NO CHANGES IN NEOCORTICAL CELL VOLUMES OR GLIAL CELL NUMBERS IN CHRONIC ALCOHOLIC SUBJECTS COMPARED TO CONTROL SUBJECTS

KATRINE FABRICIUS*, HENNING PAKKENBERG and BENTE PAKKENBERG

Research Laboratory for Stereology and Neuroscience, Bispebjerg University Hospital, Bispebjerg Bakke 23, DK-2400 Copenhagen NV, Denmark

(Received 8 December 2006; first review notified 9 January 2007; in revised form 17 January 2007; accepted 23 January 2007; advance access publication 6 March 2007)

Abstract — Aims: To study if the total glial cell population in the neocortex is intact in subjects with a history of severe alcohol abuse compared to control subjects. Further, to investigate whether the cortical nerve cell nuclei and nerve cell perikarya volumes are the same in chronic alcoholic subjects as in the control subjects. Methods: Using the stereological method, the optical rotator in a vertical design, the perikaryon cell volume and nuclear cell volume in the neocortex and its four subdivisions were measured in 11 alcoholicics and 10 control subjects. Using the Cavalieri estimator of volumes and the optical dissector for cell counting, we estimated the total number of glial cells in the neocortex and compared previous stereological results for chronic alcoholic subjects. Results: We found the mean neuronal cell volumes to be unaffected by severe alcohol abuse (p = 0.86) and a normal total number of glial cells (p = 0.39) in chronic alcoholic subjects compared to control subjects. Conclusion: Only glial cells and dendritic/synaptic changes have so far been reported in stereological studies of the brains of alcoholic subjects. We thus have increasing evidence that it may be possible for some individuals to return to their previous cognitive abilities after cessation of alcohol which may give hope and encouragement for chronic alcoholic subjects to stop the abuse.

INTRODUCTION

The consequences of alcohol misuse are serious and in many cases life threatening. Alcohol abuse increases the risk for certain cancers, especially those of the liver, oesophagus, throat, and larynx and may cause liver cirrhosis, immune system problems, brain damage, and harm to the fetus during pregnancy (review Knight and Longmore, 1994). Cognitive impairments are found in 50–70% of recent detoxified alcoholic subjects with symptoms ranging from mild impairment of memory and concentration to the demential symptoms such as those seen in Wernicke–Korsakoff syndrome (Carlen et al., 1981; Carlen and Wilkinson, 1987; Charness et al., 1989). Observations made with CT and MRI generally support evidence of a relationship between prolonged alcohol consumption and structural cerebral and cerebellar atrophy, observed as widening of cortical sulci and enlargement of the brain ventricles (Jernigan et al., 1991; Pfefferbaum et al., 1992; Mann et al., 2001). Nevertheless, stereological studies have shown that alcohol abusers do not have a reduced number of neurons in the neocortex compared to normal controls subjects, while others have reported reduction of white matter volume in brains of alcoholic subjects (Harper et al., 1985; De la Monte, 1988; Harper and Corbett, 1990; Jensen and Pakkenberg, 1993; Harding et al., 1997). However, this reduction in white matter volume is not followed by a reduction of the total length of myelinated nerve fibres in the subcortical white matter (Tang et al., 2004), thus alcohol abuse does not lead to degeneration of myelinated axons. It has been suggested that the degeneration observed in the cerebellar regions in alcoholic subjects might be due to thiamine deficiency and liver disease, and represent the same disease as Wernicke’s Encephalopathy (WE), an acute transient stage of alcohol related neurological symptoms including confusion and abnormally gross muscle control (Charness, 1993; Butterworth, 1995). Support for this hypothesis includes a study based on densities by Baker et al. (1999) showing no consistent changes in the number of neurons or changes in the structural volume of any cerebellar region in chronic alcoholic subjects without the clinical sign of WE. In contrast, alcoholic patients with thiamine deficiency had a 43% Purkinje cell density reduction in the cerebellar vermis. Baker suggested that chronic alcohol consumption per se does not necessarily damage cerebellar tissue, but that an unknown factor is required to trigger cerebellar degeneration in chronic alcoholic subjects (Baker et al., 1999). Finally, a stereological study by Andersen (2004) found that alcoholic subjects without WE had a reduction of Purkinje cell volume in the cerebellum, but no loss of cerebellar neurons (Andersen, 2004), and Korbo (1999) in a pilot study has shown that the cognitive impairments seen in alcoholic subjects is not followed by a simultaneous loss of neurons in the hippocampus (Korbo, 1999).

