Effects of Chronic Alcoholic Disease on Magnocellular and Parvocellular Hypothalamic Neurons in Men

Abstract

Although numerous data showing severe morphological impairment of magnocellular and parvocellular hypothalamic neurons due to chronic alcoholic consumption have been gathered from animal experiments, only one study (Harding et al., 1996) was performed on post mortem human brain. This study showed a reduction in the number of vasopressin (VP)-immunoreactive neurons in the supraoptic (SON) and paraventricular (PVN) nuclei, but did not provide any data regarding the effect of chronic alcohol intake on human parvocellular neurons. In order to assess whether the changes observed in the animal model also occur in humans and provide a structural basis for the results of clinical tests, we performed immunohistochemical and morphometric analysis of magnocellular (VP and oxytocin, OT) and parvocellular (corticotropin-releasing hormone, CRH) neurons in post-mortem brains of patients afflicted with chronic alcoholic disease. We analyzed 26 male alcoholics and 22 age-matched controls divided into two age groups - "young" (<40 yr) and "old" (>40 yr). Hypothalamic sections were stained for OT, VP, and CRH. The analysis revealed: 1) decrease in VP-immunoreactivity in the SON and PVN as well as OT-immunoreactivity in the SON in alcoholic patients; 2) increase in OT-immunoreactivity in the PVN; 3) increase in CRH-immunoreactivity in parvocellular neurons in the PVN. Furthermore, the proportion of cells containing CRH and VP was increased in alcoholics. These findings indicate that chronic alcohol consumption does indeed impair the morphology of magnocellular neurons. The enhancement of CRH-immunoreactivity and increased co-production of CRH and VP in parvocellular neurons may be due to a decline in glucocorticoid production, implied by the hypoplastic impairment of adrenal cortex we observed in alcoholics during the course of this study.

Key words
Vasopressin - oxytocin - corticotropin-releasing hormone - human - alcoholic disease
Chronic alcohol exposure profoundly modifies the morphology and function of certain brain structures [1], including hypothalamic neuroendocrine centers. Due to the side effects of alcohol intake – dehydration and change in serum osmolarity – many studies focus on the hypothalamic neurohormone vasopressin (VP), which is synthesized in magnocellular hypothalamic nuclei, supraoptic (SON) and paraventricular (PVN) nuclei, and released into peripheral blood circuitry from the posterior pituitary lobe. A number of studies in rodents demonstrated the inhibition of VP synthesis by chronic alcohol intake [2,3]. In the rat model, prolonged ethanol exposure leads to a selective loss of VP-ergic neurons in the SON [4] and PVN [5], and an elevation in the VP mRNA levels in the remaining magnocellular neurons [4,6]. In the only study on the effects of chronic alcohol abuse in humans, Harding and colleagues [7] demonstrated a considerable loss of VP-immunoreactive neurons in magnocellular nuclei of alcoholics. After prolonged alcohol intake, plasma levels of VP remained unchanged in both humans [8-10] and rodents [4], but in humans, VP response to an osmotic challenge was reduced [8]. The effects of alcohol on the second population of magnocellular neurons, which produce oxytocin (OT), are much less known. No changes in OT mRNA have been reported in rats subjected to prolonged ethanol exposure [5]. In contrast, plasma levels of OT are elevated in human alcoholics [10]. Post-mortem morphological analysis, however, was not performed.

Ethanol also has an effect on parvocellular hypothalamic neurons. Of particular interest are those that produce corticotropin-releasing hormone (CRH) [11,12]. As a central neurohormone of the hypothalamic-pituitary-adrenal (HPA) axis, CRH triggers adrenocorticotropic hormone (ACTH) and glucocorticoid secretion in response to a variety of acute and chronic stimuli [13]. CRH is co-produced with VP, which potentiates effects of CRH on pituitary corticotrophs, especially under chronic stress paradigms [14-16]. Prolonged alcohol intake in rats blunts the expression of both CRH and VP mRNAs in the parvocellular PVN [17]. In actively drinking men, lower basal plasma levels of stress hormones were detected when compared to non-alcoholics, while alcoholics showed a blunted ACTH response in the CRH stimulation test [18]. However, there is no evidence that ethanol induces death or damage in CRH neurons [17].

