

Alcohol intake during prenatal life affects neuroimmune mediators and brain neurogenesis

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Summary. Several lines of evidence suggest that alcohol exposure during prenatal gestation, or during early postnatal life may be a risk factor for the manifestation of neurological and for immune-related disorders in later life. The cellular, biochemical and molecular mechanisms of ethanol toxicity, however, have not been yet clearly established. Recent studies indicated that neurotrophin signaling pathways may be involved in ethanol mediated cell death. The present investigation addressed the question of whether nerve growth factor (NGF), which is the first and best characterized member of the neurotrophin family, and NGF-target cells are affected by prenatal exposure to alcohol. The result of our study indicates that NGF synthesis and the functional activity of NGF-target cells localized in the brain are markedly influenced by ethanol intake. The possible link between such changes and the hypothesis that these alterations may contribute to certain of the neuropathology observed following alcohol exposure would be discussed.

Key words: ethanol, neurotrophins, NGF-receptors, brain, ChAT, brain development, brain damages.

Riassunto (*Effetto dell'esposizione prenatale all'alcol sui mediatori neuroimmunologici e sulla neurogenesi del cervello*). Diverse osservazioni sperimentali suggeriscono che l'esposizione all'etanolo durante il periodo gestazionale o in una fase precoce della vita post-natale costituisce un fattore di rischio per la manifestazione di disordini neurologici e di natura immunitaria nell'età adulta. I meccanismi cellulari, biochimici e molecolari alla base della tossicità da etanolo, comunque, non sono stati ancora completamente chiariti. Studi recenti indicano che il segnale cellulare trasdotto dalle neurotrofine potrebbe essere coinvolto nella morte cellulare indotta da etanolo. In questa ricerca abbiamo cercato di capire se il fattore di crescita nervoso (NGF), che è il primo e meglio caratterizzato membro della famiglia delle neurotrofine, e le cellule target dell'NGF sono sensibili all'esposizione di etanolo in età prenatale. I risultati del nostro studio indicano che la sintesi di NGF e l'attività funzionale delle cellule target dell'NGF nel cervello sono fortemente compromesse a seguito di assunzione di etanolo. Il possibile legame tra questi fenomeni osservati e l'ipotesi che questi eventi possano contribuire ad alcune alterazioni neurologiche osservate a seguito di esposizione all'etanolo saranno oggetto di discussione.

Parole chiave: etanolo, neurotrofine, recettori dell'NGF, cervello, ChAT, sviluppo cerebrale, danni cerebrali.

INTRODUCTION

Several lines of evidence indicate that alcohol exposure during prenatal gestation can influence cell proliferation and differentiation in the central nervous system (CNS) causing brain growth retardation and deficits in the limbic areas involved in cognitive functions [1-4]. The cellular, biochemical and molecular mechanism implicated in the deleterious action of alcohol intake are not fully known. Studies reported in recent years led to the hypothesis that alcohol can act on biological mediators, including growth factors synthesis and release by cells of the CNS. Nerve growth factor (NGF) is a member of the family of proteins known as neurotrophins, including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 and NGF is one of the most thoroughly studied neurotrophic factors, playing a crucial role in the survival and development of specific peripheral and brain neurons [5-10]. NGF is

produced and released by a variety of cells localized in the central and peripheral nervous system and by cells of the immune and endocrine systems [11]. Under normal conditions NGF is present in the bloodstream of rodents and humans and increases during stressful events, neuroendocrine alterations, and following neurological insults [12, 13]. Within the CNS, NGF is produced in frontal cortex, the hippocampus (HI) and the hypothalamus and exerts a trophic action on the cholinergic neurons of the basal forebrain, particularly at the level of the medial septum and the nucleus basalis of Meynert and Broca's diagonal band, both during development and in adulthood [8, 10]. Studies published in recent years have shown that alcohol abuse can also lead to immune neuroimmune-related pathologies suggesting that NGF plays a crucial role of survival, differentiation and function, not only of nerve cells, but also of immuno-competent cells [11, 12].

Aim of the present study was to address further the question of brain NGF distribution in the CNS of rodents that have experienced ethanol abuse during prenatal life and to assess whether ethanol intake affected the distribution of brain progenitor cells in postnatal life.