Possible reversibility after alcohol abuse has been reported in a neuropsychological study, that demonstrated that former alcoholic subjects with an abstinence of >5 years had the same cognitive abilities as the control group (Reed et al., 1992). Thus, the clinical picture with reversibility of the symptoms after years of abstinence fits well with none or a limited neuronal loss in the central nervous system of human alcoholic subjects.

Previously we had estimated the total number of neocortical neurons and found it to be the same in brains of subjects dying from chronic alcohol abuse as in the brains of non-alcoholic control subjects (Jensen and Pakkenberg, 1993). The total number of neocortical glial cells has not previously been estimated in relation to chronic alcoholism. The aim of this study was to find out if the total glial cell population in the neocortex is intact in subjects with a history of severe alcohol abuse compared to control subjects. Further, to investigate whether the cortical nerve cell nuclei and nerve cell perikarya...
volumes are the same in chronic alcoholic subjects as in the control subjects. The stereological tools, the Cavalieri principle, the optical dissector and the vertical rotator method, were applied. Data will be discussed and combined with stereological data from previous studies on the same material.

MATERIALS AND METHODS

Subjects

The materials comprised 10 male brains from normal subjects and 11 male brains from chronic alcoholic subjects. The brain material had previously been used in other studies (Jensen and Pakkenberg, 1993; Pakkenberg and Gundersen, 1997; Korbo, 1999; Tang et al., 2004). However, one normal male brain was removed from this study, due to technical reasons since cell volumes could not be obtained with sufficient precision. Brains were obtained from necropsies, and a complete post-mortem examination was carried out in each case, including a histological diagnosis. Selection of brains for this study was based on clinical and pathological data. Despite some chronic medical conditions, all subjects were functioning normally in their community until shortly before death. Although no cognitive assessment was performed, their local practitioner or the hospital staff regarded them as cognitively normal subjects. Alcoholic subjects were included if they were known to have a severe and lasting history of excessive alcohol intake, and if they fulfilled the DSM-III-R (American Psychiatric Association, 1987) criteria for alcoholism. None of the alcoholic subjects suffered from WE. At necropsy, all alcoholic subjects had patho-anatomical evidence of alcohol abuse and most had liver cirrhosis and/or varices of the oesophagus. In addition, ascites, pancreatitis, and alcoholic cardiomyopathy were found. Three alcoholic subjects died from acute pancreatitis, two from bleeding oesophageal varices, while the remaining subjects died from septic shock, cardiomegaly, pneumonia, pulmonary oedema, lung cancer, or colon cancer.

Normal subjects were included if they did not have prior neurological or psychiatric disorders. Eight control subjects died from acute myocardial infarction (AMI), one was killed violently, and one died from a combination of AMI and septic shock, cardiomegaly, pneumonia, pulmonary oedema, lung cancer, or colon cancer.

Neuropathology

After sampling biopsies for counting, tissue blocks were sampled from frontal, parietal, –medial temporal and occipital lobes, insula, gyrus cinguli, and hippocampus and one or two tiers of mesencephalon. The tissue was processed routinely and embedded in paraffin wax. Four-micron-thick sections were cut from all blocks for H&E stains, and for immunohistochemistry in selected areas. Eight-micron-thick sections were cut from all blocks for Klüver–Barrera staining. Immunohistochemistry included beta-amyloid (DAKO M0872 1 : 200), tau (DAKO A0024 1 : 50,000), ubiquitine (DAKO Z0458 1 : 5,000), and alpha-synuclein (NDS 18–0215 1 : 2,000). The brain stems from all control subjects were normal, including pigmentation of substantia nigra. There were no tumors or hemorrhages. No brains showed cortical spongiosis, vasculitis, or encephalitis.

