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Morphologic variations in the pineal gland of the albino rat after a chronic alcoholisation process

J. Martínez-Salvador, A. Ruiz-Torner, A. Blasco-Serra, F. Martínez-Soriano*, A.A. Valverde-Navarro

Department of Human Anatomy and Embryology, University of Valencia, Valencia, Spain

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ABSTRACT

We studied the effect of alcohol on the pineal gland of 48 male Wistar rats. Animals were divided into control and experimental groups. The experimental group underwent a previous progressive alcoholisation period with ethanol diluted in water at a concentration of 40%. Animals were sacrificed at 3, 6, 9 and 12 months, and the ultrastructure, karyometric indices, and number of synaptic bodies in the pineal gland were analysed. The results showed progressive morphologic alterations in the ethanol-treated animals, which culminated in fatty degeneration of the pineal parenchyma after 6 months. The karyometric indices decreased in both the central and peripheral areas compared with the control group. Moreover, the seasonal rhythmicity observed in the controls disappeared in the experimental groups, whose number of different populations of synaptic bodies (synaptic ribbons and synaptic spherules) considerably lowered with inversion of their normal seasonal rhythm. These results support that chronic alcoholisation leads to fatty degeneration of the pineal parenchyma, and a considerable alteration in nuclear functional rhythms and synaptic bodies.

1. Introduction

Most research on the functional connection between the pineal gland and alcoholisation has focused on alcohol effects on melatonin secretion. The commonest data reported establish a connection among alcohol ingestion, variations in melatonin plasma levels, and darkness. These data have been reported for not only laboratory animals (Moss et al., 1986; Rudeen and Symmes, 1980), but also for humans (Ekman et al., 1993; Wikner et al., 1995; Kühlwein et al., 2003).

The effect of ethanol on melatonin secretion in mice and rats has been well established (Czarnecka et al., 1999; Kühlwein et al., 2003; Danel et al., 2009), as has the effect of chronic alcoholisation on the protective and antioxidant effect melatonin (Crespi, 2012; Genç et al., 1998; El-Sokkary et al., 1999; Fonzi et al., 1994; Raghavendra and Kulkarni, 2001). Likewise, alterations in the chronobiology of hormone secretion and sleep (Danel and Touitou, 2001), or in the inhibition of melatonin secretion (Del Rio-Hortega, 1920; Fonzi et al., 1994; Röjdmark et al., 1993), have been reported.

However, morphological studies are scarce. Brovina and Nastashinskaia (Brovina and Nastashinskaia, 1990) studied the pineal glands of 45 dead alcoholic humans. They divided glands into different groups according to the patient's degree of alcoholisation, ascertained variations in the density of the pinealocytes and karyometric indices, and showed the influence of alcohol ingestion on these parameters.

The existence of circadian and seasonal rhythms in the nuclear activity of the pinealocyte of some rodents has been established (Cimas et al., 1992; Matsushima et al., 1983). This rhythmicity is reflected in variation in the number of synaptic bodies (SB), especially in synaptic ribbons (SR) and synaptic spherules (SS). These ultrastructural organelles are located inside the cytoplasm of pinealocytes of lower vertebrates, birds and mammals, together with much scarcer organelles called intermediate synaptic bodies (ISB). ISB are morphologically irregular SB. The most frequent forms of ISB are the triangular synaptic bodies (TSB) and the rectangular synaptic bodies (RSB). SB have been reported to be present in hamsters (Matsushima et al., 1983), rabbits (Martínez-Soriano et al., 1984; Martínez-Soriano et al., 1999), cows, sheep and pigs (Struwe and Vollrath, 1990). SR seem to be dynamic indicators of the metabolic activity of the pinealocyte (Khaledpour and Vollrath, 1987; Martínez-Soriano et al., 1999; Martínez-Soriano et al., 2000). More recently, Spiwoks-Becker et al. (Spiwoks-Becker et al.,

Abbreviations: SB, Synaptic Bodies; SR, Synaptic Ribbons; SS, Synaptic Spherules; ISB, Intermediate Synaptic Bodies; TSB, Triangular Synaptic Bodies; RSB, Rectangular Synaptic Bodies; CAZ, Cytomatrix Active Zone; CtBP1, C-Terminal Binding Protein 1; KIF3A, Kinesin Family Member 3A; AMP, Adenosine Monophosphate; ICER, Inducible Early Repressor

* Corresponding author at: Departamento de Anatomía y Embriología Humana., Facultad de Medicina y Odontología, Universitat de València, Av. Blasco Ibáñez 15, 46010, Valencia, Spain.

E-mail address: martinfr@uv.es (F. Martínez-Soriano).

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2008) reported that pinealocyte's SR are associated with CAZ proteins, such as Bassoon, Piccolo, CtBP1, Munc13-1, and morphoprotein KIF3A, all of which are present in the synapses of sensory organs. Protein composition changes at night with an increase in Bassoon, Piccolo, and Munc13-1, whereas KIF3A predominates during the daytime. CtBP1 is equally present night and day.

Alcohol-induced disorders include the modification of hormone secretion and sleep rhythms (Danel and Touitou, 2001). However, the putative relationship between alcoholisation and morphofunctional alterations in the pineal gland has been less studied. The aim of this work was to study the variations that chronic alcohol ingestion produces on some rhythmic morphofunctional parameters of the pinealocytes, specifically on the karyometric index and synaptic bodies, over an experimental 1-year period.

