 46]. In a rat model a genetic contribution to the fetal endocrine system vulnerability to maternal alcohol consumption has been also hypothesized [54].

Concerning the observed differences in ethanol toxicity between the ethanol solution group and the red wine group it should be noted that the putative antioxidant properties of red wine polyphenols may have in some way the ability to counteract important components of toxicity induced by early ethanol exposure as previously prospected [55-58]. The results shown in this study as well as in our previous works clearly indicate tissue dependent effects, with protective effects in the Central Nervous System and thyroid and no protective effects in the liver, testis and adrenal glands [13, 26, 27]. It could be possible the red wine mediated differences observed in this study are due to the source (locally produced *vs* innervating cell derived) of the neurotrophins in these peripheral tissues. However, drinking ethanol in any form during pregnancy (including red wine) gives serious damage to the fetus and then the only indication is to quit any kind of alcoholic beverages during gestation and lactation.

At the present time it is not known what are the consequences of changes neurotrophic factors in the thyroid, testis and adrenal glands as shown in this preliminary study with also a limited number of experimental subjects or it is not known whether there are any structural or hormonal changes in these aged endocrine glands after neonatal alcohol or red wine exposure and additional studies are needed. In conclusion as ethanol exposure in fetus during pregnancy and lactation is a putative neurodevelopmental model of FASD, and given previous data that cells of target organs of ethanol intoxication have disrupted neurotrophic factors synthesis, release and utilization, these results represent a further step in the attempt to unravel the molecular events involved in endocrine gland changes. Therefore, this study may attract a significant interest by those working on the animal models of FASD.

Acknowledgements

Mauro Ceccanti and Marco Ceccanti were supported by Sapienza Università di Roma, Rome, Italy. Marco Fiore, Sara De Nicolò, George Chaldakov, Paola Tirassa and Valentina Carito were supported by CNR, Rome, Italy. Rosanna Mancinelli and Giovanni Laviola were supported by ISS, Rome, Italy.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Received on 18 July 2013.
Accepted on 30 October 2013.

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