In order to provide a structural basis for the results of clinical tests and to compare animal models with changes in humans, we performed a morphological analysis of magnocellular (VP and OT) and parvocellular (CRH) neurons in post-mortem brains of patients afflicted with chronic alcoholic disease. We studied VP- and OT-immunoreactivity in magnocellular neurons from the hypothalamic nuclei and in the posterior pituitary. To characterize the activity of the HPA axis in alcoholics, we analyzed the content of CRH-immunoreactive product and its co-localization with VP in the hypothalamic PVN, as well as the morphology of the adrenal gland.
Sections of hypothalami and adrenal glands were deparaffinized in xylene and rehydrated through a graded ethanol series. The sections were then immersed in Ehrlich’s hematoxylin for 10 min, differentiated in 70% ethanol and rinsed in distilled water. After that, the sections were counterstained in alcoholic eosin solution containing 1% eosin for 3 min. Following washing under running water, sections were dehydrated, cleared in xylene and cover-slipped.

Immunohistochemistry

Consecutive sections of pituitaries and hypothalami containing the dorsolateral part of the SON and PVN from each subject were stained with antisera to VP, OT, or CRH. After deparaffinization and rehydration, slides were preincubated for an hour at room temperature (RT) in 1% Triton X 100-phosphate buffered saline containing 5% normal goat serum (PBS-NGS). Overnight incubation with one of the primary rabbit polyclonal antibodies – anti-VP or anti-OT (Chemicon International, CA; 1:1,000), or anti-CRH (Peninsula Laboratories Inc., Division of Bachem, CA, USA; 1:1,000) in working solution (1% NGS, 0.3% Triton on PBS, pH 7.4) was performed in humid chambers at 4°C.

After that, sections were washed in PBS for an hour and incubated in working solution with biotinylated goat anti-rabbit IgG (Vector Elite kit, Vector Laboratories, Inc., Burlingame, CA; 1:500) for two hours at RT. After washing in PBS for an hour, ABC complex (Vector Laboratories, CA) in PBS was applied (1 h, RT). Finally, after washing in PBS, the reaction was visualized with freshly prepared glucose-oxidase-diaminobenzidine (GOD-DAB) as described by Zaborsky and Heimer [21]. After rinsing in distilled water, sections were dehydrated through ascending concentrations of ethanol, cleared in xylene and mounted with embedding medium (OTC, Germany). For control incubations we used either normal rabbit serum, diluted 1:1,000 in PBS-Triton instead of the primary antibodies or anti-OT (anti-VP or anti-CRH), preabsorbed with synthetic OT (VP or CRH) (Sigma).

Double immunostaining for CRH and VP was performed as previously described [22]. Briefly, sections were incubated with the rabbit polyclonal anti-CRH overnight at 4°C. After that, biotinylated goat anti-rabbit IgG was applied followed by avidin-biotinhorseradish peroxidase complex (Vector Laboratories, 1:100). Immunoprecipitates were visualized by DAB (Sigma). Next, sections were treated for 15 min in 0.1 M glycine buffer at pH 3.4 in order to remove the immunocomplexes. Subsequently, sections were incubated overnight in the rabbit polyclonal anti-VP and developed by Cy3-labeled goat anti-rabbit Fab fragment (Dianova) at 1:200. After washing, sections were mounted onto glass slides in the mixture of veronal-glycerol (2:8). We counted the total number of CRH-positive neurons and the number of CRH neurons in the VP-immunopositive product from the PVN. Then the percentage of single-labeled and double-labeled CRH-positive cells was calculated. Sections were examined with an Olympus BX50 microscope. An Olympus DP10 digital camera with DP-Soft 3.0 software was used for microphotography.

Quantification of immunoreactivity in neurons of hypothalamic nuclei

Areas and spectrum of the optical density of DAB-immunoreactive products in the neurons and posterior pituitary lobe were defined in immunocytochemically stained VP, OT, or CRH sections with the ImageTool (University of Texas Health Science Center, USA) and ImageJ (NIH) software packages. Four sections of each sample that were stained either with VP, OT or CRH antibodies were processed for semiquantitative analysis. Correlation of measured area (in pixels) and average density of the gray level were calculated (in arbitrary units, a.u.) for an estimation of the relative optical density of immunoreactive products. On the sections stained for VP, OT or CRH, areas of the cell somata and the nuclei of the neurons were also measured using ImageTool. After that, absolute and relative (in %) areas of immunoreactive products were calculated.

Statistics

The distinctions in the mean all measured parameters between the subjects as a whole and among the different age groups were tested using MS Excel 7.0. T-test was used for statistical analysis; p < 0.05 was considered significant. Data are presented as the mean ± SE of values as indicated.

Results

Effects of chronic alcoholic disease on magnocellular neurons of the SON and PVN

Histological examination of the SON and PVN revealed pyknosis, cytokeratolysis (Fig. 1a) and pericellular edema (Fig. 1b) in a fraction of magnocellular neurons.

After immunohistochemical staining for OT and VP, similar cellular distribution within the SON and PVN was observed in both control and alcoholic groups. The largest part of the SON – the dorsolateral (prechiasmatic) part – contained predominantly VP-positive neurons (Fig. 1c); only few OT-positive neurons were present in the cap of this part (Fig. 1d). The proportion of OT and VP-immunoreactive neurons was relatively equal (Fig. 1g, i).

