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Influence of ethanol consumption on brain nerve growth factor and its target cells in developing and adult rodents

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Summary. - In the present study the effect of alcohol consumption on brain nerve growth factor (NGF) and the NGF-target cells of adult male rats and pups exposed prenatally to ethanol is evaluated. It is hypothesized that NGF, a trophic agent for the survival and maintenance of basal forebrain cholinergic neurons, might be affected by the neurodegenerative events which occur during ethanol consumption. To test this hypothesis, we used adult rats exposed to ethanol for 16 weeks and pregnant rats exposed to ethanol for six days. Our experiments show that ethanol ingestion causes a reduction of NGF in the hippocampus, of choline acetyltransferase activity in both the septum and hippocampus, and in the distribution of NGF receptor in the basal forebrain cholinergic neurons. The results indicate that the damaging effect of alcohol on forebrain cholinergic neurons is also associated with impairment of central NGF-target structures.

Key words: ethanol, NGF, brain, rodents, choline acetyltransferase (ChAT), forebrain cholinergic neurons (FCN).

Riassunto *(Effetti dell' etanolo sui livelli dell'NGF e sulle cellule NGF recettive nel sistema nervoso centrale del ratto).* - Sono stati studiati gli effetti di un abuso di alcool sui livelli del fattore di crescita della cellula nervosa e sulle cellule bersaglio di tale fattore nel sistema nervoso centrale di ratti adulti e di ratti neonati sottoposti a trattamento prenatale con etanolo. E' stato ipotizzato che il NGF, fattore neurotrofico responsabile della sopravvivenza e del mantenimento dei neuroni colinergici del prosencefalo basale, possa essere coinvolto durante gli eventi neurodegenerativi che si osservano in seguito ad un abuso di etanolo. Per verificare questa ipotesi, abbiamo utilizzato sia ratti adulti sottoposti a somministrazione cronica di etanolo per 16 settimane, sia ratti neonati sottoposti a trattamento prenatale con etanolo. I nostri esperimenti hanno messo in evidenza che la somministrazione cronica di etanolo induce una riduzione dei livelli di NGF e della colina acetiltransferase in alcune regioni del sistema nervoso centrale oltre a una riduzione della distribuzione del recettore per il NGF nei neuroni del prosencefalo basale. Questi esperimenti indicano che gli effetti dell'etanolo sui neuroni colinergici del prosencefalo basale sono associati con un'alterazione della sintesi di NGF e un indebolimento delle strutture bersaglio del NGF.

Parole chiave: etanolo, NGF, sistema nervoso centrale, roditori, colina acetiltransferasi (ChAT).

Introduction

Ethanol abuse produces a wide range of effects in the central nervous system (CNS). Although there are no specific CNS "receptors" for ethanol, this agent is able to penetrate neuronal cell membranes.

During pregnancy, alcohol assumption exerts a detrimental effect on the growth and development of the fetus and newborn human and animal subjects [1]. Alcohol, like other drugs with a molecular weight between 600 and 1000 Da, passes freely across the placental barrier and concentrations of alcohol in the fetus are at least as high as in the mother [1, 2]. The common pattern of mental and physical abnormalities in humans has been termed collectively the "foetal alcohol syndrome" (FAS). Mental retardation is the most pronounced behavioural effect of this syndrome [3, 4]. Although biochemical, hormonal and neuronal alterations have been implicated in these deficits, the mechanism of the alcohol pathogenesis remains unclear. One of the neuropathological and neurochemical hallmarks of ethilism is a selective alteration of basal forebrain cholinergic neurons (BFN) such as a reduction in cell size, loss of dendritic branching or a decrease of choline acetyltransferase (ChAT) enzymatic activity [5-7].

Several *in vitro* and *in vivo* studies have established that cholinergic neurons of the BFN are responsive to nerve growth factor (NGF) [8-11]. It has been shown that NGF administration to developing rodents prevents the degeneration of sensory and sympathetic neurons occurring in this period [12, 13].

Moreover, NGF is able to prevent the progressive loss of cholinergic neurons occurring as a result of experimentally-induced axotomy [14] or in neurodegenerative diseases associated with aging [15, 16].

These observations led us to investigate whether this neurotrophic factor, by influencing neuronal survival and through its cholinotrophic effects, can prevent or

counteract the neurotoxic effects of ethanol in both the developing and the adult stages. The results of chronic alcohol intake in adult rats and preliminary results regarding the effects of ethanol during pregnancy are presented here.