MATERIALS AND METHODS

Animals and housing

Adult pregnant Sprague-Dawley rats were purchased from Charles River, Italy. The animal was kept under standardized conditions with pellet food and tap water available *ad libitum*. Animal care procedures were implemented according to the intramural committee and institutional guidelines that are in compliance with the national and international laws and policies (EEC Council Directive 86/609, OJL 358, I December 12, 1987). At day 15 of pregnancy one group of rats (no. = 6) received a single administration of intragastric ethanol (20%, v/v), 4 g/kg body weight (bw), under anesthesia. A second group of pregnant rats (no. = 8) were similarly treated, but the ethanol was replaced by an isocaloric equivalent of sucrose (4% v/v, 2g/kg body weight) as control. This stage of pregnancy was chosen because previous studies have shown that brain cells are particularly affected during this particular prenatal developmental age.

At birth each litter was reduced to 8 pups and divided in two different groups: (no. = 4)-sucrose group, 0.15 ml/10 g body weight (bw) of a 5% w: v sucrose solution, equivalent caloric for ethanol intake. At 4 weeks of postnatal life, sucrose and ethanol-treated rats were deeply anaesthetized with sodium pentobarbital and animals sacrificed for biochemical and structural studies.

NGF determination

Brain tissues were dissected and processed for quantification of endogenous NGF by a highly sensitive and specific two-site enzyme immunoassay, described in detail previously [14]. Briefly, 96-well immunoplates (NUNC) were coated with 50 μ l per well of 0.4 μ g/ml monoclonal anti-mouse-betaNGF antibody 27/21 (Boheringer, Mannheim, Germany). Parallel wells were coated with mouse IgG for evaluation of non-specific signals. After an overnight incubation at room temperature (20 °C) the plates were washed three times with washing buffer and the samples were incubated in the coated wells (50 μ l each) overnight at room temperature. After an additional three washes the immobilized antigen was incubated with 0.5 mU per well monoclonal antibody 27/21 conjugated with b-D-galactosidase (Boheringer, Mannheim, Germany) for 2 h at 37 °C. The plates were again washed with washing buffer, and then finally incubated with chlorophenol-red-b-D-galactopyranoside (Boheringer, Mannheim, Germany) in substrate buffer for another 2 h at 37 °C. The colorimetric reaction product was measured at 570 nm using a microplate reader (Dynatech MR 5000, PBI International). NGF concentrations were determined

from the regression line for the NGF standard (ranging from 1.56 to 50. pg/well purified mouse NGF) incubated under similar conditions in each assay. Recombinant BDNF is not recognized in the ELISA at concentrations up to 20 ng/ml [14].

Data are presented as means \pm SEM for each tissue and animal group investigated. Means of alcoholics and controls were compared by analysis of variance (ANOVA) and statistical significance was accepted at a level of $p < 0.05$.

ChAT and low-affinity

NGF-receptor immunohistochemistry

For these studies rats were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS), pH 7.4, the brains removed and placed in a cryoprotectant solution of 20% sucrose in PBS, serial coronal sections (30 μ m) were cut on a cryostat and used for routine histological analysis and stained with toluidine blue, or for immunohistochemistry to identify NGF-target cells.

For immunohistochemical localization in brain NGF-responsive cells we used ChAT monoclonal antibody (Mab17 kindly provided by Dr. Costantino Cozzari, Institute of Cellular Biology, CNR Italy) and low-af-

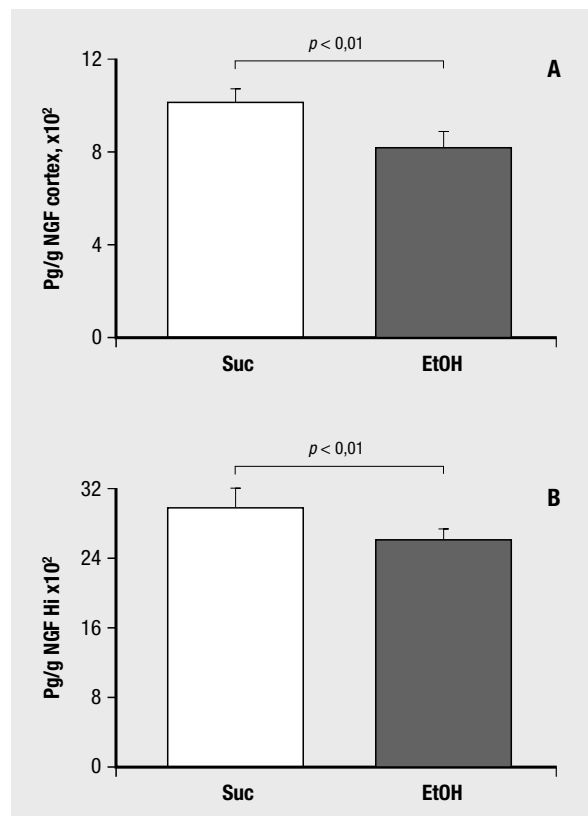


Fig. 1 | Levels of NGF in the cortex (A) and HI (B) of rats exposed at 15 days of prenatal life to sucrose (Suc) or ethanol (EtOH). Note the decrease of NGF concentration in these two brain regions after prenatal ethanol intake. These differences are statistically significant, $p < 0.01$.

