Research Report

The vasopressinergic innervation of the lateral septum of the rat after chronic alcohol consumption and withdrawal

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Abstract

We have recently reported that ethanol ingestion induces morphological changes in the vasopressinergic neurons of the supraoptic nucleus and that withdrawal from alcohol partially reverses these alterations. Since the production of vasopressin is not restricted to the magnocellular neurons of the hypothalamus, we investigated the effects of long-term ethanol intake and withdrawal on the lateral septum, an area heavily innervated by vasopressinergic fibers. Besides, as ethanol leads to a decrease of the plasma levels of testosterone, a hormone which plays a pivotal role in the development and maintenance of the vasopressinergic innervation of the lateral septum, we included groups of alcohol-fed animals submitted to testosterone replacement both in physiological and supraphysiological doses. In ethanol-treated rats there was a marked reduction in the number of vasopressin-immunoreactive fibers in the lateral septum. Following ethanol withdrawal a partial recovery in the number of vasopressin-immunoreactive fibers was observed. In both groups of ethanol + testosterone-treated animals the vasopressinergic innervation was increased when compared to the alcohol-fed group, although a complete reversal was not achieved. Therefore, two mechanisms might be regarded as underlying the impoverishment of the vasopressinergic innervation in the lateral septum after prolonged alcohol consumption: alcohol-induced cell death in the bed nucleus of the stria terminalis, from where these fibers arise, and/or alcohol-induced decrease in testosterone plasma levels.

Key words: Vasopressin; Lateral septum; Ethanol; Withdrawal; Immunocytochemistry; Rat

1. Introduction

Chronic ethanol consumption induces profound alterations in the structure of the central nervous system (CNS) which comprise degenerative changes in cell organelles and neuronal processes, loss of synaptic contacts and cell death [3,4,30]. From a functional point of view, prolonged ethanol ingestion is known to impair the mechanisms of synthesis, transport and release of peptides in the CNS [18,28,32]. We have recently demonstrated, furthermore, that under conditions of chronic ethanol exposure the neurohypophysial hormone-producing magnocellular neurons of the hypothalamus undergo neuronal degeneration and a marked enlargement of their cell bodies [25]. This

increase in neuronal volume, which was proved to be partially reversible after ethanol withdrawal, was particularly evident in cells secreting vasopressin (VP) [38].

In addition to the classic endocrine functions of VP, mediated by V2 receptors [23], it is now well established that this peptide also acts as a neurotransmitter at V1 receptors in the CNS [2,20], thereby influencing several central processes mainly related to adaptive behaviour [8,14,20,24]. Interestingly, some reports advanced that the V1 receptor-mediated action in the lateral septum (LS) is crucial for the maintenance of ethanol tolerance [40], therefore suggesting that chronic ethanol ingestion might alter VP synthesis and release [40,41].

In order to investigate this hypothesis we selected the LS because this component of the limbic system is heavily innervated by VP fibers which originate from the bed nucleus of the stria terminalis (BNST) [1,10,37,39]. Furthermore, the VP axons in the LS

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display a sexual dimorphic pattern of innervation [11] as they are much denser in males than in females, which was demonstrated to be dependent on the imprinting effects of androgens during development [9] and adulthood [12,13]. Because ethanol is known to decrease plasma levels of testosterone [6,7,16,42], we thought it would be of interest to ascertain if long-term ethanol ingestion leads to alterations in the central VP innervation and, if so, to evaluate the degree of reversibility of such alterations after ethanol withdrawal or hormonal replacement.

2. Materials and methods

Male Wistar rats derived from the colony of the Gulbenkian Institute of Science (Oeiras, Portugal) were maintained throughout the experiment under standard laboratory conditions (light/dark cycle of 12:12 h and temperature of 20–22°C). Solid diet and water were available *ad libitum* until they were 2 months old. At this age rats were assigned to five experimental groups and placed in individual cages. At 12 months of age the groups were reduced to a standard composition of four rats each, by selecting animals at random. Rats were treated as follows:

2.1. Ethanol-treated group

Rats were given as the only available liquid source a progressively increasing aqueous ethanol solution: starting with a 5% (v/v) solution, the final 20% (v/v) solution was reached 2 weeks later and was maintained up to 12 months of age. Food was freely available throughout the experimental period. The amounts of food and ethanol solution consumed were measured every other day.

