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Morphological Investigation of Alcohol and Drug-related Changes in the Central Nervous System

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The doctoral thesis is available at the library of Rīga Stradiņš University and on the RSU home page: www.rsu.lv

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ABBREVIATIONS

CNS – central nervous system

CC – lat. *cortex cerebri*

CS– lat. *corpus striatum*

SN – lat. *substantia nigra*

ROS – reactive oxygen species

BBB – blood-brain barrier

ECM – extracellular matrix

NSE – neuron specific enolase

GFAP – glial fibrillary acidic protein

TGF-β – transforming growth factor β

NGFR – nerve growth factor receptor

MMP – matrix metalloproteinase

Cu/Zn SOD – Cu/Zn superoxide dismutase (SOD1)

TUNEL – TdT-mediated dUTP nick-end labeling or terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labeling

EDX – energy dispersive X-ray microanalysis

1. INTRODUCTION

The use of addiction-inducing psychoactive substances is linked to the abnormal functional activity of the central nervous system (CNS), by leading to changes and, often, permanently damaging its structural components. Each year, chronic alcoholism causes about 2.3 million premature deaths worldwide (World Health Organization, 2009), including 3.5% of all deaths in the United States (Mokdad *et al.*, 2004). The European region has a higher proportion, with more than 1 in every 10 deaths in Europe attributed to alcoholism (Rehm *et al.*, 2009). Nervous addiction-induced diseases, including alcohol abuse cases, have been widely reviewed (Moselhy *et al.*, 2001; Kosten and O'Connor, 2003; Harper and Matsumoto, 2005; Spanagel, 2009). The basic functions of neuronal and glial cells of various brain regions are affected and completely disarranged in the brain under alcohol exposure (Miguel-Hidalgo *et al.*, 2002; Ikegami *et al.*, 2003). However, causes of ethanol-related brain damage remain not well understood (Syapin, 2011; Tamrazi and Almast, 2012). All these trends of the above-mentioned multidisciplinary studies suggest that additional evidence should be obtained on damage of cellular constituents of the brain in chronic alcoholics and drug addicts. Moreover, this should be related to the concept of selective response to injury induced by psychoactive substances in the central nervous system.

A wide range of neurologic disorders is associated with subcortical white matter defects and myelin damage that can be caused by alcohol (Fields, 2008). The reported alcohol-induced changes in brain tissue in both the gray and the white cortical matter as well as the subcortical area vary from reduction of gray cortical matter and neuronal loss up to significant reduction of white matter volume (Kril *et al.*, 1997; Harper and Kril, 1989; Badsberg *et al.*, 1993; Fein *et al.*, 2002; Harper, 2009; Bühler and Mann, 2011; Kim et al., 2014). However, it has been shown that the impairment of brain function in chronic

alcoholics is not accompanied by the death of cortical neurons but by dendritic changes together with possible axon degeneration (Harper and Corbett, 1990; Ullén, 2009; Rasakham et al., 2014), and changes in homeostatic regulation of synaptic plasticity mediated by astroglial cells (Aschner *et al.*, 2002; Benarroch, 2005; Volterra and Meldolesi, 2005).

Further enigmas, apart from preferential white or gray matter injury in alcohol-induced brain damage, are questions related to the topography of brain regions predominantly affected in alcoholic subjects. Recent clinical and instrumental studies, including functional MRI (magnetic resonance imaging) of alcohol-dependent individuals, demonstrate that the *corpus striatum* (CS) region is particularly vulnerable (Wise, 2004; Schacht *et al*., 2011; Schneider *et al*., 2001; Kalivas and Volkow, 2005). The *striatum* area has an essential role in motor control, decisional performance (Balleine *et al*., 2007; Surmeier *et al*., 2009) as well as in craving and motivation to procure the drug in addicted humans (Volkow *et al*., 2009). The dopaminergic neurons of *substantia nigra* are involved in the regulation of corticostriatal synaptic plasticity (Reynolds and Wickens, 2002; Kreitzer and Malenka, 2008) and the latest findings reveal morphological changes in this region in drug-dependent individuals (Todd *et al*., 2013).

Alcohol consumption in conjunction with genetic, biological and chemical factors may cause oxidative stress-related intracellular changes of neurons (Kruman *et al*., 2012; Ji, 2012). In comparison with other tissues, the antioxidant activity of the brain is lower and overexpression of the reactive oxygen species (ROS) may significantly contribute to the neuronal cell death and development of neural disorders (Mattson, 2000). Increased SOD activity could be associated with activation of a cellular defense mechanism against the presumptive damage by ROS (Uttara *et al*., 2009). Appearance of ROS overexpression along with activation of metalloproteinases (MMPs), in general, and MMP9, in particular, has been evidenced in striatal brain-blood barrier

(BBB) damage (Kim *et al*., 2003). Moreover, MMP activation due to oxidative stress has been shown to cause neurodegeneration (Wright and Harding, 2009). Some of the still unanswered questions are: 1) what are peculiarities of damage mechanisms taking place in the CNS as a consequence of chronic consumption of psychoactive substances; 2) what is the relative contribution of oxidative stress, caused by consumption of psychoactive substances, to the brain matter changes, as compared to other damaging factors and, finally, 3) what is the action of MMP *in vivo* conditions in chronic consumption of psychoactive substances, and, specifically, in the prefrontal cortex, CS, and SN gray matter, and the white matter associated with these three regions. The role of the transforming growth factor beta (TGF-β) secreted by neurons is important for the regulation of neurotrophic factors (Krieglstein *et al.*, 2002); furthermore, *in vitro* analyses show TGF-β acting as a neuroprotective factor (Roussa *et al.*, 2004).

Ions of metals such as manganese (Mn) and iron (Fe) may have a pathogenic effect and induce neurodegeneration. In addition, it has been found that changes in the concentrations of trace elements can be detected by the concentrations of phosphorus (P), potassium (K), calcium (Ca) and chloride (Cl) ions (Johansson and Strömberg, 2002; Dučić *et al.*, 2013). Endotheliocytes, basal membrane and astrocytes of the BBB have an essential role in the normal functional activity of the brain. Astrocytes are known to express different types of neurotransmitter receptors, the activation of which is linked to intracellular changes in Ca level (Haydon and Carmignoto, 2006; Winship *et al*., 2007). It is also acknowledged that zinc (Zn) and copper (Cu) ions accumulate in SN and promote the death of dopaminergic neurons of this region in Parkinson`s disease. Knowledge about distribution and concentration of trace elements in tissues is of great importance, since trace elements are involved in many biological functions of living organisms in a more complicated way than it has been previously thought (Serpa *et al*., 2008).

Changes in the activity of ion channels as well as an elevated permeability of the axonal membrane, caused by unphysiological increases in intra-axonal Na and Ca ions, are pivotal in mediating axonal injury and degeneration (Medana and Esiri, 2003).

Currently, the available information about high-quality measurements of adaptive changes in structural components of the brain of chronic alcoholics and drug addicts, obtained immunohistochemically and electron-microscopically, remains scarce. The importance of this study is underlined by a need to understand structural changes in the brain and changes in the intercellular communication that may be caused by consumption of addiction-inducing substances.

1.1. The aim of the study

Structural and ultrastructural characterization of the brain tissue in chronic alcoholics and drug addicts by applying the methods of the light microscopy, immunohistochemistry and EDX microanalysis.

