Quantitative histochemical study of cytochrome oxidase in the dLGN of aging rats

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Abstract

We carried out a quantitative histochemical study of the enzyme cytochrome oxidase (CO) in neurons of the dorsal lateral geniculate nucleus (dLGN) of male Wistar rats aged 3, 18, 24 and 28 months. The results show that the activity of cytochrome oxidase decreases significantly between 24 and 28 months. We also checked whether a correlation existed between neuronal size and enzymatic activity. Low correlation coefficients were obtained which were between 0.4139 at 3 months and 0.2092 at 28 months. Nevertheless, we observed a certain relationship between both parameters, and therefore we classified the neurons as light, moderate and dark according to their optical density, which correlates with enzyme cytochrome oxidase activity, and as small, medium and large depending on their size. We found that light neurons were scarcely represented in the dLGN. At the age of 3 months, the most frequent neurons were moderate, medium-size ones, and dark, small ones. The population of moderate neurons increased with age, reaching 74.5% at the 28th month, 52.2% of which corresponded to medium-size neurons. In the same group dark neurons decreased, falling to a total of 15.3% made up of medium and large-size ones. These results could be interpreted as reflecting a decrease in the bioenergetic competence of the neurons of this nucleus in old age.

Keywords: Aging; Cytochrome oxidase; Densitometry; dLGN
1. Introduction

One hypothesis proposed to explain the factors which trigger cellular aging suggests that free radicals can induce alterations in the main biological molecules, with the consequent deterioration in cellular function [1,2]. These alterations can be reduced by the action of various antioxidant agents, such as the enzymes superoxide dismutase, catalase and glutathione peroxidase, which are present in practically every tissue, although their levels are much lower in the central nervous system [3]. Studies in vivo have demonstrated that superoxide dismutase and catalase activity decrease with age, [4–7], the brain being especially sensitive to these changes [8].

Much research has focused on mitochondria, since it is in these organelles that free radicals are generated [6,9]. Some researchers have analyzed the changes which take place in the components of the mitochondria electron-transfer chain with the passage of time. Thus, Benzi et al. [10] observed a decrease in the forebrain of old rats in the concentrations of cytochrome $aa_3$, without changes in ubiquinone or cytochrome b.

Martinez et al. [11] reported a parallel decrease in the content of lipid peroxide and cytochrome c oxidase in synaptic mitochondria from mouse brain, as well as an increase in the concentration of glutathione with aging. On the other hand, some authors have observed changes in the lipid composition of mitochondrial membranes with an increase in total cholesterol, a decrease in phospholipids, especially in cardiolipin, and an increase in the cholesterol/phospholipid ratio [12].

As the enzyme cytochrome oxidase is considered an appropriate metabolic marker of neuronal functional activity [13], the aim of this study was to analyze its activity in dLGN neurons of young, middle-aged and old rats (3, 18 and 24–28 months-old, respectively). With this in mind we applied quantitative histochemical rather than biochemical methods in order to minimize damage to the physical integrity of the structure studied.

2. Material and methods

2.1. Materials

A total of twelve apparently healthy male Wistar rats, distributed in groups of four were used. The animals were submitted to prior exploration, and those with any kind of anomaly which could interfere with our study were disconed; their ages were 3 months, young adults; 18 months, mature adults; 24 months, showing the first signs of aging; and 28 months, which is considered very old according to their survival rate [14]. Their weight was between $247 \pm 7.5$ g and $412 \pm 10.3$ g. The animals were kept in a controlled environment at a temperature of $23 \pm 1°C$ and a light/dark cycle of 12 h/12 h. They had access to water and food ad libitum and the
Venice Conference guidelines were followed for their care [15]. All the animals were anaesthetised with chloral hydrate at 8% (0.1 ml/30 g weight) and then decapitated.

2.2. Cytochrome oxidase histochemistry

Once decapitated, the unfixed brains were quickly removed, immersed in buffered 0.1 M phosphate (pH 7.4) at 4°C, and subjected to increasing concentrations of sucrose (10, 20, 30%) for 48–72 h until they sank. In a cryostat, at −21°C, the brains were transversally sectioned at 30 μm from the rostral to the caudal region of the nucleus and incubated at 37°C in darkness for 45 min following our modified version of the Wong–Riley method [16]. The incubation medium prepared immediately before its use contained: 41.1 mg 3,3′-diaminobenzidine (DAB), 75 ml 0.1 M phosphate buffer, 18.6 mg cytochrome C type III (Sigma) and 3.33 g sucrose. Catalase was used (200 g/ml) to eliminate the presence of endogenous peroxidase. The experimental sections were simultaneously incubated under the same conditions and in a horizontal position to avoid any variation in staining intensity. Finally, sections were rinsed three times in 0.1 M phosphate buffer at 4°C, dehydrated and mounted in Eukitt.

2.3. Quantitative analysis

A total of between 150 and 170 neurons with a visible nucleolus per animal were used to measure the levels of CO. We used a Nikon photometer attached to an IBAS 2000 (IPS) image analyzer with a ×100 objective and a 520 nm filter. Lighting and magnification conditions were kept constant. The system was calibrated by measuring the grey levels in a non-reactive area (reference point) in each of the sections measured. Neuronal profiles were drawn with a light pen using a digital image with a resolution of 512 × 512 pixels (1 pixel = 0.1611 μm at ×100). The light transmission was estimated from the midgrey level of each neuron, 0 being black and 225 white. Optical density (OD), which reflects the intensities of CO reaction products, was calculated from the transmission percentage (T) using the following formula:

\[ \text{OD} = -\log_{10} T \] (adimensional parameter)

2.4. Statistical treatment

The results obtained were analyzed by applying the Kruskal–Wallis non-parametric test \( (P < 0.0001) \) and a multiple comparison test to detect significant differences \( (P < 0.01) \) between the groups. Similarly, via linear regression, we analysed possible correlations between the optical density values of cytochrome oxidase and neuronal size in each age group. Finally, an interval table was calculated for these two parameters and a Chi-square test carried out to see whether any degree of correlation existed between both parameters.
3. Results

We have illustrated the OD distribution values for each age in the histogram in Fig. 1. The most frequent range is between 0.4 and 0.6 at all the ages studied, although the proportions are different.