2. Materials and methods

2.1. Animals

Forty-eight male Wistar rats that weighed 130 ± 20 g were used for this experiment. Only male rats have been used to avoid the complex interactions that melatonin, alcoholization and the estrous cycle of female rats may have had in the experiment (Chuffa et al., 2013).

Animals were housed in the Central Research Unit of the University of Valencia, with a controlled cycle of 12 h light-12 h darkness, and constant temperature (22 ± 2 °C) and humidity (55 ± 10%). All the animals came from litters born on similar dates. Animal experimentation was carried out in accordance with the European Communities Council Directive and was approved by the Ethics Committee of the University of Valencia. Animals were weighed weekly to determine any possible differences between groups.

Rats were divided into two groups of 24 animals (control group and experimental group). The control group received a standard diet and water *ad libitum*. The experimental group also received a standard diet, but an increasing solution of absolute alcohol was added to their drinking water until a maximum dilution of 40%. This dilution was maintained for 1 year.

At 3, 6, 9, and 12 months six animals from each group were sacrificed (i.e., January, April, July and October), and their pineal glands were removed.

2.2. Alcoholisation process

The aversion that animals generally feel towards alcohol stops them from consuming it voluntarily. Consequently, ethanol was gradually introduced into the rats' diet. The adaptation period lasted 35 days, due to the high concentration of ethanol (40%) that had to be ingested.

Graduated glass drinkers were used to accurately monitor the quantity of ingested ethanol solution. In order to avoid the possible volatilisation of part of the ethanol, the alcohol solution was changed daily.

2.3. Electron microscopy study

Animals were sacrificed after anaesthesia with an intraperitoneal injection of sodium Nembutal. Afterwards, they were perfused, first with physiological serum, and then with Karnovsky solution (Karnovsky, 1965). Each experimental group was sacrificed at the same time as its corresponding control group. Immediately before sacrifice, a blood sample was taken to determine the blood alcohol level.

Pineal glands were removed and post-fixed in osmium tetroxide for 90 min, dehydrated with graded series of acetone, stained with 5% uranyl acetate and 1% phosphotungstic acid in 70% acetone, and finally embedded in Epon resin. Tissue was then cut transversely with an ultramicrotome into semithin sections (1 μm) and stained with Toluidine Blue.

Measurements of pinealocyte nuclei from 100 peripheral (cortical) and 100 central (medullar) have been reported to be sufficiently representative of each animal. Four semithin sections of the pineal gland's *pars distalis* of each rat were used to measure the 200 nuclei (50 nuclei were measured per section; 25 nuclei were measured per pineal layer). All the four selected sections were at least 15 μm away from the preceding one to avoid including the same pinealocyte nucleus in more than one section. Only clearly visible pinealocyte nuclei were considered. Nuclear measurements were recorded with the VISILOG programme for the imaging analysis.

Nuclear volume (V) was calculated using Jacobj's formula (1935) (Jacobj, 1935) with the following karyometric indices: longer diameter (A), shorter diameter (B) and a constant (k). $V = \Pi/6 \times A \times B^2 \times k$.

The ultrathin sections used to study SB were mounted on 300-mesh copper grids and stained with 20% uranyl acetate in 100% methanol for 1 min, followed by lead citrate for 5 min (Reynolds, 1963).

The number of SB was calculated by counting those observed in eight grid squares, each measuring 65 × 65 μm (total area 33800 μm^2, magnification ×12000) and expressed as 20000 μm^2.

2.4. Statistical analysis

The statistical data analysis was done after a descriptive study. A comparative analysis of the variables was carried out by contrast and significance tests. Any P-values lower than 0.05 were considered statistically significant. An analysis of variance (ANOVA) was used when comparing the means of more than two variables.

3. Results

3.1. Rats alcoholised for 3 and 6 months

Ribosomes were scarce in the three-month experimental group, but there were numerous vesicles that folded over to form pseudomyelinic shapes. However, the most important signs appeared after 6 months of alcoholisation. In this last group, presence of abundant small, fusiform, and triangular shaped cells was noted in the intercellular and perivascular spaces. There were large vacuolisations and dense granules of a lysosomal nature in the cytoplasm of these cells, which were adipose-grained in nature (Fig. 1). The perivascular space also contained an amorphous fibrillar-grained material of an unknown nature.

In this group evident and numerous signs of alterations of the myelin layers of the fibres that adhere to pineal parenchyma were observed, such as presence of vesicles, granulations, disintegration, and breakage of myelin layers (Fig. 2). These findings, which were frequent

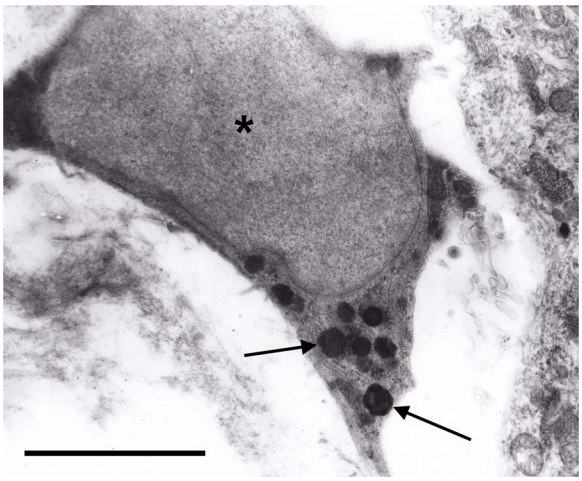

Fig. 1. Vacuolizations (*) and dense lysosomal granules (arrows) in the cytoplasm of the cells after 6 months of alcoholization. Scale bar = 125 nm.