1.2. Hypotheses

1. Morphological alterations in CNS may be attributed to changes in neural tissue affected by toxic, addiction-inducing psychoactive substances; alterations caused by toxicity of alcohol and narcotic drugs (opioids, benzodiazepine, carbamazepine, methcathinone) are characteristically featured.
2. The expression of selected immunohistochemical markers is variable; a combination of markers can elevate usefulness of results regarding the morphological changes in CNS in users of psychoactive substances.
3. To investigate the toxic effects of alcohol and narcotic drugs (opioids, benzodiazepine, carbamazepine, methcathinone), a number of morphological

research techniques with a subsequent integrated evaluation of the obtained results can be applied.

1.3. Objectives

1. To carry out the investigation of *cortex cerebri, corpus striatum* and *substantia nigra* regions at the cellular and ultrastructural level in order to obtain changes representing the role of psychoactive substances assessed by light microscopy, immunohistochemistry and EDX microanalysis.
2. To estimate the expression of the oxidative stress marker, and thereby morphologically and statistically analyze the changes of cortical and subcortical regions in response to consumption of psychoactive substances.
3. To assess the expression of MMP9, and its distribution to contributing the structural changes of the brain caused by consumption of alcohol and narcotic drugs.
4. To study SOD1 and MMP9-related structural changes and their interaction, to test for any correlations between these results and those obtained by electron microscopy.
5. To clarify morphologically the ultrastructural changes in the gray and white matter of the brain triggered by the use of psychoactive substances.
6. To determine the role of tissue mediators in processes occurring in neurons and glial cells in three specific brain regions in case of consumption of psychoactive substances.
7. To explore the role of chemical elements in structural changes in case of consumption of psychoactive substances as well as to examine the applicability of EDX technique for the purposes of measuring the concentration of trace elements in the brain.

1.4. Novelty of the study

This study attempted to elucidate a preferential cellular vulnerability in three different brain regions of chronic alcoholics and drug users, structurally and ultrastructurally. Currently, there are no data regarding quantitative and/or semiquantitative estimations of protective and alterative changes of structural components of the brain in alcoholics and drug users assessed by use of immunohistochemistry and electron microscopy. We demonstrated a high degree of protective reactivity in neurons localized in the brain regions of chronic alcoholics and drug users analyzed in this study. Moreover, based on our results regarding regional deviations of antioxidant activity, in general, and reduction of SOD1 immunoreactivity in the white matter of SN, which was paralleled by severe damage of myelin structure documented electron microscopically, in particular, we provide the evidence regarding the selective vulnerability of neural constituents. We found that a combination of morphological assays jointly assessed by statistical analysis provided relevant results on the neurobiology of brain in case of chronic alcoholism and drug addiction.

1.5. The structure of the doctoral thesis

The promotional thesis is written in Latvian. It has a classical structure. The paper includes an introduction, a literature review, an overview on materials and methods, results, discussion, conclusions, and the list of literature. The promotional thesis has been written on 161 pages including 4 tables and 61 figures. 337 literature sources have been used for analysis.

1.6. Personal contribution

The author was involved in all stages of the study, including the study design and selection of the immunohistochemical markers. The literature

review, sampling, instrumental measurements, morphological examinations and analysis, taking the photomicrographs as well as the statistical analysis and interpretation were performed by the author.

1.7. Ethical concerns

The study protocol was approved by the local Ethics Committee of Riga Stradins University on 17.12.2009.

2. MATERIALS AND METHODS

2.1. Study methodology and principles of group selection

For this study, brain and liver autopsy tissues from 46 chronic alcohol users, 10 drug addicts and 12 control subjects were obtained between 2007 and 2012 from the Pathology center of Riga East Clinical University hospital and the Latvian State Centre for Forensic Medical Examination.

The study was subdivided into the following 5 steps:

I. *Material sampling.*

First, patient records obtained from the Pathology center of Riga East Clinical University hospital were analyzed between 2007 and 2010; 13 subjects diagnosed with chronic alcoholism were selected. Autopsy samples were obtained from the Latvian State Centre for Forensic Medical Examination after macroscopic evaluation made by a certificated pathologist. Brain, heart, liver and pancreatic tissues were obtained from subjects assumed to have been chronic alcoholics, and after notification of needle marks in the subjects assumed to have been drug addicts following recommendations of Ji (Ji, 2012). Prefrontal (CC), *substantia nigra* (SN) and *corpus striatum* (CS) brain tissue was obtained according to the location of studied region as shown in maps of human brain in the atlas by Dauber (Dauber, 2007). For better evaluation of appropriateness of the particular samples, blood and urine test results (such as an increased concentration of alcohol and/or other addiction-inducing substances), obtained from the Centre for Forensic Medical Examination, were taken into consideration. Chronic alcoholic subjects were selected according to criteria established by Harper (Harper *et al.*, 2003). In all cases, a morphological examination of liver tissues and evaluation of the expression of immunohistochemical markers were performed in order to select the cases suitable for inclusion in this study.

The subjects were divided into the following groups:

1. Chronic alcoholics: 34 male subjects aged 33 – 66 (73.9%), 12 female subjects aged 44 – 60 (26.1%). The length of life of these subjects averaged 47 ± 10 years of age. Alcohol was detected in blood samples of 17 of 25 chronic alcoholics (68%) (0.53 – 4.07 ‰); no other toxic substances were detected neither in blood samples nor urine samples.

2. Drug addicts: nine male subjects aged 22 – 45 (90%) and one female subject aged 22 (10%). The length of life of these subjects averaged 34 ± 8 years of age. Alcohol was detected in blood samples of five drug addicts (0.59 – 1.67 ‰). Opioids (morphine, methadone, tramadol) were detected in blood samples of drug addicts. Benzodiazepine and carbamazepine were detected in the single samples, whereas, methcathinone was detected in blood of two drug addicts.

For a control group, 12 subjects aged 17 to 37 were selected. An additional morphological examination of brain and liver tissues done by a certified pathologist was performed to omit cardiovascular, respiratory or liver pathologies. The control group consisted of 10 male subjects (83.3%) and two female subjects (16.7%). The length of life of control subjects averaged 29 ± 6 years of age.

Immunohistochemically-detected brain changes correlated to the age of subjects were studied in chronic alcoholics and subdivided as follows: 1– young alcoholic subjects (under the age of 34), 2– older alcoholic subjects (above the age of 60).

II. *Biomarker selection.* Within the frame of the 2nd step, markers for further immunohistochemical analysis were selected. Selection of biomarkers and appropriate antibodies used in this study was based on the literature analysis distinguishing their use in studies of the structure of nerve and glial cells and of the processes within them.

III. *Brain region selection.* Selection of three particular regions of the brain was made based on the literature analysis. Thereafter, further examination was undertaken by the light microscopy, electron microscopy (TEM, SEM) and EDX microanalysis.

IV. *Data analysis.* Statistical data analysis-based evaluation of the informativeness of the techniques was applied throughout the study. Apart from descriptive morphology, statistical analysis was performed in order to estimate immunohistochemistry data. It was evaluated semi- and quantitatively according to the established scoring system. Different statistical tests were used in order to compare specific brain regions and the expression of selected markers within a single group and between different study groups, with an aim to obtain an analysis-based estimate of the informativeness of markers and techniques applied throughout the present study.

V. *Element analysis.* Additionally, analysis of alterations in concentrations of chemical elements, including metals, was performed by EDX method. The aim was to explore the applicability of this particular technique for further morphology-based CNS studies and to explore, whether the EDX data can supplement the results obtained by conventional morphology techniques.