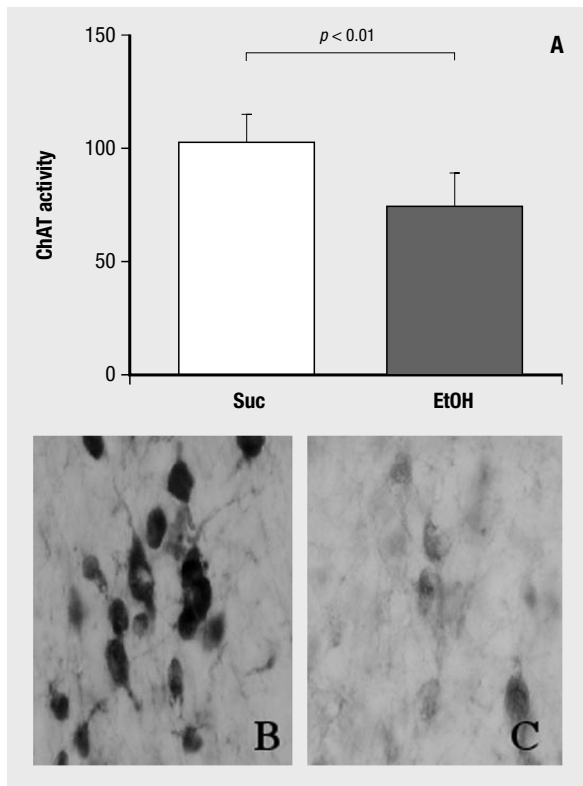


Fig. 2 | ChAT activity in the HI (A) of rats exposed at 15 days of prenatal life to sucrose (Suc) or ethanol (EtOH). Note the decrease, compared to control, of this cholinergic enzyme after ethanol intake (A). Figures B,C show ChAT-immunoreactive neurons in the septum of rats exposed to sucrose (B) or ethanol (C). Magnification X-260.

finity (p-75) monoclonal antibody, (kindly provided by Dr. E.J. Johnson from Washington University, St. Louis, MO, USA). Brain sections, including the septum, were incubated overnight with the mentioned antibodies and processed for immunoperoxidase with the ABC Vectastain Kit (Vector Lab. Inc. Burlingame, USA) following the manufacturer's instructions. Staining specificity was assessed by omission of the primary antibody and by isotypic IgG.

Quantitative analysis of ChAT- and p75-positive neurons in the basal forebrain cholinergic neurons was carried out using a Zeiss Axiophot microscope and an image analysis program (IAS 2000, Delta System, and Rome, Italy) connected to a PC computer. The number of positive neurons present in 10-matched sections/animal containing the medial septum were evaluated in experimental and control groups (no. = 4 rats/group). The average values of the pooled cell counts from each group were compared.

RESULTS

General observations

The amount of alcohol administered to pregnant rats does not cause lethal effect on the mother and no abor-

tion. At birth all pups exposed to alcohol appear normal, they weighted less compared to the pups exposed to sucrose. Two weeks after birth no differences in bw and other somatic features were observed between the two groups of rats.

Effect of ethanol on NGF level in the cortex and HI

The cortex and HI produce and store the largest amount of NGF in the brain. As shown in Fig. 1A and Fig. 1B, respectively, the concentration of NGF in these two brain structures of rats exposed to ethanol during prenatal life are significantly lower compared to the concentration of NGF in the same brain structure of non-ethanol exposed rats.

Effect of ethanol on NGF-target cells

Because one prominent action of the NGF produced in the HI is to provide trophic support of NGF-target neurons and to regulate the biosynthesis of the cholinergic enzyme ChAT, we measured the activity of this enzyme in the HI. As reported in Fig. 2A, ethanol exposure during prenatal life reduces significantly the presence of ChAT activity in this brain region. Rats exposed to ethanol also showed a marked decrease in ChAT immunoreactivity in cholinergic neurons located in the septum (Fig. 2B-C).