2.2. Pair-fed control group

Animals with free access to water were given an amount of dry food which was equivalent to that consumed by animals of the ethanol-treated group plus a quantity which provided a caloric intake equal to that supplied by the quantity of ethanol solution drunk by the ethanol-treated rats. The additional amount of pellets was calculated taking into account the caloric value of ethanol (1g–7 kcal) and the volume of alcohol consumed by ethanol-treated rats (10 ml ethanol – 7.9 g).

2.3. Withdrawal group

Rats were ethanol-treated, as described above, up to 9 months of age and thenceforward switched to standard laboratory food and water; the shift from the ethanol solution to water was performed gradually over a 2-week period by progressively reducing the ethanol concentration of the drinking solution.

2.4. Ethanol + physiological dose of testosterone treated group

Animals were ethanol-treated up to 12 months of age as described above. Four weeks before the end of the experiment a capsule made of a Silastic tube filled with testosterone propionate (Sigma; 2.5 cm; 1.5 mm i.d.; 2.4 mm o.d.) was implanted subcutaneously under ether anesthesia, an amount which is known to restore the normal plasma levels of testosterone [13,15].

2.5. Ethanol + supraphysiological dose of testosterone treated group

Animals were ethanol-treated up to 12 months of age. Four weeks before the end of the experiment three capsules, similar to the above described, were implanted subcutaneously under ether anesthesia.

In all groups studied liquids consumed were supplemented with 300 mg/100 ml of vitamins (Vitamin Diet Fortification Mixture, ICN) and 500 mg/100 ml of minerals (Salt Mixture, ICN). Body weights were recorded on the day of sacrifice.

Rats were anesthetized with ether and perfused transcardially with 4% paraformaldehyde in phosphate buffer at pH 7.6. It should be emphasized that with the fixative used the quality of immunostaining in the LS was similar to that obtained with other fixatives commonly employed to stain this area [11–13], namely the mixture of 2.5% glutaraldehyde with 1% paraformaldehyde or simply 5% glutaraldehyde. Brains were removed from the skulls and immersed for 48 h in the same fixative; then, 50 μ m thick serial coronal sections were cut on a Vibratome.

Sections containing the LS, selected according to the Paxinos and Watson atlas [31], were processed immunocytochemically using the Avidin-Biotin technique. The rabbit VP antiserum (Truus 18-09; MBR) was used at a dilution of 1:30,000 in 0.05 M Tris (Sigma), pH 7.6, containing 0.5% Triton (BDH). Sections were incubated overnight at 4°C. Biotinylated goat anti-rabbit antibody (Vector) at 1:400 dilution was used as the secondary antibody. Sections were then treated with Avidin-Biotin horseradish complex (Vector) diluted 1:800. In these two last steps the incubation was carried out for 1 h at room temperature. Following the treatment with the peroxidase complex, sections were incubated for 10 min in diaminobenzidine (Sigma) to which 0.01% H₂O₂ was added. Sections were rinsed for at least 30 min between each step with 0.05 M Tris. Control incubations without primary antiserum or with preabsorbed antiserum always gave negative results. All procedures were performed on a rocking table. Stained sections were mounted on gelatin-coated slides and air dried. They were then dehydrated and coverslipped using 'Eukitt'.

The seminal vesicles of all animals were dissected and weighed after the removal of their contents. The weight of the seminal vesicles was used as a reliable, although indirect, indicator of long-term plasma testosterone levels [13,34].

The density of VP-containing fibers in the LS was estimated independently by two investigators, in a blind study, using a semi-quantitative approach. The following four categories of fiber density were distinguished: +, very few fibers; ++, moderate number of fibers; +++, fibers forming a network; + + + +, fibers forming a dense network.

To test for the effect of treatment on the weight of the seminal vesicles a one-way analysis of variance (ANOVA) was performed. Subsequent multiple comparisons were performed using linear polynomial contrasts. Differences were considered to be significant at $P < 0.05$.

3. Results

The pattern of VP-immunostaining in the brain of the normal rat was identical to that described by other authors [1,10,37,39]. VP-containing fibers were visible in the LS of all groups analysed. In general, small variations in the density of the fiber network were noted within each group, although individual differences were larger in the experimental groups.