2.2. Conventional light microscopy and immunohistochemistry

ABC kit (StreptABC Complex/HRP Duet, Sigma-Aldrich, St. Louis, MO), HRP Polymer system (CellMarque, Rocklin, CA, USA) and EnVision system (DacoCytomation, Glostrup, Denmark) were used in this study.

Mouse monoclonal antibody anti-NSE (Novocastra, Newcastle, UK, Leica Biosystems, 1:100, clone 5E2), anti-GFAP (Novocastra, Newcastle, UK, Leica Biosystems, 1:100, clone GA5) and anti-NGFR (DacoCytomation, Carpinteria, CA, USA, 1:50, clone NGFR51) were applied to randomly

selected samples of the brain tissue. For the visualization of activated microglia, an immunohistochemical reaction with anti-CD68 antibody (DacoCytomation, Glostrup, Denmark, 1:50, clone PG-M1) was performed on tissue samples of the brain selected through a screening procedure.

In order to distinguish changes caused by oxidative stress, anti-SOD1 antibody was used for analysis of the brain tissue (Novocastra, Newcastle, UK, Leica Biosystems, 1:100, clone 30F11). To detect the expression of growth factors and metalloproteinases, antibodies such as anti-TGF-β1 (Novocastra, Newcastle, UK, Leica Biosystems, 1:40, clone TGFB17) and anti-MMP9 (Novocastra, Newcastle, UK, Leica Biosystems, 1:40, clone 15W2) were applied.

The TUNEL method was applied in order to identify TUNEL-positive cells in the tissues using In Situ Cell Death Detection Kit, POD kit and including Enzyme solution, Label solution and Converter-POD (Roche, Mannheim, Germany).

For visualization of antigen-antibody reactions, 1) biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin (ABC kit), 2) HiDef Detection™ HRP Polymer system, (CellMarque, Rocklin, CA, USA), with amplification of primary antibody and polymer labeling, or simply, polymer conjugation (En Vision system) with enzyme were used.

Expression of antigens was detected and estimated as cytoplasmic, membrane or nuclear.

Histological sections of 4 – 5 μm were cut from formalin-fixed, paraffin-embedded tissues and mounted on slides. Sections were deparaffinized in xylene and hydrated in a series of graded ethanols. The endogenous peroxidase activity was blocked with either 30% hydrogen peroxidase in methanol (20 – 40 min) or Dual Endogenous enzyme block. For antigen retrieval the sections were boiled in 0.01M citrate buffer (15 min). For ABC or En Vision system non-specific binding was blocked with 1% BSA (bovine

serum albumin fraction V) (Roche, Mannheim, Germany) TRIS solution 1 h (RT). Thereafter, consecutive sections were successively incubated for 12 hours overnight (2 – 6 C°) with the primary antibodies (following manufactures recommendations). After rising in PBS solution, sections were incubated with secondary antibody (biotinylated goat anti-mouse/rabbit Ig) or HRP polymer (Labeled polymer-HRP) 30 min (RT), or with HiDef Detection™ Amplifier 10 min (RT). After rinsing, incubation with streptavidin (ABC and HiDef Detection™ HRP Polymer system) 30 min (RT) or HiDef Detection™ HRP Polymer Detector 10 min (RT) was applied, respectively. The antigen sites were then visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, St. Louis, MO) or DAB kit (UltraMarque™ HRP Detection system or DAB+Chromogen and DAB+Substrate buffer) 10 min. The sections were counterstained with Mayer's hematoxylin, washed with tap water, dehydrated, cleared and mounted in PERTEX (mounting medium for light microscopy, Histolab, Gothenburg, Sweden).

Cells that were labeled by the above-mentioned antibodies and displayed brown reaction products were considered immunopositive. Sections from cases with known antibodies positivity were used as positive controls. Immunohistochemical controls that included omission of the primary antibodies or substitution of it with TRIS solution were used as negative control.

Histological slides for quantization of TUNEL-positive cells were deparaffinized and hydrated, treated for 40 min with 0.3% H_2O_2 in TRIS. For antigen retrieval the sections were boiled in 0.01M citrate buffer (15 min). Sections were kept in 1% BSA for 1 hr and afterwards incubated at 37 °C with TUNEL mix (TUNEL Enzyme solution: TUNEL label, dilution 1: 9) for 1 hr in a humid chamber. After washing with TRIS buffer sections were incubated with Converter-POD solution for 30 min (37 °C) rinsed and incubated with DAB. Tissue sections were counterstained with Mayer's hematoxylin.

Perl`s reaction, in order to detect iron (III), was performed randomly on tissues of chronic alcoholics, drug addicts and control subjects.

Expression of antigens was estimated at the autopsy sections of particular regions of grey and white matter of the subcortical nuclei as well as CC, and the autopsy sections of lateral ventricles; an evaluation of the expression in different types of cells was also carried out. Immunohistochemical analysis included evaluation of immunoreactivity of neuronal and glial cells (astrocytes and oligodendrocytes); analysis of endotheliocytes and pericytes was also carried out. The immunoreactivity of SOD1 and TGF-β1 in liver tissue was measured as well.

The expression of antigens was estimated in 10 randomly selected areas of the sample (magnification ×400), using Leica light microscope (LEICA, LEITZ DMRB, Germany) and included SN, CS nuclei region and CC with its associated white matter.

The immunostaining intensity for antibodies was semiquantitatively assessed using the following scoring system: 0 – no staining, 1 – low, 2 – moderate, 3 – intensive staining. The extent of the TGF-β1, SOD1 and MMP9 immunostaining, defined as the percentage of positive staining areas, was scored from 0 – 100%. It was multiplied with intensity and defined as expression. The total number of neurons appearing within the microscopic field reflecting a certain brain region was estimated quantitatively.

2.3. Statistical analysis

Data were presented as medians with interquartile range (IQR (25%; 75%)). The Chi-Square test for categorical variables was performed. Comparison of immunostaining values between groups was made with the Mann-Whitney U or Kruskal-Wallis tests, within group with the Wilcoxon Signed Ranks or Friedman's tests. Spearman's rank correlation coefficient was

used to estimate a relation between TGF-β, SOD1 and MMP9 expression. The correlation was considered strong in cases when the correlation coefficient equaled or exceeded 0.7. A P-value of less than 0.05 was considered statistically significant. The SPSS 20.0 version was used for all calculations.

2.4. Transmission electron microscopy

1 mm^3 tissue autopsy material pieces were processed for conventional transmission electron microscopy and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer 2 – 4 hr (4 ºC), postfixed in 1% OsO_4 1 hr (4 ºC), rinsed in PBS without sucrose and afterwards dehydrated in a series of graded ethanols and acetone, and embedded in epoxy resin.

Tissues were stained with 2% uranyl acetate in 70º ethanol and dehydrated in ethanol series. Thereafter, samples were infiltrated and embedded in Epoxy resin in acetone (Epoxy embedding medium kit: Epoxy embedding medium, Epoxy embedding medium hardener DDSA [2-dodecenylsuccinic anhydrite], NMA [methylnadic anhydrite], (Sigma-Aldrich, Buchs, Switzerland). Afterwards, tissue pieces were embedded overnight in Epoxy mixture without accelerator (Epoxy embedding medium accelerator DPM-30 [2,4,6-Tris (dimethylaminomethyl)phenol]), then tissue samples were embedded in Epoxy mixture with accelerator in molds and polymerized at 60°C for 24 – 48 hours. Ultrathin, 60-nm-thick fine sections were cut with LKB ultramicrotome, collected on Formvar-coated 200-mesh copper or nickel grids, and examined in a JEM 1011 transmission electron microscope (JEOL, Japan) at magnification ×2000 – ×40000.