Estimation of neocortical volumes

The brains were fixed in 0.10 M sodium phosphate buffered formaldehyde (pH 7.2, 4% formaldehyde) for at least 5 months to insure proper fixation. The meninges were removed and the cerebellum and brainstem detached at the level of the third cranial nerve. The right or left hemisphere was chosen randomly, and the frontal, temporal, parietal, and occipital regions delineated and painted on the pial surface in different colours. Each hemisphere was then embedded in a 6% agar and sliced coronally with 7.0 mm spaces, starting randomly at the frontal pole within the first 7 mm. The neocortical volume was estimated by Cavalieri’s point-counting principle (Gundersen and Jensen, 1987):

\[ V_{\text{reg}} := T \cdot a(p) \cdot \sum_{i=1}^{n} P_i \]

where \( T \) is the mean coronal slice thickness, \( a(p) \) is the area associated with each point in the grid, \( P_i \) the number of points hitting the region in the \( i \)th slice and \( n \) the number of equidistant slices through the hemisphere. An average of 270 points were counted on the neocortical section areas providing a total volume with a coefficient of error (CE) of 3% (Gundersen and Jensen, 1987; Gundersen et al., 1999).

The mean cortical thickness was estimated as the contour of the cortex divided by the surface area. Estimation of the surface area was not strictly based on unbiased calculations, but estimated by counting intersection points between the boundary and test lines in a non-uniform design (non-rotated slices) (Gundersen, 1985; Regeur and Pakkenberg, 1989; Toft et al., 2005). This has previously shown to result in a bias around 3%, and was therefore neglected (Oster et al., 1993).

The vertical optical rotator principle

Most stereological procedures require isotropy (meaning that all directions are equally probable). A design with completely random orientation combined with uniform random sampling generates isotropic uniform random (IUR) section planes (Gundersen et al., 1988a). Due to the anisotropy of most biological structures, isotropy must often be guaranteed before cell volumes are estimated. A modification, the so-called vertical design, was first described by Baddeley et al. (1986). Four requirements must be fulfilled:

Requirement 1. A vertical axis must be generated in the tissue.

\[ V_{\text{reg}} := T \cdot a(p) \cdot \sum_{i=1}^{n} P_i \]
Requirement 2. All sections must be cut parallel to the vertical axis, and all subsequent measurements must be made with respect to the axis, which must be identifiable in all sections.

Requirement 3. The vertical sections must have random positions and random (i.e. 2-D isotropic) orientations.

Requirement 4. On the vertical section, a test line is given a weight proportional to the sine of the angle between the test line and the vertical direction (Baddeley et al., 1986).

Due to the use of the optical rotator method, the fourth requirement in the vertical design is replaced by a new principle based on the Pappus–Guldinus theorem, which provides an estimation of the volume of an object obtained by rotating a planar figure around an external axis in the plane (Jensen and Gundersen, 1993). In this way it is possible from planar information (one section) to get an estimate of the 3-dimensional volume of e.g. a neuron. A vertical section is a plane section perpendicular to a known horizontal plane. As mentioned, the external axis must lie in the vertical section. The horizontal plane is a reference plane defining the orientation of the section. The horizontal plane can be generated artificially by the observer or given by the tissue. In this study the vertical axis was parallel to the y-axis on the monitor. Random rotation is accomplished by the random rotation of the bars and each section was cut parallel to the long axis of the bars. On the computer screen, the top and the bottom boundary points of the sampled cells were defined by the operator, and between these two points the computer generates three lines perpendicular to the vertical axis. The operators indicate the interception of the lines with the cell boundary and the computer then generates a volume estimate from the formula:

\[ V = \sum l_i^2 \times t \times \frac{\pi}{2} \]

where \(l_i\) is the distance between the intersections and the vertical axis and \(t\) is the distance between the test lines defined by \(t = h/3\), where \(h\) is the height of the profile projected on the vertical axis.

An example of the design is illustrated in Fig. 1.