Fig. 1. Photomicrographs of the lateral septum of control (A), ethanol-treated (B), withdrawal (C), and ethanol + testosterone in physiological doses (D) rats stained for vasopressin. Note that the fiber density (arrows) and boutons (arrowheads) are markedly decreased in the ethanol-treated rat when compared with all other animals. In the withdrawal and ethanol + testosterone animals the fiber density and boutons is smaller than in the control. A slight increase in the density of boutons can be seen in the ethanol + testosterone rat when compared with the withdrawal. LV, lateral ventricle. $\times 270$.

Table 1

Vasopressin (VP) fiber density in the lateral septum and weight of seminal vesicles (SV) of male rats under various experimental conditions

Treatment	VP fiber density	SV
Control (C)	++++/++++/++++/++++	566.5 $\pm$ 39.1
Ethanol (E)	+ / + + / + / +	308.5 $\pm$ 29.7
Withdrawal (W)	+++ / +++ / +++ / +++	464.3 $\pm$ 72.6
Ethanol + Phys. Test. (E + T1)	+++ / +++ / +++ / +++	657.3 $\pm$ 77.3
Ethanol + Supraphys. Test. (E + T2)	+++ / ++++ / ++++ / ++++	939.5 $\pm$ 137.0

Each entry of '+' signs represents the VP fiber density in one rat: +, very few fibers; ++, moderate number of fibers; +++, fibers forming a network; +++, fibers forming a dense network. The weight of seminal vesicles, expressed in mg, are presented as mean $\pm$ S.D. Post hoc linear polynomial contrasts: C vs. E, C vs. E + T2, E vs. E + T1, E vs. E + T2, W vs. E + T2, E + T1 vs. E + T2, $P < 5 \times 10^{-4}$; W vs. E + T1, $P < 0.001$; E vs. W, $P < 0.005$; C vs. W, C vs. E + T1, $P < 0.05$.

In control animals a dense network formed by numerous VP-immunoreactive fibers was readily visible in the LS (Fig. 1A). Conversely, in alcohol-treated animals the number of VP-immunoreactive fibers was markedly reduced and a fiber network was not noted (Fig. 1B). The pattern of innervation of the LS in withdrawn animals (Fig. 1C) differed from that observed in ethanol-treated and control rats: in the withdrawal group the number of labelled fibers was markedly higher than in ethanol-treated rats but smaller than in control animals. A similar, although more conspicuous finding was depicted from the analysis of the LS from animals submitted to ethanol + testosterone treatment in physiological doses (Fig. 1D). The treatment with a supraphysiological dose of testosterone did not produce any further increase in the density of the VP fiber network of the LS. It must be stressed, furthermore, that in both testosterone-treated groups and in withdrawn rats the fiber network did not display the complexity observed in control animals.

The weights of the seminal vesicles are shown in Table 1. ANOVA revealed a significant effect of treatment on the weight of the vesicles ($F = 45.93$; $P < 5 \times 10^{-5}$). Alcohol-fed animals had the lightest seminal vesicles, which reflects low plasma levels of testosterone. A significant recovery in the weight of the vesicles was observed in withdrawn animals. The weight of the seminal vesicles of the rats submitted to physiological doses of testosterone was higher than that of controls, which suggests that the plasma levels of testosterone reached at least normal levels following the treatment employed. Because the seminal vesicles of the animals injected with higher doses of testosterone were heavier than those of animals treated with physiological doses, it is likely that, in the first named group, the plasma levels of this hormone are higher too.

4. Discussion

The VP neurons of the BNST and their projections to the LS display sexual dimorphic characteristics which are known to be affected by testosterone levels during development and adulthood [9,12,13]. Following alcohol consumption plasma testosterone levels are decreased [6,7,16,42]. This alteration has been ascribed to changes in the testicular Leydig cells [16], as a consequence of increased cellular peroxidation [33] and/or to a reduction in the circulating levels of luteinizing hormone (LH) [42], resulting from the alcohol-induced decrease in the synthesis and release of LH-releasing hormone [6]. Due to the role played by testosterone in the regulation of VP synthesis [26,27], it seems likely that the decreased VP innervation of the LS in alcohol-treated rats might depend on the reduction in

the levels of testosterone which occurs in this situation. The reduction in the weight of the seminal vesicles of alcohol-fed animals, suggesting a long-lasting decrease in testosterone levels, lends strong support to this hypothesis. In keeping with this assumption is the partial recovery of the VP innervation in the LS of withdrawn animals in which higher levels of testosterone are likely to be present, as suggested by the increased weight of their seminal vesicles.