2.5. Scanning electron microscopy and EDX

According to the protocol of tissue preparation for SEM, samples were fixed in 2.5% glutaraldehyde solution and postfixed in 1% OsO_4. The tissue

dehydration was made using increasingly concentrated solutions of acetone, dried by the critical point method (drying device E3000, Agar Scientific Ltd, U.K.) using liquid CO_2, mounted onto metal stub, covered with gold or carbon layer (for SEM or EDX, respectively) using an automated sputter coater (JFC-1300, JEOL, Japan). Tissue samples were examined under a JSM-6490LV scanning electron microscope (JEOL, Japan) at voltage of 25kV using SEI or BEI mode and magnification ×3.000–×20.000.

3. RESULTS

3.1. Conventional light microscopy

Histological analysis, performed on routinely stained sections obtained from brain tissue of chronic alcoholics revealed of vascular stasis, perivasal hemorrhages, diffuse perivascular swelling, often fibrosis of pia mater and areas of demyelinization of the white matter. Analysis of the liver tissue showed total or partial lobular steatosis, periportal as well as portal fibrosis and leukocytes infiltration. In addition to a diffuse hepatic steatosis, portal liver cirrhosis was often detected.

3.2. NSE immunohistochemistry

Anti-NSE antibody was used to perform immunohistochemical reactions on the selected tissue sections obtained from chronic alcoholics, drug addicts as well as from control subjects. NSE expression was detected in perikarya and processes of the neurons. The immunoexpression of NSE was different in SN and CS regions.

3.3. GFAP immunohistochemistry

Due to the complex histo-architecture of the brain, a specific marker – monoclonal anti-GFAP antibody – was used for detection of astrocytes. The analysis was performed for selected cases of chronic alcoholic subjects and drug addicts. An especially high intensity of GFAP was found in CC astrocytes in the white matter as well as particular regions of the gray matter of the brain. Similarly, a high GFAP intensity was detected in the astrocytes of the CS region of both the white and the gray matter of alcoholics and drug addicts. The localization of anti-GFAP antibody had a diffuse character and was seen both,

in the cell bodies and their processes. A high GFAP intensity was also detected in the terminal expansions of astrocytes located close to capillaries.

3.4. Iron histochemistry

Perl`s reaction was selectively performed on tissues of chronic alcoholic subjects, drug addicts and control subjects. Cells containing iron granules were stained blue along this reaction. There were very few Fe positive cells in samples obtained from chronic alcoholic subjects. Much more heavily deposited Fe granules located close to the capillaries were found in drug-addict subjects.

3.5. CD68 immunohistochemistry

High CD68 intensity in the gray and white matter of all studied brain regions was detected in alcoholics. CD68 positive cells were often located close to capillaries as well as neurons.

3.6. TGF-β1 immunohistochemistry

The median values of TGF-β1 expression were higher in SN and CS regions of alcoholics and control subjects; no differences were found in CC region (Fig. 3.1). In alcoholics as compared with controls, lower median values of TGF-β1 expression were found in nerve fibers of the white matter in SN: 0.95 (0.60; 1.50) and 1.40 (0.80; 2.10), and CC: 0.90 (0.60; 1.00) and 1.60 (0.90; 2.00), respectively ($p<0.001$).

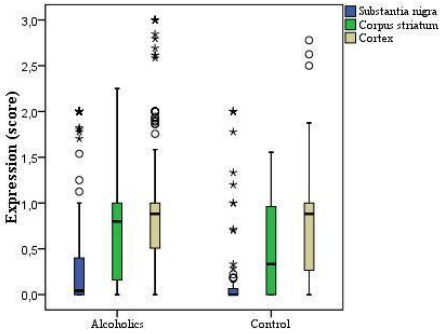

Fig. 3.1. Distribution of neuronal TGF-β1 expression in nigral, striatal and cortical brain regions

In drug addicts (when compared to control subjects), higher median values were detected only in neurons of the CC region: 1.20 (0.84; 2.66) and 0.88 (0.26; 1.00), (p=0.011); similarly, these were higher in the white matter as well: 0.90 (0.50; 1.60) and 1.40 (0.80; 2.10), (p<0.001), respectively. In chronic alcoholics neuronal TGF-β1 expression revealed statistically significant correlation with SOD1 expression in SN region (r=0.199, p=0.038), whereas, a negative correlation was found in the CC region. In the CS region, a negative correlation between neuronal MMP9 expression and TGF-β1 expression (r=- 0.366, p<0.001; positive correlation between TGF-β1 and SOD1 expression (r=0.332, p=0.005), as well as a negative correlation between TGF-β1 and MMP9 expression appearing in the nerve fibers (r=-0.303, p=0.004) was found in chronic alcoholics.

3.7. NGFR immunohistochemistry

The intensity of NGFR was comparatively lower in the white and gray matter regions of SN both in alcoholics and drug addicts, when compared to control subjects. Chronic alcoholic subjects compared with controls revealed lower number of NGFR-positive neurons (7.3%, (n=8) and 11.4%, (n=19),

p<0.001) and NGFR-positive nerve fibers (29.1%, (n=32) and 44.3%, (n=31), p=0.037) in SN region.

3.8. SOD1 immunohistochemistry

The comparison of chronic alcoholic subjects and control subjects revealed statistically significant differences in expression, characteristic of neurons in SN and CC regions; the median values of SOD1 expression were higher in chronic alcoholics (Fig. 3.2). Similar results were obtained by comparing drug addicts with control subjects: SN 1.00 (0.40; 1.00) and 0.14 (0.00; 0.57), CC 2.22 (1.73; 2.79) and 0.86 (0.51; 1.47), p<0.001. Results of SOD1 expression in oligodendrocytes, astrocytes and neuropil of the gray matter as well as oligodendrocytes, astrocytes and neuronal processes of the white matter showed higher median values in CS, CC and the gray matter of the SN in alcohol users. In contrast, lower median values of SOD1 expression were found in the white matter of SN: 0.70 (0.50; 0.90) and 1.20 (0.80; 1.50), p<0.001.

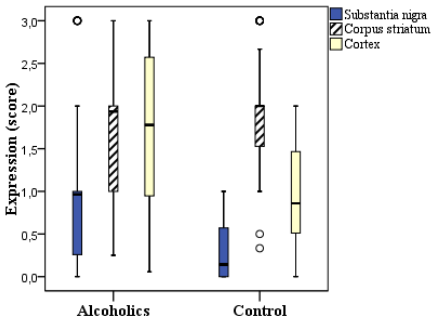

Fig. 3.2. Distribution of neuronal SOD1 expression in nigral, striatal and cortical brain regions

In comparison to control subjects, higher median values of SOD1 expression were found in the nerve fibers of the white matter of drug addicts:

SN 0.90 (0.70; 1.20) and 1.20 (0.80; 1.50), p=0.005; CC 0.60 (0.40; 0.80) and 0.35 (0.30; 0.40), p<0.001.

3.9. MMP9 immunohistochemistry

Median values of MMP9 expression in neuronal perikarya of all the regions were relatively higher for alcoholics than the control subjects (Fig.3.3.).