Materials and methods

Animal breeding and alcohol administration

Adult male and nulliparous Sprague-Dawley rats were used for this study. The animals were kept on a standard rat food diet and on a 12:12 L/D cycle. Under mild ether anesthesia, 24 adult male rats for each group were gavaged once a day for 16 weeks with 40g/kg of alcohol or with an isocaloric sucrose solution.

Females were gavaged once a day with 30% v/v ethanol (group E) made from 95% stock reagent in deionized water on days G 12-18 or with an isocaloric sucrose solution (group S). Group C were not gavaged and were allowed free access to food. More females were assigned to group E than the other groups to compensate for the loss of dams in this group due to ethanol toxicity.

Birth was designated postnatal day-0 (PNO); on this day all offspring were examined for external abnormalities and the litters were weighed and adjusted to four males and four females where possible. Physical characteristics - weight, age of eye opening and of hair growth - were measured. The rats were sacrified for biological and biochemical studies at PN3, PN7, PN14 and PN21.

NGF bioassay

The cortex and the hippocampus were omogenated in 2:1 water volume, centrifuged at 12.000 rpm for 10 min and the supernatant was immediately used for biological assay. Neurons dissociated from the rat superior cervical ganglion (SCG) were used because more than 90% are receptive to NGF [17,18], whereas other growth factors present in the HI have no effect on sympathetic neurons. Nerve cells from SCG of 17-18-day-old rat fetuses were mechanically dissociated and cultured in collagenated Falcon tissue dishes containing Dulbecco's basal medium only, or the medium with additional amounts of NGF. In the absence of NGF, only a small number of neurite-bearing cells appeared in the culture, whereas the addition of as little as 0.02ng of NGF yielded a detectable increase in number of neurites. By adding increasing amounts of NGF, the number of responding cells also increased, resulting in a dose-response curve which was used as a standard curve for evaluating the amount of NGF present in the tissue samples to be tested [19].

In order to assess whether the neurite-promoting activity was caused by NGF or by other factors known to be present in the brain, extracts were preincubated with 10 mg of purified NGF antibodies. All samples were tested in triplicate and presented ± SEM.

Immunohistochemistry

Rats treated with alcohol and sucrose were perfused under Nembutal anesthesia via the ascending aorta with 200 ml of 0.1 M PBS, pH 7.4, containing 0.5% sodium nitrite, followed by 500 ml of 4% paraformaldehyde in phosphate buffer. The brains were then removed, immersed in the same fixative for six more hours, and then in 20% sucrose in PBS for 24 h. Each brain was then mounted on the stage of a freezing microtome and 60 consecutive frontal sections of 40 cm thickness were cut, spanning from the striatum to the caudal hypothalamus, as listed in Paxinos and Watson [20] and immunostained for ChAT and NGF receptor (NGF-r) as described previously [17]. For routine histology, sections were stained with toluidine blue and examined under a standard Zeiss microscope.

Biochemical determinations

ChAT enzymatic activity was determined in the entire cortex, HI, striatum, ST and cerebellum. After an overdose of ether, brain tissues were quickly dissected out and ChAT activity determined according to the method of Fonnum [21]. The values are expressed as units of enzymatic activity per milligram of tissue. One unit of ChAT was defined as a micromole of acetylcholine formed per minute at 37 °C.

Statistical analysis

The data are presented as the mean ± SEM. The significance of differences between groups was assessed by Student's t test.

Results

Chronic consumption of ethanol in adult rats

No somatic or behavioural differences were noticed between the two groups. Likewise, no gross pathology was observed in the brain, digestive tract, liver or lymphoid tissues after alcohol or sucrose consumption. Sucrose treatment did not affect NGF-r expression of ChAT enzymatic activity when compared to untreated controls.

Biological assays showed that the level of NGF in the HI of alcoholic rats was reduced by as much 45%, although no differences were observed in the cortex. The induced neurite outgrowth was inhibited by NGF antibodies, suggesting that the observed effect was caused by NGF and not by other growth factors.

The ChAT enzymatic activity in forebrain cholinergic tissues is reduced in the septum (- 23%, $p < 0.01$) of alcohol-treated rats, the HI (- 15%, $p < 0.02$) and the cortex (- 9%, p = not significant). In the striatum no differences were observed.

Morphometric examination carried out on ChAT and NGF-r immunostained forebrain cholinergic neurons (FCN) showed that neuron and neurite profiles localized in the ST and NB of alcohol-exposed rats are less densely immunoreactive. Following alcohol consumption, many ChAT-positive neurons appear vacuolated and shrunken in comparison to the FCN of sucrose-treated or untreated controls. Likewise, FCN of the medial septum expressing NGF-r show loss of immunoreactivity following chronic consumption of alcohol.