**Sampling procedure for cell volume estimation**

From every second neocortical slice, starting randomly, transcortical wedges were sampled uniformly and systematically from every slice containing the frontal, temporal, parietal and occipital regions. Each wedge was cut into 2 mm parallel bars providing 25 to 50 bars per region. These were subsampled systematically, uniformly and randomly, resulting in each region being represented by 6 to 10 bars. Each bar was then rotated randomly around its vertical axis and embedded in Historesin (Reichert-Jung, Heidelberg, Germany). One 35-µm-thick vertical section was cut from each bar, stained with a modified Wölbach's Giemsa stain, and used for counting and measuring in optical dissectors. The volume of each neuron in the four cortical regions was estimated by the vertical rotator method using the CAST-GRID software program (Olympus (Visiopharm), Denmark). For each cortical region, the nucleus and perikaryon volume was estimated for all neurons sampled in the optical dissectors.

Changes in tissue volume before and after the histological processing, were quantified and found to be the same in the two experimental groups: a swelling of 7.5 and 6.1% respectively. Since this was not statistically significantly different, we did not correct for tissue changes.

To estimate the total cell body volume in the neocortex, the total estimated number of neurons in the cortex (neu, cortex) was multiplied with the mean perikaryon volume in the cortex:

\[ \text{Volume(cell body)} = V(\text{mean per neu, cortex}) \times N(\text{neu, cortex}) \]

**Estimation of the total number of neurons**

A previous study by Jensen and Pakkenberg, (1993) of the same brains estimated the total number of neurons using the Cavalieri principle and optical dissectors. Knowing the height

---

**Fig. 1.** Schematic and real-time image of the vertical design on stained sections. The vertical axis is parallel to the y-axis on the monitor. The nucleolus is used as reference point. Top and bottom boundary points are defined by the operator, and the computer then generates three test lines with a constant known distance in a uniform position perpendicular to the vertical axis. The distance between the lines is \(t = h/3\), where \(h\) is the height of the profile projected on the vertical axis. Intersections between the lines and the cell boundary are defined by the operator (the X), and the computer then generates an estimate of the cell body volume. To the left, a schematic drawing; to the right a computer image (right-side image from Toft et al., 2005).
of the counting frame, \( h \), the area of the frame \( a(\text{frame}) \) and the volume of the disector \( v_{\text{dis}} = h \times a(\text{frame}) \), the total number of particles \( N \), in a specimen of a given volume, \( V_{\text{ref}} \), is given by:

\[
N = \left( \frac{\sum Q}{\sum v_{\text{dis}}} \right) \times V_{\text{ref}}
\]

for \( \sum Q \) equal to the total number of counted particles (Gundersen et al., 1988b) and used to estimate the total glial number in this study.

**Statistical analysis**

Variation within groups was indicated by the coefficient of variation while the CE was used to estimate the precision of the measurements. The CE was calculated according to the formulas in Gundersen et al., 1999, and only used on total neuron numbers. The coefficient of variance (CV) of the logarithmic size distribution, denoting distributional CV or DCV, expresses the intra-individual variation in neuronal size to avoid confusion with the ordinary inter-individual CV. DCV was computed as: DCV: = \text{antilog} (SD (\log v)) − 1.

Differences between groups were tested using an unpaired two-sided Student’s \( t \)-test with a significance level of 0.05. For data where normality tests failed, the non-parametric Mann–Whitney \( U \)-test was used.

**RESULTS**

The mean geometric volumes of the neuronal nuclei and perikarya volume in the control and alcoholic subjects in the four sub-cortical regions of the neocortex are shown in Table 1. Also shown, is the total number of neocortical neuron cells from a previous study, excluding one male as mentioned earlier, showing no statistically significant differences either in total numbers (\( P = 0.48 \)) or in the four subdivisions of the neocortex. In this study of the glial cell counts, the mean values were \( 33.8 \times 10^9 \) (mean CE = 0.09) for control subjects and \( 30.5 \times 10^9 \) (mean CE = 0.09) for alcoholic subjects, which was not statistically different (\( P = 0.39 \)). This was also true for the four sub-regions (statistical data not shown).

Figure 2 illustrates the absolute size distributions of the nerve cell perikaryon and cell and nuclei volumes in neocortex in the two groups.

The mean volume of the sum of neuronal cell perikarya in the neocortex was 34.5 cm\(^3\) and 35.1 cm\(^3\) for controls and alcoholic subjects, respectively. There was no statistical difference between the two groups (\( P = 0.84 \)) (Fig. 3).