In controls, a clear reduction of VP- and OT-immunoreactivity in the SON and PVN was found in patients in the old group (Table 1). Analysis of the SON in the young alcoholic group revealed no changes in OT and VP immunoreactivity (Table 1). However, profound reduction of OT and VP-positive profiles was observed in the SON of the old group (Fig. 1d, f; Table 1). In the PVN from alcoholics, VP-immunopositive product was significantly reduced in both age groups (Fig. 1h; Table 1). In contrast, pronounced increase in OT immunoreactivity was observed in elder patients (Fig. 1j; Table 1).

Effects of chronic alcoholic disease on the posterior pituitary

Relatively equal and moderate distribution of VP and OT immunoproducts was observed in controls (Fig. 2a, c), with no difference in the young and old groups in optic immunoreactivity density (Fig. 2e). In alcoholics, irregular distribution of VP signal with “lump-like” accumulations in terminals occurred in both groups (Fig. 2b). General reduction of optic density of VP-immunoreactivity was found in the young group (Fig. 2e). In spite of this, OT
content remained unchanged (Fig. 2e), but "coarse-like" distribution of OT-immunoreactivity was often observed (Fig. 2d).

CRH neurons of the PVN in alcoholics
Small and medium-sized CRH-positive neurons were located along the third ventricle in the PVN from non-alcoholic patients (Fig. 3a). The intensity of CRH-staining varied from poor to very strong: weakly and moderately stained cells prevailed (Fig. 3b).

In controls, the number of CRH-positive neurons and the intensity of the signal were clearly increased in elder patients (Table 1). In alcoholics, the population of CRH-positive neurons in the PVN was much larger in both age-matched groups (Fig. 3c). At the same time, CRH-positive perikarya and processes were heavily stained (Fig. 3d) and cellular profiles were enlarged (Table 1).

We performed double-immunostaining to evaluate co-expression of CRH and VP in the same parvocellular neurons. In controls, VP-immunoreactivity was found in 11.2 ± 1.74% CRH-positive neurons from young patients (Fig. 3e, f) and in 13.21 ± 1.65% CRH-positive neurons in the old group. In alcoholics, the proportion of double-labeled neurons was significantly increased in both age groups: 18.38 ± 3.54% in young patients (p < 0.05; Fig. 3g, h) and to 19.4 ± 2.41% in elder patients (p < 0.05).
Table 1  Relative areas (in %) of vasopressin (VP)-, oxytocin (OT)- and corticotropin-releasing hormone (CRH)- immunoreactive products in neurons of the supraoptic (SON) and paraventricular (PVN) nuclei in alcoholics

<table>
<thead>
<tr>
<th>Nucleus/neurohormone</th>
<th>Age ≤ 40 yr</th>
<th>Alcoholics</th>
<th>Age &gt; 40 yr</th>
<th>Alcoholics</th>
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<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>Alcoholics</td>
<td>Control group</td>
<td>Alcoholics</td>
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<tr>
<td>SON</td>
<td></td>
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<tr>
<td>VP</td>
<td>81.54 ±0.69</td>
<td>81.55 ±0.45</td>
<td>78.52 ±0.54b</td>
<td>77.93 ±0.45b</td>
</tr>
<tr>
<td>OT</td>
<td>77.46 ±0.61</td>
<td>76.54 ±0.65</td>
<td>75.09 ±0.72b</td>
<td>73.37 ±0.76b</td>
</tr>
<tr>
<td>PVN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP</td>
<td>82.99 ±0.67</td>
<td>80.54 ±0.64a</td>
<td>81.13 ±0.61b</td>
<td>78.27 ±0.67a'b</td>
</tr>
<tr>
<td>OT</td>
<td>78.66 ±0.76</td>
<td>79.18 ±0.56</td>
<td>74.14 ±0.87b</td>
<td>76.31 ±0.68a'b</td>
</tr>
<tr>
<td>CRH</td>
<td>66.28 ±0.41</td>
<td>73.85 ±0.41a</td>
<td>68.31 ±0.37b</td>
<td>69.23 ±0.32a'b</td>
</tr>
</tbody>
</table>

*a p < 0.05 in the same age group; b p < 0.05 between different age groups.