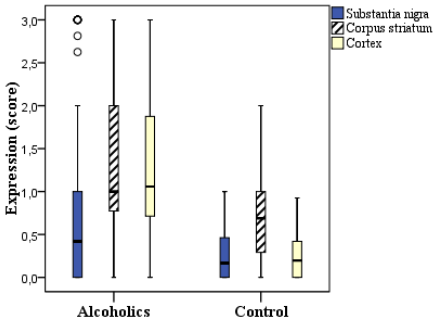

Fig. 3.3. Distribution of neuronal MMP9 expression in nigral, striatal and cortical brain regions

A correlation between MMP9 expression in neurons and nerve fibers of the white matter in SN was detected neither in alcoholics nor in control subjects. However, such a correlation proved to be characteristic of CS: (r=0.298, p<0.001) and (r=0,417, p=0,003); CC (r=0.376, p<0.001) and (r=0.382, p=0.003) both in chronic alcoholics and control subjects. Statistically significant positive correlations between neuronal SOD1 and MMP9 expression in all the studied brain regions of both chronic alcoholics (SN (r=0.532, p<0.001), CS (r=0.327, p<0.001) and CC (r=0.306, p<0.001)) and drug addicts (SN (r=0.463, p<0.001), CS (r=0.269, p<0.001) and CC (r=0.323, p<0.001)) were obtained. Statistically significant positive correlation between SOD1 and MMP9 expression was found in neurons and the white matter in SN of chronic alcoholics (r=0.316, p<0.001), whereas, it was negative – in CS (r=-0.183, p=0.021). SOD1 and MMP9 correlations in SN of alcoholics, obtained by the

application of immunohistochemical and statistical methods, explained the changes obtained in nerve fibers of the white matter of this region electron-microscopically. A negative correlation between SOD1 and MMP9 expression was observed in neurons and the white matter in SN of drug addicts ($r=-0.413$, $p<0.001$).

In SN neurons, there was a weak correlation found between the age and SOD1 expression ($r=0,139$, $p=0,039$). Analysis of other regions did not reveal any correlations; no correlations regarding MMP9 expression were found as well. A comparison of young chronic alcoholic subjects (under the age of 34) and a group of older chronic alcoholic subjects (above the age of 60) revealed no correlations in neurons regarding SOD1 expression and MMP9 expression. Still, a correlation regarding TGF-β1 expression exists, and the median values decrease with age for SN in contrast to increasing median values for CC region.

3.10. TUNEL reaction

No TUNEL-positive neuronal nuclei were found in alcoholics; in contrast, a low TUNEL-positivity was found in neurons of drug addicts (<5%, n=20). The highest number of TUNEL-positive nuclei was found in CS (20%, n=70); these nuclei were comparatively less found in CC (10%, n=10). In the white and gray matter of SN, a higher number of TUNEL-positive glial cell nuclei were found (5%, n=20), in contrary to CS region, where a number of TUNEL-positive neurons is higher (20%, n=70) than a number of glial cells (5 – 10%, n=70). In contrary, in the white matter of CC, the highest TUNEL-positivity was found in oligodendrocytes (30%, n=10).

3.11. Hepatic TGF-β1 and SOD1 immunohistochemistry

Alcoholic and control subjects were selected for analysis that included the use of anti-TGF-β1 and anti-SOD1 antibodies. In hepatocytes of chronic

alcoholic subjects, TGF-β1 expression varied from weak to strong, whereas, the rate of SOD1 – from medium to strong. In control subjects, SOD1 and TGF-β1 intensity varied from weak to medium.

3.12. Transmission electron microscopy

Scattered, mainly medium-sized irregularly shaped nerve cell bodies were seen in the dorsal striatum region, whereas the larger and medium-sized ones were present in the *substantia nigra* region. The neuron nucleus revealed a rounded envelope contour and a fine granular chromatin pattern, and one single and large nucleolus was usually present in each nucleus. Some neuronal nuclei revealed convolution of the nuclear envelope, but chromatin always demonstrated a fine granular pattern. The rough endoplasmic reticulum was abundant in the majority of cases, and presented with regularly shaped paralleled cisternae. Sometimes, these cisternae were slightly expanded. Golgi complexes were purely or moderately developed and localized in close vicinity to the rough endoplasmic reticulum cisternae. Neuronal somata showed rounded or oval, swollen mitochondria with a matrix of low electron density. Mitochondrial cristae patterns varied very greatly and some mitochondria had a reduced number of cristae. In most cases the cristae were narrowed and paralleled, while others were vacuolated. Only scattered lipofuscin inclusions were demonstrated in the neuronal cells' perikarya. Dendritic processes often did contain lipofuscin inclusions. Some cortical neurons contained more prominent inclusions with a combination of fingerprint-like profiles and a dense matrix. The *substantia nigra* neurons confirmed the presence of melanin granules, also distinguishable at a light microscopic level. We did not detect any ultrastructural features of the neuronal death. Morphology of the neuronal perikarya reflected survival of synthetic functions and cell energetics.

Prominent differences between neuronal somata from various regions of the brain were not demonstrated.

Axoplasm often displayed low electron density and contained occasional organelles and their remnants. Same axons revealed a varying ultrastructure with partially good preservation of the axoplasm content, including the cytoskeleton. Traditionally, axons demonstrated enlarged mitochondria often occupying the whole axoplasm area (Fig. 3.4. B).

We observed relatively darker dendritic cytoplasm with swollen mitochondria and an altered number and structure of cristae within them. Dendritic end parts were often accompanied by empty synapses. Dendrites showed cytoplasmic synaptic vesicles, prominent presynaptic and postsynaptic parts with condensed, electron-dense membranes. Mitochondria were middle-sized and swollen, relatively darker than those seen in axons.

The nucleus of an astrocyte usually revealed an oval or irregular shape. The euchromatin was dominating, and small chromatin clusters were distributed evenly. Occasionally, small nucleolus was distinguishable. The cell cytoplasm was electron-lucent and contained a reduced number of cytoskeletal elements. Mitochondria were rounded and swollen, basically, medium-sized.

We observed that oligodendrocytes characteristically displayed spherical nuclei. Occasionally, a wavy contour of the nuclear envelope was noticed. Clumps of peripheral heterochromatin appeared in the cell nucleus; electron-dense, large nucleoli were demonstrated. A narrow rim of electron-lucent cytoplasm enveloped the cell nucleus. Some oligodendrocytes accumulated polymorphous inclusions localized in the perinuclear region. Prominent and often expanded cisternae of rough endoplasmic reticulum were also seen. Mitochondria usually appeared as rounded, medium-sized, swollen bodies (Fig. 3.4. A).

Membranes of the myelin sheath enveloping these damaged axons revealed a loss of regularity. Occasionally, the protrusions within the myelin

sheath were observed with or without cytoplasmic insertions. The myelin sheath was irregularly folded and split, often revealing vacuoles between the myelin lamellae (Fig. 3.4. C). Characteristically, the most pronounced detachment and/or crossing of the myelin membranes took place at the innermost aspect of the myelin coat, still, sometimes, involving the total thickness of the sheath (Fig. 3.4. D). On the contrary, a general parallel patterning of the myelin membranes accompanied by rare vacuole-like protrusions was detected in the samples of control subjects.

Microglial cells nuclei were irregularly shaped and usually displayed electron-dense peripheral heterochromatin clusters. The cytoplasm of microglial cell was electron-dense. It contained cisternae of the rough endoplasmic reticulum and Golgi apparatus, small lysosomes and inclusions.