The number of densely stained ChAT immunoreactive neurons in the ST is reduced by 26% and by 16% in the NB of alcohol-exposed rats.

The reduced immunoreactivity and ChAT enzymatic activity caused by alcohol consumption are re-established following intracerebroventricular (icv) administration of NGF.

Ethanol administration during pregnancy: preliminary results

The measure of body weight from birth to weaning in rats treated prenatally with alcohol shows significant reduction at PN6 ($p < 0.0004$), PN8 ($p < 0.005$) and PN14 ($p < 0.005$) ($n = 6$). The ethanol pups show, although not significantly, a delay in eye opening and hair growth.

The biological assays show that there is no difference between experimental and control pups, in the level of NGF in the cortex and hippocampus at PN3, while we observed a slight difference in the level of NGF in the hippocampus of prenatally exposed rats at PN7 (0.7 ng/ml versus 1.2 ng/ml of the control pups; $n = 4$). ChAT enzymatic activity, measured at different development stages, reveals a reduction of the enzymatic activity in prenatal ethanol-exposed pups at PN7 in the striatum (1.25 U/ml *vs* 2.5 U/ml of controls) and in the septum (2.7 U/ml *vs* 3.3 U/ml; $n = 4$), while there is no difference in the same regions later in development.

Discussion

The main object of this investigation was to study the correlation between alcohol ingestion and NGF and NGF target cells in the CNS of adult and young rats.

Several studies have shown that chronic ethanol consumption both in human and animal models causes neurochemical, behavioural and neuropathological effects in the central nervous system [22]. Chronic ethanol consumption has been reported to cause enduring cognitive impairments. These cognitive disfunctions have been attributed to deficiencies in the cholinergic and aminergic neurotransmitter systems as well as to hippocampal damage. For example, chronic ethanol intake causes a loss of neuronal cell bodies in the hippocampus, the basal forebrain neurons and the cerebellum of young and adult rodents [22-25]. It has been shown that prolonged oral administration of ethanol profoundly reduces markers of cholinergic function in the brain, including aceltylcholine (ACh) content, release and synthesis, ChAT and acetylcholine esterase (AChE) activities and Ch-uptake. These changes occur widely throughout the brain and increase with the duration of ethanol intake [23]. Moreover, it has been demonstrated that in the hippocampus the decreased dendritic spine density, observed after long-term ingestion of ethanol, regains its normal appearance when ethanol is withdrawn [25].

These results, although providing evidence that nerve cells of the CNS have the ability to overcome the neuroanatomical and neurochemical damage caused by alcohol ingestion, also suggest a possibility that ethanol might affect the synthesis of neurotrophic factors present in the CNS.

Since FCN express NGF-r and are receptive to the action of NGF [10, 26], the question as to whether a link exists between the deficit induced by alcohol intake and brain NGF levels was raised. Our studies clearly indicated that ethanol, both in the adult and during development, affects NGF level in different brain regions, thus suggesting the hypothesis that ethanol interferes with the synthesis and/or activity of NGF [27, 28]. This hypothesis was further strengthened by the observation, reported in a previous *in vitro* study, that alcohol intake results in a dose-dependent inhibition of NGF-induced neurite outgrowth [29] and that *in vivo* alcohol exposure induces deficits on young and adult Purkinje cells [23], which are known to express NGF-r.

The neurotoxic effects of prenatal exposure to ethanol on the cholinergic system have also been examined by other authors. Using the developing chick embryo as the animal model, it has been shown that NGF is able to enhance the expression of cholinergic neurons in both the brain and spinal cord of experiment animals, shown by higher levels of ChAT activity than in controls. Moreover, co-administration of this growth factor with ethanol prevents the ethanol-induced decrease in ChAT activity [30].

These findings suggest that the impairment of cholinergic enzymatic activity and NGF-r expression, observed after chronic alcohol intake and prenatal exposure to ethanol, might be the result of a reduced brain NGF level. The fact that the decrease of ChAT activity in the ST and NB of adult rodents is restored after icv injection of NGF [28] supports this hypothesis.

In conclusion, although both our results and those of others suggest that alcohol ingestion causes specific deficits in the CNS, and alteration in brain NGF level, it

should be taken into consideration that a high level of circulating alcohol is associated with a broad spectrum of actions affecting the nervous, endocrine and immune systems. More systematic studies are therefore required for a thorough understanding of the dynamic destructive and compensatory changes that are produced in the CNS following alcohol intake and to determine the role that reduced level of NGF plays in the neuropathogenesis of ethilism.

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