Finally, the neocortical surface area, cortical thickness and cortical volumes of the neocortex and its four sub-regions showed no statistically significant differences between the two groups, Table 1.

**DISCUSSION**

Examples of some stereological findings in previous studies of the brains of chronic alcoholic subjects compared to control subjects are shown in Table 2. In the brains of these chronic alcoholic subjects, we found no significant changes in either cell volumes or in the total glial cell numbers. A number of data exists for the estimation of cell size in the central nervous system. However, modern stereological tools have only been available in recent years, which is why most data on cell volumes have been obtained from 2-dimensional area profiles giving obvious difficulties in interpretation of results due to the 3-dimensional cell volumes. Stereological methods may provide estimates of neuronal numbers and cell volumes without the methodological assumptions of conventional morphometric techniques. Using the rotator method, an estimate of the volume of nucleus and perikaryon of hundreds of sampled neurons can be obtained in a day, making it possible to obtain the mean cell volume and
The absolute size distribution. Previously, alcohol abuse has resulted in a decrease in Purkinje cell perikaryon volume by 24% and Purkinje cell nuclei volume by 16% compared to non-alcoholic control subjects. In this study we found the geometric mean nuclei and geometric mean perikarya neuronal volumes and their size distribution in the neocortex to be the same in chronic alcoholic subjects and control subjects. This suggests that the cognitive impairments that are often present in chronic alcoholic subjects cannot be explained by changes in cell volumes.

The major limitation in this study, as with most studies estimating volumes, includes that the volume of histological fixated cells might be biased to an unknown degree by factors involved in fixation procedures, even though these parameters are later measured using stereological methods based on unbiased principles. However, this material has been preserved within the same time interval (min. 4 years ± 1.2 years) and the average cell volumes are in agreement with other studies of brain tissue independent of storage time.

Note that differential cell shrinkage can never be excluded. It should, however, be distinguished between neurons and glial cells but a possible counting bias can never be excluded. It is indicated in parentheses and was calculated as: antilog (SD (log n)) - 1. For the surface area, cortical volumes and cortical thickness, the CV (CV = SD/mean) is indicated.

A previous study–recalculated (from Jensen and Pakkenberg, 1993).

Table 1. Bilateral mean number of neurons, glial cells, geometric mean volumes of nuclei and perikarya and surface areas, cortical volumes as well as the average cortical thickness in controls and alcoholic subjects