Endothelial cells were characterized by a variably flattened form and electron-dense cytoplasm. Endotheliocytes nuclei were enveloped in a wavy enclosure. An endothelial cell surface exhibited short irregular projections of microvilli, and the plasma membrane revealed an average amount of small pinocytotic vesicles. The cytoplasm showed rare, occasionally swollen mitochondria. At intersections between endothelial cells, primarily primitive junctions were observed, with tight junctions being rare. The basement membrane showed variable thickness, sometimes exhibiting a multilammellar appearance. Astrocyte foot processes demonstrated large, vacuolated mitochondria, vacuoles and a decrease in a number of cytoskeleton elements.

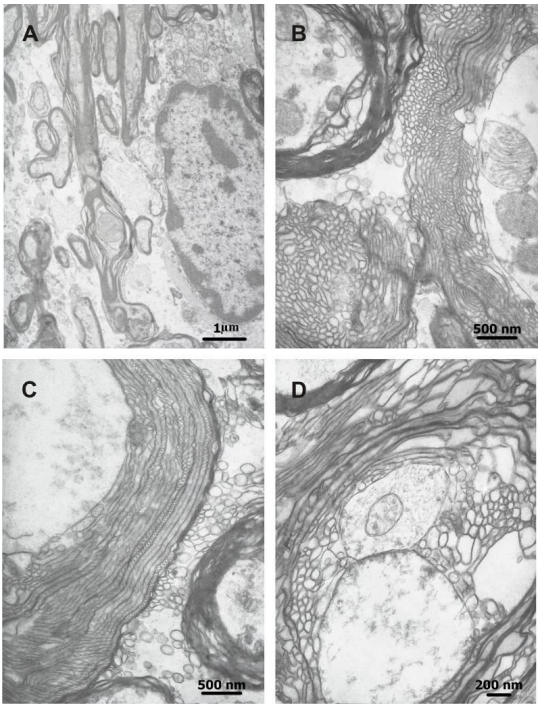

Fig. 3.4. Alcohol-related changes in *corpus striatum* (A) and *substantia nigra* (B, C, D) in human brain. (A) The micrograph demonstrates an oval oligodendrocyte nucleus surrounded by a narrow rim of cytoplasm containing short cisternae of rough endoplasmic reticulum, ribosomes, swollen mitochondria and myelinated axons with swollen mitochondria. (B) Myelinated axons demonstrating a varying degree of myelin layer affection. (C) An enlarged swollen axon with electron-lucent cytoplasm, swollen mitochondria. (D) Myelinated axons demonstrating heterogeneity of ultrastructure of myelin.

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3.13. Scanning electron microscopy

We found that the white matter structure and its conditioning, in general, and the basal ganglia region, in particular, are essential for assessment of the alcoholic brain damage. Therefore, we inserted SEM images illustrating the spatial relationships between the main constituents. These groups of cells or nuclei demonstrated neurons displaying a conical-like shape (Fig. 3.5.A). The

size of perikaryon was set at 20 μm, and the initial segment of axon often was detectable. The cytoplasmic part of a neuron had relatively high volume with well-defined nucleus in its central region. Astrocytes revealed similar or slightly smaller cell bodies and were localized in the close vicinity of the vascular wall (Fig. 3.5.B). Neuropil contained randomly oriented and greatly varying neuronal cell processes (diameter 1.3 – 2.9 μm). Frequently, the processes demonstrated local expansions. In the white matter, there were well definable oligodendrocytes with the cell size of about 7 μm. Nuclei within them were relatively large, occupying the majority of the cytoplasm. The number of oligodendroglial cells found was relatively small compared to the fraction of the white matter appearing in the studied visual field. Endotheliocytes, in turn, were tightly interconnected, and, sometimes, encircled by pericytes.

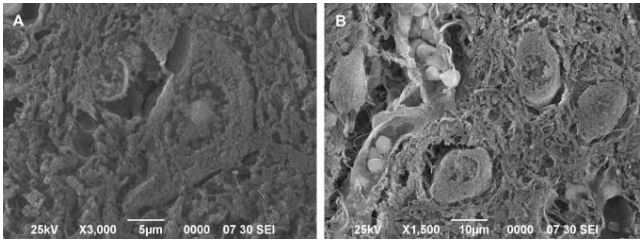

Fig. 3.5. (A) Electron micrograph demonstrating a nigral neuron revealing a large rounded nucleus. (B) Astrocytes appear close to capillary.

3.14. EDX

Chemical element analysis in nerve cells from the SN region of alcohol abusers shows varying concentrations of the main constituent elements O, P, S and in particular, Ca. Additionally, a probable presence of Mg and Cu is observed close to the lower detection limit for these elements.

4. DISCUSSION

We performed a complex immunohistochemical and ultrastructural study on brain tissue obtained from the cortical, striatal and nigral regions of alcoholics and drug users, and compared these samples with controls. In order to foresee vulnerability of neural elements reflected by their oxidative state more clearly, we used a complex histopathological estimation and statistical analysis. We found that a combination of morphological assays jointly with statistical data provided relevant results on the neurobiology of brain in case of alcohol and drug addiction.

The results obtained in our study showed increased TGF-β1 expression intensity in chronic alcohol abusers in all regions of investigation in comparison to control group. Our results reveal tendencies opposing those in the study of Cippitelli and colleagues with laboratory animals (Cippitelli *et al.*, 2010) where neurons of *gyrus dentatus* and hippocampus showed decreased TGF-β1 expression under alcohol exposure. Study of Kim and Piras revealed increased TGF-β1 levels in plasma samples of chronic alcohol abusers (Kim *et al.*, 2009; Piras *et al.*, 2012); in contrast, our study shows an increase of TGF-β1 in the tissues of particular brain regions. Similarly, a study by Roussa that was done on *in vitro* models provided an idea regarding protective function appearing in case of alcohol intoxication *in vivo* conditions (Roussa *et al.*, 2004). Interestingly, TGF-β1 expression estimations in the neuropil of SN in chronic alcohol abusers revealed lowered values when compared to controls. Similarly, we observed statistically significant differences in TGF-β1 expression in the white matter fibers of SN and CC regions where chronic alcohol abuser median values were lower than those obtained from control group. These differences were not observed in the CS region. The results showed that increased CS expression in the neuron' perikaryon does not always correlate with expression in the neuron processes. Furthermore, there were

significant neuronal TGF-β1 expression differences in different brain regions. It is possible that increased TGF-β1 expression in neuron cell bodies observed in our study is an attempt to compensate low TGF-β1 in SN and CC white matter fibers. In addition, it is also possible that increased TGF-β1 expression in neuron cell bodies caused by oxidative stress has affected MMP9 expression as well (Horssen *et al.*, 2006, Mentlein *et al.*, 2012). Interestingly, results obtained from drug abusers when compared with control, showed statistically significant differences in TGF-β1 expression in neurons only from CC region where, similarly to chronic alcohol abusers, median values were higher. We found significant differences in TGF-β1 expression in the white matter of SN region in chronic alcohol and drug abusers, thus providing an opportunity to estimate pathways and effects of particular psychoactive substances. We observed positive TGF-β1 and SOD1correlation in chronic alcohol abuser SN neurons, whereas this correlation was negative in neurons of the CC region. Negative TGF-β1 and MMP9 correlation was detected in alcohol abuser CS neurons as well as in the white matter fibers. Interestingly, the control group also showed positive TGF-β1 and SOD1 correlation, but this was not observed between TGF-β1, SOD1 and MMP9. This multifactorial involvement shows complicated regulatory mechanisms occurring in the nerve tissue, and, similar to Pages and his colleagues' report (Page-McCaw *et al.*, 2007), our observations indicate that MMP9 can activate latent TGF-β1 protein, leading to its increase in SN and CC neurons. Our observations regarding chronic alcoholic subjects showed significantly elevated expression of NGFR in striatal and cortical neurons in comparison with controls, contrary to SN. In cases of drug addiction, basal ganglia neurons demonstrated increased NGFR expression when compared with controls. Median values of neurotrophin receptor expression in specific regions of CNS suggest that alcohol and drug exposure variably affects receptors for neurotrophic factors. The axons of the SN demonstrated low NGFR expression, whereas CC white matter showed