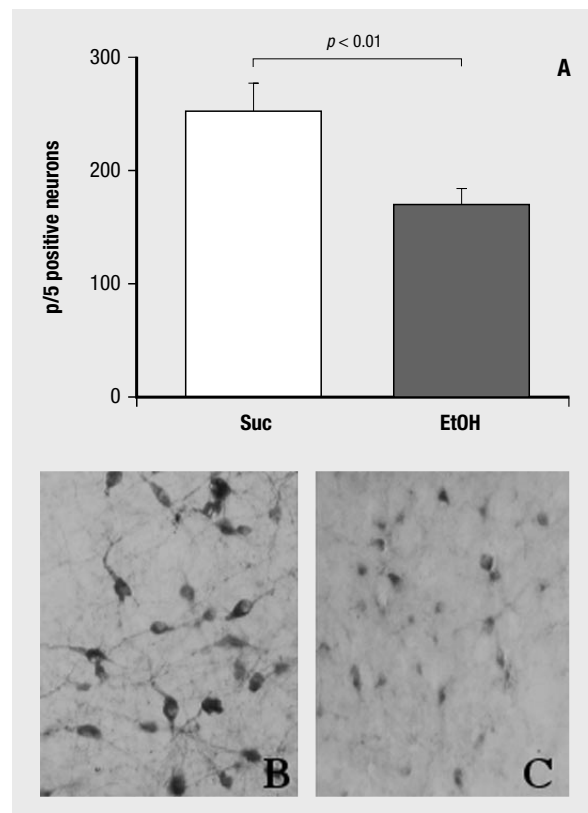


Fig. 3 | P75-immunoreactive neurons in the septum of rats exposed at 15 days of prenatal life to sucrose (Suc) or ethanol (EtOH). Note the reduction in the number of these NGF-receptive neurons after ethanol intake (C) compared to control (B). Magnification X-120.

Effect of ethanol on NGF-receptor expression

To further characterize the role of ethanol on NGF-target cells, we analyzed the presence of NGF receptor-immunoreactivity in neurons of the septum, which receive trophic support from the NGF produced in the HI. As shown in *Fig. 3A-C*, the number of p75-positive, NGF-receptive neurons located in this brain region of rats exposed to alcohol are markedly reduced compared to sucrose-exposed rat brains.

Effect of ethanol on HI progenitor cells

The dentate gyrus of the HI generates new granule neurons also during the postnatal life. To further explore the effect of prenatal ethanol intake in brain cell plasticity, we investigated whether prenatal alcohol exposure affects brain progenitor cells. Histological and immunohistochemical analyses revealed that cells located in the dentate gyrus of ethanol-exposed rats display characteristics of cells death, not evident in the dentate gyrus of rats exposed to sucrose. The effect of ethanol on cell survival is more clearly evident when rats were received BrdU, a marker for the identification of cell proliferation. As reported in *Fig. 4A*, the dentate gyrus of rats exposed to ethanol displays a reduced number of BrdU-positive cells compared to the dentate gyrus of sucrose-exposed rats *Fig. 4B*, indicating alcohol intake can interfere with the proliferating and/or survival activity of this brain progenitor cells.

DISCUSSION

The aim of this study was to investigate the effect of prenatal alcohol exposure on NGF expression in the HI and on the cholinergic enzyme, ChAT activity in septal neurons, since survival and functional activity of these neu-

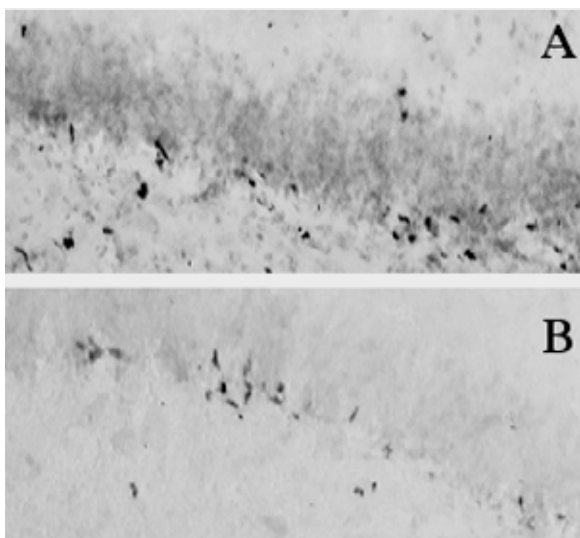


Fig. 4 | Immunolocalization of proliferating cells in the hippocampal formation showing a reduced number of BrdU-positive cells in the dentate gyrus of ethanol exposed rats (B) compared to the presence of BrdU-positive cells in the dentate gyrus of sucrose exposed rats (A). Magnification X-180.