However, it is not likely that the amelioration observed in the VP innervation of the LS of withdrawn animals commensurates with the length of the withdrawal period. Indeed, none of the causes which could determine the axonal regrowth are expected to remain active long after abstinence is settled. On one hand, there is evidence that serum testosterone levels are restored after 2 weeks of withdrawal [5]; on the other, it is not conceivable that in the LS a compensatory axonal regrowth takes place following an eventual alcohol-induced degeneration. Indeed, a significant neuritic reorganization was never detected in any of the numerous brain regions studied in this condition, even after 6 months of abstinence [3,30]. It cannot be neglected, furthermore, that the period of ethanol intake differs between alcohol-treated and withdrawn animals, and that this could interfere with the outcome of the results. However, there is ample evidence demonstrating that most of the deleterious effects of prolonged ethanol ingestion are already established after 4 months of treatment [4,30].

In an attempt to further clarify the role of low testosterone levels as an underlying cause for the alcohol-induced changes on VP innervation of the LS, a group of alcohol-treated animals was submitted to physiological doses of testosterone. Here, a striking increase in the density of VP innervation of the LS was found, which reveals that even in the mature brain and under the action of an aggressive agent such as ethanol, neurons projecting to this area display remarkable recovery capabilities. Our finding partially fits previous observations made in the LS of long-term castrated and aged rats which lose almost all visible VP innervation; in fact, following an identical testosterone treatment, i.e. equal doses and equal duration, these animals display a VP fiber network similar to that of young male rats [13,15].

However, the density of the network of alcohol-fed animals treated with physiological doses of testosterone is still decreased as compared to control animals. To test for a dose-dependent phenomenon, a group treated with a supraphysiological dose of testosterone was evaluated. As no further increase in the density of the vasopressinergic network was observed, it can be inferred that the testosterone-induced reversal effect is not an additive phenomenon. In other words, it is not conceivable that the lack of a complete

restoration of VP immunoreactive fibers in the LS following alcohol treatment can only be attributed to a reduction in VP synthesis resulting from low plasma levels of testosterone. We would argue, rather, that it might also depend on neuronal degeneration in the BNST, from where most of these axons derive. To corroborate this assumption an unbiased stereological study of this nucleus is required, which cannot be performed in immunostained material.

Long-term ethanol consumption is known to alter neuronal structure and function, although a marked regional vulnerability has been described [3,4,25,28,30]. As regards neurotransmission, there is evidence that alcohol intake interferes with the synthesis of neurotransmitters in general: whilst the synthesis is decreased in most of them [28], in others, such as opioid peptides, it is increased [19,36]. Interestingly the VP system was found to be deeply affected by alcohol intake [18,19,22,35] and, furthermore, its extrahypothalamic component is known to be more vulnerable than the hypothalamic one [18]. In fact, the alcohol-induced damage in the BNST is more striking than that observed in the paraventricular (PVN) and supraoptic (SON) nuclei [18]. However far we may yet advance an explanation for these regional differences, it is tempting to suggest that the damage inflicted in the VP component of the BNST derives both from a direct effect of alcohol and from the alcohol-induced testosterone decay. On the contrary, in the PVN and SON, testosterone-induced effects are apparently non-relevant. Be this as it may, this assumption fits the idea that alcohol effects upon the VP innervation of the septum is a complex and non-specific phenomenon.

It has been suggested that the presence of normal concentrations of VP in certain regions of the brain, namely in the septal area, facilitates both consolidation and retrieval of memory [14,24]. Hence, it seems sound to suggest that the reported reduction of VP in the LS might concur to explain the impairment of mechanisms of learning and memory, known to occur after prolonged ethanol ingestion [17,29]. Consequently, it is worth noting that changes in the VP extrahypothalamic system may also play a role in the mechanisms of ethanol tolerance, as it is well established that this situation very much depends on the referred cognitive functions [41].

The underlying causes for the development of tolerance after ethanol intake has long captured the attention of many researchers. In spite of this, the fine mechanisms of its development still remain cryptic. Hoffman and colleagues first advanced that VP would probably play a key role in the maintenance of tolerance to ethanol [21]. However, in a subsequent report [18], in which *in situ* hybridization techniques were employed, these authors claimed that following alcohol consumption the synthesis of VP in the BNST was

decreased. This observation led them to question their first hypothesis and to suggest that the presence of high levels of VP in the LS would not be required to modulate tolerance. Our present results lend support to this hypothesis.

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