strong expression in alcoholics. The NGFR expression was increased in the SN white matter of drug addicts. Recent data show that neuronal activity accompanied by increased secretion of neurotrophins, can regulate interactions between cellular activity and synaptic plasticity (Berry *et al.*, 2012). It is reported that NGFR is a co-receptor for myelin-associated glycoprotein signaling and has the potential to be a therapeutic target for promoting nerve regeneration (Wong *et al.*, 2002).

We performed a complex immunohistochemical and ultrastructural study on the brain tissue obtained from the cortical, striatal and nigral regions of addicts, and compared these samples with controls. In order to foresee vulnerability of neural elements reflected by their oxidative state more clearly, we used a complex histopathological estimation and statistical analysis. We found that a combination of morphological assays jointly with statistical data provided relevant results on the neurobiology of brain due to psychoactive substances. The presence and wide distribution of SOD1 in different cell types within the CNS is an important factor effectively catalyzing transformation of the superoxide radicals into hydrogen peroxide and molecular oxygen. According to the literature immunolocalization of SOD1 in different cell types is assumed to be a response reaction to oxidative stress damage (Peluffo *et al.*, 2005; Yon *et al.*, 2008). Immunohistochemical analysis of SOD1 showed increased antioxidant activity within the CNS basal ganglia region including gray and white matter (Sommavilla, 2012). The deficiency/excess of antioxidants in chronic alcoholics and drug users can promote fragility of neurons related to oxidative damage and the process of neurodegeneration. SOD1 expression in neuronal somata was statistically different within alcoholics and drug users when different brain regions analyzed in this study were taken into consideration (Maier and Chan, 2002). Moreover, the distribution of SOD1 expression correlations, reflecting glial cells particularly oligodendrocytes, found in the gray matter, had the same tendency (Ward *et al.*,

2009). We used our results regarding regional deviations of antioxidant activity, in general, and reduction of SOD1 immunoreactivity in the white matter of SN which was paralleled by severe damage of myelin structure documented electron microscopically, in particular, to provide evidence regarding the selective vulnerability of neural constituents in alcoholics. Our findings suggest that the endogenous antioxidant system is an important behavior regulator operating in the brain tissue. Ultrastructural analysis has shown that organelle damage in neurons and glial cells has a selective pattern with clear mitochondrial involvement. It has been believed that free radicals are produced by several ethanol-related mechanisms (Matsuda *et al*., 2009) observed in the cortex of human alcoholics due to impaired oxidative phosphorylation and massive production of free radicals.

Similarly to SOD1 immunolabeling, MMP9-expression in neuronal somata was statistically different within alcoholics and controls groups when different brain regions analyzed in this study were taken into consideration. We suggested that increased MMP9 expression in the white matter is not related to prominent BBB alteration. It is highly possible that MMP9, generally referred to as ECM degrading protease, can cause myelin damage (Milward *et al*., 2008). The presence of elevated expression of MMP9 in oligodendrocytes and microglia correlates with the myelin sheath damage demonstrated in these alcoholic subjects ultrastructurally. Kobayashi and others (Kobayashi *et al*., 2008) demonstrated that MMPs promote peripheral myelin damage through myelin basic protein (MBP) degradation that partly coincides with our results on chronic alcoholism related changes in myelination based on MMP9 activity. According to Moore (Moore *et. al*., 2011) the action of MMPs is regulated by the endogenous tissue inhibitors of metalloproteinase (TIMPs) secreted by astrocytes, thus regulating inhibition of MMPs. Prominent SOD1 expression within astrocytes might be associated with alterations in the inhibitory function of this enzyme related to MMPs (Mentlein *et al*., 2012; Morita-Fujimura *et al*.,

2000). An interesting finding of our study is the co-localization of increased MMP9 reactivity and elevation of immunoexpression of SOD1 associated with ROS. Simultaneously, specifying regional peculiarities in alcoholics, we suggest that the behavior of the white matter in *substantia nigra* stands apart from the other findings of this study. Indeed, we found the elevation of MMP9 expression along with a decrease of SOD1 immunoreactivity as compared with controls. The presence of ROS indicates that overexpression of MMP9 plays a significant role in consumption of psychoactive substances and excessive formation of free radicals. An interplay between overexpression of SOD1 and MMP9, especially with regard to their activity in oligodendrocytes and microglial cells in the white matter regions, does not exclude a possibility that oxidative stress and MMP-related processes orchestrate myelin layer changes and degradation. It is highly possible that decrease of the white matter volume shown in chronic alcoholism (De la Monte, 1998) occurs due to microglial activation and consequent myelin alteration. Despite the fact that we failed to evidence a direct MMP9 mediated myelin damage, in general, our immunohistochemical findings on elevation of MMP9 expression in the white matter of studied brain regions, microglia and oligodendrocytes are in accordance with the ultrastructural alterations of myelin reported in this study. Interestingly, ependimocytes showed negative MMP9 expression, whereas in alcoholics MMP9 staining was positive. In accordance with Tateno and Saito (Tateno and Saito, 2008), heavy alcohol consumption induces changes in lateral ventricles, including decrease of neurogenesis which has been shown both in *in vitro* studies and in animals.

There was some variability in the number of TUNEL-positive cells in various brain regions. Despite cell death mechanisms related to neurodegeneration that have been reported worldwide, it has been revealed that cell death mechanisms may be dysregulated in alcoholics. This impaired regulation may reflect molecular adaptations that counteract alcohol

neurotoxicity in cells that survive after chronic alcohol exposure (Johansson *et al*., 2009). Examination of *bulbus olfactorius* and TUNEL-positive neurons in the CC region with light and electron microscopy methods indicate that ethanol-induced neurodegeneration is necrotic (Obernier *et al*., 2002). Our data of TUNEL-positive staining in brain tissue in cases of chronic alcoholism and drug addiction are useful for further examination of the brain damage and neuronal cell death due to neurotoxicity.

Permanent alcohol abuse may lead to chronic liver injury such as liver steatosis, fibrosis and cirrhosis. Hepatic SOD1 and TGF-β staining was significantly higher in case of chronic alcoholism compared with the control group. Our observations suggest that liver stellate cells can transform into myofibroblast-like cells as well as take part in the formation of ECM, thus playing a role in damaging the functioning of the liver and the development of fibrosis (Jester *et al*., 1999; Herrman *et al*., 2007; Gressner *et al*., 2007). A strongly positive hepatic SOD1 staining found by us supports the role of oxidative stress in fibrosis.