rons are NGF-dependent [10, 13]. The result of this study showed that alcohol intake during prenatal life reduces the basal level of NGF in the HI, NGF receptor immunoreactivity in septal cholinergic neurons and the presence of ChAT activity in the septum. This observation suggests that alcohol assumption during prenatal life might impair irreversibly the NGF signaling pathways. This hypothesis is consistent with our previous studies showing that prenatal ethanol exposure reduces NGF synthesis in the HI and expression of p-75 NGF-receptor in the offspring [6, 15]. Since NGF is known to be implicated in preventing neuronal damages and in protecting cell death, the reduced synthesis and release of NGF represent negative event for neuronal growth, survival, and differentiation. Our observation therefore suggests that the reduced presence of NGF concurs to the development manifestation of neurological deficits induced by ethanol intake.

Moreover, because brain cholinergic neurons are critically involved in learning and associative processes, the reduced activity of this cholinergic enzyme indicates the existence of a link between brain NGF synthesis and release and functional activity of brain cholinergic neurons. This hypothesis is consistent with findings obtained with aged animals showing that impairments of the brain cholinergic pathway can be attributed at an insufficient production and/or uptake of NGF. Thus, the deficit in learning and cognitive abilities resulting after ethanol intake might be also associated to a reduced synthesis of NGF, a molecule which plays a crucial role in the regulation of basal forebrain cholinergic pathways and learning processes [8-10].

Several lines of evidences suggest that deficits in cell survival and/or cell migration during brain development is a prominent risk factor for the manifestation of neurological disorders in later life [16, 17]. Given the role of NGF in neurogenesis in shaping hippocampal plasticity, it is possible that NGF-hippocampal interaction underlies the decrease of cell proliferation.

We have recently reported that NGF is involved in the survival and differentiation of brain progenitor cells [18, 19]. Given that alcohol intake induces down-regulation of NGF availability, it is possible that a low presence of NGF can cause attenuation in neurogenesis. This interpretation predicts that animals treated with alcohol prior to BrdU injection should show similar changes. Our results indeed indicate that rats prenatally exposed to alcohol also display a decrease in cell proliferation in the dentate gyrus. The observation that reduced NGF presence in the HI of alcohol-exposed rats prompted us to investigate whether low presence of NGF might reduce the properties of progenitor cells present in the dentate gyrus. The observation that the number of these cells are reduced in the dentate gyrus of rats exposed during fetal life to alcohol suggests that the neurotoxic action of alcohol include also impairment of functional activity of brain progenitor cells, occurring, most probably through a mechanism involving down-regulation of NGF and NGF-receptors expression. The report that ethanol exposure of neonatal rats reduced the expression of both p75 and trkA NGF receptors on the Purkinje cell dendrites further supports the hypothesis that ethanol in-

terferes with neurotrophic support of these brain cells by reducing the levels of available NGF receptor. A number of studies have reported that injured brain induced by neurotoxic compounds or surgery intervention can lead in HI-based learning, it is reasonable to hypothesize that one mechanism by which alcohol affects neurogenesis is to provoke changes in NGF synthesis.

Alcohol consumption is known to be associated with decreased cellular immune response and increased susceptibility to infections [20, 21]. It is also known that a number of immuno-competent cells are able to produce NGF and/or are receptive to the action of this growth factor [22, 23] and that ethanol intake, both during prenatal, or postnatal life can severely affect the functional activity of the immune system. We have recently reported that human macrophages exposed *in vitro* to ethanol are characterized by altered ability to produce and to respond to the action of NGF [22]. This finding along with the observations that the exposure to etha-

nol induced in M/M cell population a sharp decrease of phagocytosis and bactericidal activity [24] suggests that the lack of NGF synthesis might lead to an impairment of innate immunity in alcohol addiction.

In conclusion, the evidence that alcohol-induced decrease of hippocampal NGF down-regulates ChAT activity and reduces cell neurogenesis in the dentate gyrus, suggests that the altered brain basal level of NGF need to be considered as an important mechanism implicated in neurological and neuro-immune based deficits during development and most probably also in adult life.

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