Fig. 2  VP- and OT-immunoreactivity in the posterior pituitary in alcoholics. a–d The posterior pituitary of a 42-year old control (a, c) and a 47-year old alcoholic (b, d) patients. Relatively equal and moderate distribution of VP- (a) and OT- (c) immunoreactivity is observed in the control patients. In the alcoholic patient, VP- (b) and OT- (d) immunoreactivity is distributed irregularly, with an accumulation in “coarse-grain” or “lump-like” (b, arrows) axonal terminals. Immunohistochemical staining with anti-VP and anti-OT antibodies, developed by GOD-DAB method. Scale bar for all panels = 100 μm.

e Mean of relative optical density (OD) of immunoreactive products in posterior pituitaries of control and alcoholic patients. Significant reduction of OD for VP (P < 0.05) occurred in the elder alcoholic group.

Effects of chronic alcoholic disease on the adrenal gland

In alcoholic patients, a dramatic reduction in the width of adrenergic cortex with alteration in zonation was observed (Fig. 4c). Detailed examination revealed pyknosis (Fig. 4d, e) and vacuolated dystrophy (Fig. 4f) of corticocytes in zona fasciculata and zona glomerulosa.

Discussion

In agreement with Harding and colleagues [7], who reported neuronal death in the SON and PVN, we found signs of neuronal degeneration – pyknosis, cytokaryolysis, and pericellular edema – in those brain regions. Neuronal loss of VP and OT-ergic neurons with compensatory up-regulation of VP and OT mRNAs expression in surviving neurons was also reported in animal mod-
We observed substantial decrease in VP immunoreactivity in neurons of the hypothalamic nuclei and accumulation of VP-immunoproduct in "coarse-grained" and "lump-like" structures in the posterior pituitary. These observations, suggesting inhibition of VP synthesis and impairment of VP release, are in line with the blunted response of VP to osmotic challenges in alcoholic patients [8]. Similar changes have been found in OT-ergic population of the SON. However, clear elevation of OT-immunoreactivity was found in the PVN, especially in elder patients. Activation of OT synthesis in the PVN may contribute to abnormal elevation of plasma OT in alcoholics during periods of abstinence lasting 4 to 28 days [10]. The abnormal increase in OT may be due to alcohol withdrawal, as OT is typically released in response to acute stress paradigms [23–25]. However, it is unlikely that the OT rebound would last a whole month. Enhanced OT production may contribute to compensation of water loss [26,27] and development of alcohol tolerance [28].

According to clinical studies, chronic alcohol consumption suppresses cortisol plasma levels [18], which is consistent with the
adrenal cortex hypoplasia observed by us and reported by other groups in humans and animal models [29–31]. Interestingly, while prolonged ethanol intake leads to decrease in CRH mRNA levels in parvocellular neurons in rats [17,32], we observed a profound increase in CRH immunoreactivity in parvocellular neurons from the PVN of alcoholic patients. This increase may be explained by several mechanisms. First, low levels of circulating glucocorticoids may tonically upregulate CRH expression; second, ethanol withdrawal (the patients were admitted to hospital after long-term drinking) may exaggerate CRH production and release [33–35]; and third, depression (25–50% of actively drinking alcoholics are depressed [36]) may activate CRH synthesis [37–40].

Co-expression of CRH and VP in parvocellular neurons of the PVN is well-documented in rodents [13] and reported in humans [41,42]. Chronic alcohol consumption and alcohol withdrawal do not affect VP mRNA levels in parvocellular neurons in rodents [17]. In alcoholic patients, we found an increase in the number of double-labeled cells in both, the young and the old groups. Upregulation of VP expression usually occurs in situations of insufficient feed-back of glucocorticoid signaling, such as after adrenalectomy or prolonged stress [14–16]. Hypoplasia of adrenal cortex in chronic alcoholics observed in our study suggests that silencing of negative glucocorticoid feed-back signaling in chronic alcohol consumption can lead to tonic activation of VP expression in CRH-ergic neurons.

The changes observed in magnocellular neurons were age-dependent. In the control group, VP and OT content in the SON and PVN was ubiquitously lower in old patients (>40 years). Consistently, decrease in VP and OT content in both nuclei (except OT in the PVN) was much more drastic in alcoholics in the old group than in younger patients. We observed an elevation in CRH immunoreactivity in the PVN of the control old group in agreement with reported activation of CRH expression during ageing in rodents and humans [43,44]). However, elevation of CRH immunoreactivity was more profound in the young alcoholic patients.

In conclusion, the analysis of magnocellular hypothalamic nuclei in alcoholic patients revealed a decrease in VP content in SON and the PVN, and OT content in the SON; all three findings were more pronounced in the elder patients (>40 years). In spite of this, content of OT was elevated in the PVN of the old group. We found an increase in CRH immunoreactivity in individual cells in parvocellular neurons as well as an increase in the number of CRH-positive neurons co-expressing VP. These changes are likely induced by insufficient levels of plasma glucocorticoids due to hypoplastic impairments of the adrenal cortex.
Acknowledgements

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