Despite the establishment of selective vulnerability to alcohol-related injury in the CNS, accurate ultrastructural details valuable for the understanding of pathogenic events are still lacking. Alcohol-induced changes studied by electron microscopy mainly reflect the ultrastructural details of neuronal injury (Khan *et al.*, 2001). To some extent our data are in accordance with findings reported by Ikegami (Ikegami *et al*., 2003), who performed a study on human alcoholic brains. We have not observed ultrastructural evidence of neuronal somata damage due to chronic alcohol abuse in the brain regions selected for analysis. However, neuronal processes appear to react in a different way. In the majority of cases, axonal degeneration was a rare ultrastructural finding of our study, but evidence of myelin layer impairment in chronic alcoholics was observed. Irregularities of the myelin sheath and splitting of myelin membranes were detected. We found that paranodal loops

were attached to the axolemma and strongly varied in their shapes. Sometimes myelin membranes revealed ballooning and crossing. Schulte (Schulte *et al*., 2010) reported that even a relatively subtle damage of local white matter fiber microstructure in alcoholics showed a relation with behavioral measures of interhemispheric information. Alcohol-induced damage of callosal fiber integrity has been reported by (Wang *et al*., 2010; Pfefferbaum *et al*., 2010). Our observations add some new insights into the transmission of signals in brain circuits under exposure to alcohol that normally enable the integration of highly lateralized visual and motor processes. These ultrastructural abnormalities of the myelin layer may result in reduced conduction velocity in the myelinated axons of the alcoholic subjects. A dendritic network providing an enormously large surface area for the reception of information is essential for interaction with other neurons. The ultrastructural changes observed in alcoholic subjects screened in our study are in agreement with results obtained by Tang (Tang *et al*., 2001), and allow speculation that degenerative changes in dendrites reduce the synaptic receptive area and alter neuronal circuitry. In the evaluation of dendritic spines as these appeared in the present study, we followed a paper published by Sorra and Harris (Sorra and Harris, 2000). We have also demonstrated occasional smooth endoplasmic reticulum profiles, sections of cytoskeleton elements, ribosomes, and the virtual absence of mitochondria within dendritic spines. Astrocytes are considered to be crucial for homeostatic regulation of synaptic plasticity. Slezak and collaborators (Slezak *et al*., 2003) reported that astrocytes change the number of synaptic connections directly, via synaptogenic signals, or indirectly, by modifying the morphology of neuronal axons and dendrites. Astrocytes are known to supply neuronal energy requirements, capture and metabolize glutamate, and release neurotropic factors required for neuronal sprouting. With the increasing knowledge of the importance of glial cells in the functioning of the brain, Fabricius *et al.* (Fabricius *et al*., 2007) proposed a hypothesis of the necessity

of estimating the number of glial cells in long-lasting alcohol abuse, and also found that the total glial cell numbers in alcoholics were not statistically and significantly different, in comparison with controls. In our study we have shown that astrocytes, in general, and astrocytic cell processes, in particular, were reduced in number or greatly shortened and with reduced ramifications.

Ultrastructural analysis of drug addicts in the basal ganglia regions, accompanied with clinical and instrumental methods, provided new insights into the vulnerability and changes of cellular structures focusing our attention on the target cells – oligodendrocytes apart from neurons (Stepens *et al*., 2013). Oligodendroglial cells showed heterogeneity in their response. Loose myelin arrangement with incisures of Schmidt-Lanterman demonstrating areas of residual cytoplasm was a common finding. In accordance with Baumann and Pham-Dinh report (Baumann and Pham-Dinh, 2001), myelin uncompaction, myelin breakdown, and morphological anomalies of myelin trigger changes of nerve conduction velocity. The mitochondrial abnormalities included their enlargement mainly due to swelling, and reduction of a number of cristae. Mitochondrial pathway of damage may lead to a switch in cell function and survival in the *globus pallidus* area. The mitochondrial changes found *in vivo* study in dopaminergic neurons of the SN region reflect the involvement of mitochondria in producing neuronal selective vulnerability and pathology (Song *et al*., 2004). Similar changes of mitochondria appeared in this study, revealing the vulnerability of glial cells under drug influence, including methcathinone exposure.

In accordance with findings reported by Weiss (Weiss *et al*., 2009), the plasma membrane of endothelial cells revealed an average amount of pinocytotic vesicles, however, a high number of mitochondria, associated with a strong metabolic activity was not detected. Ultrastructural changes of astrocytes and foot processes found in this study suggest their vulnerability and involvement in complicated BBB mechanisms of protecting the brain cells

from psychoactive substances (Zlokovic, 2008). Various ‘neurovascular unit’ components play a role in the resulting reaction to toxic substances and suggest the existence of complex processes and selective cell sensitivity.

EDX microanalysis revealed varied Ca concentrations in SN gray matter of chronic alcoholics. It is known (Haydon and Carmignoto, 2006; Winship *et al*., 2007) that activation of astrocytes are related to intracellular Ca changes. Still further studies are needed in order to detect trace elements in different regions of the brain.

5. CONCLUSIONS

1. Differentiated vulnerability of cortical and subcortical neurons and glial cells was found as a consequence of the use of psychoactive substances detected by structural and ultrastructural analysis.

2. Immunohistochemical changes of SOD1 expression are indicative of oxidative stress caused by exposure to alcohol and drugs; this marker is statistically significantly increased in neurons and glial cells of the *substantia nigra* and *cortex cerebri* as well as in nerve fibers in the *corpus striatum* and *cortex cerebri*, and is decreased in nerve fibers and glial cells of *substantia nigra* in alcoholic subjects.

3. A statistically significant increase of MMP9 expression in neurons and glial cells of alcoholics and drug users clearly suggests a role of metalloproteinase-9 in remodeling processes occurring under the exposure of psychoactive substances.

4. SOD1 and MMP9 expression varies and shows statistically significant neuronal and nerve fibers correlation in the *substantia nigra* region in alcoholics. Parallel myelin layer-related ultrastructural changes revealed in the white matter of *substantia nigra* are usable for further analysis of chronic alcohol consumption effects in CNS.

5. Ultrastructural analysis of cytoplasmatic organelles, especially the endoplasmatic reticulum, mitochondria and cytoskeleton of the cellular constituents of the human brain as well as affects of dendritic ramification and synapses reflects their reactivity and selective vulnerability due to consumption of addiction-inducing substances.

6. A strong correlation was found between the decrease in expression of the oxidative stress marker in the white matter of *substantia nigra* and the local mediator TGF-β1 and NGFR that enhance neuronal protectivity. A decreased number of TUNEL-positive nuclei of astrocytes and oligodendrocytes in the

white matter of *substantia nigra* point at some peculiarities in the cell death mechanisms operating in this region in the case of excessive alcohol consumption.

7. EDX is a suitable method for detection of high tissue concentrations of chemical elements like carbon (C) and oxygen (O) and low concentrations of phosphorus (P), sulphur (S) and calcium (Ca). Monitoring of the changes of Ca concentration accompanied by TEM is a suitable tool for examination of synaptic plasticity.

6. LIST OF PUBLICATIONS AND PRESENTATIONS ON THE STUDY THEME

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2. Skuja S., Groma V., Smane L. Alcoholism and cellular vulnerability in different brain regions. // Ultrastructural Pathology, 2012; 36(1):40–47.

3. Stepens A., Groma V., Skuja S., Platkājis A., Aldiņš P., Ekšteina I., Mārtiņsone I., Bricis R., Donaghy M. The Outcome of the Movement Disorder in Methcathinone Abusers: Clinical, MRI, and Manganesaemia Changes, and Neuropathology// European Journal of Neurology, 2013; 1–7.

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Scientific conference/congress theses

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