<table>
<thead>
<tr>
<th>Region</th>
<th>Neurons (10^6) (CE)</th>
<th>Glial cell Numbers (10^6) (CE)</th>
<th>Volume Nucleus (μm^3) (DCV)</th>
<th>Volume Perikaryon (μm^3) (DCV)</th>
<th>Surface areaa (cm^2) (CV)</th>
<th>Cortical Volumea (cm^3) (CV)</th>
<th>Cortical thickness (mm)a (CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls, N = 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal</td>
<td>7.57 (0.11)</td>
<td>14.2 (0.09)</td>
<td>327 (0.31)</td>
<td>2140 (0.39)</td>
<td>698 (0.09)</td>
<td>190 (0.15)</td>
<td>2.8 (0.30)</td>
</tr>
<tr>
<td>Temporal</td>
<td>5.29 (0.10)</td>
<td>7.15 (0.09)</td>
<td>274 (0.29)</td>
<td>1696 (0.42)</td>
<td>452 (0.14)</td>
<td>111 (0.08)</td>
<td>2.6 (0.20)</td>
</tr>
<tr>
<td>Parietal</td>
<td>5.31 (0.09)</td>
<td>7.09 (0.09)</td>
<td>223 (0.27)</td>
<td>1333 (0.44)</td>
<td>471 (0.25)</td>
<td>104 (0.09)</td>
<td>2.5 (0.32)</td>
</tr>
<tr>
<td>Occipital</td>
<td>4.28 (0.11)</td>
<td>5.36 (0.09)</td>
<td>129 (0.22)</td>
<td>615 (0.40)</td>
<td>265 (0.11)</td>
<td>56 (0.13)</td>
<td>2.1 (0.11)</td>
</tr>
<tr>
<td>Total Neocortex</td>
<td>22.5 (0.10)</td>
<td>33.8 (0.09)</td>
<td>250 (0.27)</td>
<td>1545 (0.41)</td>
<td>1886 (0.11)</td>
<td>462 (0.08)</td>
<td>2.5 (0.16)</td>
</tr>
<tr>
<td>Alcoholics, N = 11 Frontal</td>
<td>8.04 (0.11)</td>
<td>11.77 (0.09)</td>
<td>305 (0.24)</td>
<td>2078 (0.37)</td>
<td>719 (0.11)</td>
<td>197 (0.09)</td>
<td>2.7 (0.14)</td>
</tr>
<tr>
<td>Temporal</td>
<td>4.93 (0.11)</td>
<td>6.75 (0.09)</td>
<td>245 (0.29)</td>
<td>1609 (0.45)</td>
<td>399 (0.14)</td>
<td>118 (0.07)</td>
<td>3.0 (0.17)</td>
</tr>
<tr>
<td>Parietal</td>
<td>5.64 (0.10)</td>
<td>6.67 (0.09)</td>
<td>230 (0.20)</td>
<td>1377 (0.37)</td>
<td>444 (0.16)</td>
<td>119 (0.05)</td>
<td>2.7 (0.16)</td>
</tr>
<tr>
<td>Occipital</td>
<td>4.83 (0.11)</td>
<td>5.24 (0.10)</td>
<td>135 (0.19)</td>
<td>606 (0.55)</td>
<td>290 (0.13)</td>
<td>64 (0.09)</td>
<td>2.2 (0.15)</td>
</tr>
<tr>
<td>Total Neocortex</td>
<td>23.4 (0.10)</td>
<td>30.46 (0.09)</td>
<td>239 (0.23)</td>
<td>1501 (0.43)</td>
<td>1779 (0.17)</td>
<td>498 (0.05)</td>
<td>2.7 (0.07)</td>
</tr>
</tbody>
</table>

Fig. 3. Total neuronal perikaryon volume from alcohol (•) and control (○) subjects in neocortex. There was no significant difference between the two groups (P < 0.05). Horizontal bars indicate the group mean.
energy requirements, and they capture and metabolize the excitatory amino acid glutamate. In addition, astrocytes are able to release neurotropic factors required for neuronal sprouting. Oligodendrocytes are responsible for myelin production and maintenance; they provide metabolic support for neurons and express a variety of voltage-dependent and ligand-gated ionic membrane channels, allowing the reception of signals from neurons (Peinado, 1998). Microglial cells, the main cell type responsible for the immune network of the brain, play a key role as phagocytes. They are also able to release cytokines and growth factors, which modulate the metabolism and functions of other glial cells and neurons. With the increasing knowledge of the importance of the glial cells on the function of the brain, severe and long-lasting alcohol abuse might influence the number of glial cells even in the absence of changes in other glial cells and neurons. With the increasing knowledge of the importance of the glial cells on the function of the brain, severe and long-lasting alcohol abuse might influence the number of glial cells even in the absence of changes in total neuron numbers. However, this was not the case. The total glial cell numbers had a mean value of 33.8 × 10⁹ for control subjects and 30.5 × 10⁹ for alcoholic subjects, which was far from being statistically and significantly different. This was also true for the four sub-regions.

The functional changes observed in alcoholic subjects might be explained by dendritic changes together with receptor and neurotransmitter changes (Tang et al., 2004) or a decrease in the number of synapses per neuron due to a glial cell loss (Pfitzer and Barres, 1997; Korbo, 1999; review Slezak and Pfrieger, 2003; Slezak et al., 2006). If only glial cells and dendritic/synaptic changes are observed, regeneration may occur though it can take years. We thus have increasing evidence that it may be possible, at least for some individuals, to return to normal cognitive abilities after cessation of alcohol which may give hope and encouragement for chronic alcoholic subjects to stop the abuse.

Ethics
The study was approved by the Danish Ethical Committee, Jr. (KF) 11-025/04

Acknowledgements — The excellent technical assistance of Susanne Sørensen and Hans Jørgen Jensen is gratefully acknowledged. Also, the financial support provided by the Lundbeck Foundation is highly appreciated.

REFERENCES