Between the 24th and the 28th month a significant decrease (0.6417 ± 0.019 and 0.5058 ± 0.008, $P < 0.01$ respectively) is observed in the OD mean values (Table 1).

As regards the relationship between OD levels and cellular size at each age, we found low correlation coefficients ($r$ values): 0.4139 at 3 months and 0.2092 at 28 months.

Table 1

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>OD + SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.6466 ± 0.027*</td>
</tr>
<tr>
<td>18</td>
<td>0.6287 ± 0.013*</td>
</tr>
<tr>
<td>24</td>
<td>0.6417 ± 0.019*</td>
</tr>
<tr>
<td>28</td>
<td>0.5058 ± 0.008</td>
</tr>
</tbody>
</table>

* $P < 0.01$, as compared to 28-month-old rats.
Table 2
Relationship between the optical density values of cytochrome oxidase and cellular size at each age

<table>
<thead>
<tr>
<th>Area (µm²)</th>
<th>Age (months)</th>
<th>Optical density</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;0.40 light</td>
<td>0.40–0.60 moderate</td>
</tr>
<tr>
<td>Small &lt; 100</td>
<td>3</td>
<td>2.4</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.6</td>
<td>22.8</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.0</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Medium 100–160</td>
<td>3</td>
<td>14.8</td>
<td>24.3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.6</td>
<td>20.3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.2</td>
<td>29.2</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>10.2</td>
<td>52.2</td>
</tr>
<tr>
<td>Large &gt; 160</td>
<td>3</td>
<td>5.3</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>1.9</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.9</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.0</td>
<td>12.7</td>
</tr>
<tr>
<td>Total (%)</td>
<td>3</td>
<td>22.5</td>
<td>41.4</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>3.2</td>
<td>54.4</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3.1</td>
<td>55.9</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>10.2</td>
<td>74.5</td>
</tr>
</tbody>
</table>

Chi-square test (χ²).
χ² (3 months old) = 36.5606, P < 0.0001 (S).
χ² (18 months old) = 7.88213 (NS).
χ² (24 months old) = 16.1502, P = 0.0028 (S).
χ² (28 months old) = 15.011, P = 0.004 (S).

months. However, since some relationship between both parameters was found, we proceeded to classify the neurons according to their OD values as light (< 0.40), moderate (0.40–0.60) and dark (> 0.60). They were also classified with regard to their cellular size as small (< 100 µm²), medium (100–160 µm²), and large (> 160 µm²) (Table 2). The Chi-square test confirmed the existence of a relationship between both parameters, except at age 18 months.

At 3 months the greater percentage of neurons are moderate (41.4%), 24.3% are medium size, followed by the dark ones (36.1%), 23.7% of which are small-sized. At this age, the least abundant neurons are light (22.5%), 14.8% of which are medium size. At 18 months, the populations of moderate and dark neurons increase to 54.4% and 42.4%, respectively; 20.3% of the moderate ones are medium-sized and 21.5% of the dark ones are also medium. On the other hand, the number of light neurons falls. At 24 months, the percentages are similar. Finally, at 28 months, 74.5% of neurons are moderate, 52.2% being medium sized, whereas the number of dark neurons decreases to 15.3% (Table 2).
4. Discussion

Among the enzymes used to analyze the changes present during cellular aging, we chose CO, a mitochondrial enzyme located in the internal membrane that is the final component of the electron transfer chain from cytochrome c to oxygen. This enzyme was selected because it is considered to be a metabolic marker of neuronal functional activity [13]. The differences in its activity, as observed in the brain, seem to indicate the heterogeneity of energy demand in the different neurons of a given region [17–19]. Thus, neuronal groups with different activity rates are found within the nervous system, and within them CO levels change considerably [19–23]. The variations in CO activity reflect differences in the concentration of the CO protein within the same neuron [24,25], and are related to the level of mitochondrial DNA and of mRNA sub-units present in the various neuronal compartments [26].

We found differences in the activity of the CO of dLGN neurons depending on the age of the rodents, a fact apparent in other species, but at different life stages [27–29]. Upon analyzing the mean values of the OD, we found that CO activity significantly decreased ($P < 0.01$) between the 24th and the 28th month. This can be attributed, according to Benzi et al. [11], to a decrease in the content of mitochondrial cytochrome $a_3$. Accordingly, Gold et al. [30], Abul-Erreish and Sanadi [31], and Benzi [32] think that this could be related to a decrease in the number of $aa_3$ molecules. On the other hand, Martinez et al. [11] have also demonstrated a decrease in the content of cytochrome c oxidase in the synaptic mitochondria of old mice parallel to a decrease in lipid peroxide, as well as an increase in the concentration of glutathione. They point out that these changes are likely to be interdependent. Other researchers have observed differences in the behaviour of this enzyme depending on the cerebral regions studied, the parieto-temporal cortex and the cerebellum being the most affected by age [33].

The histochemical quantitative studies of Kageyama and Wong-Riley [18] and Liu and Wong-Riley [29] in adult cat and monkey dLGN, respectively, have shown a relationship between CO activity levels and neuronal size. In our study of this relationship we have obtained somewhat lower coefficients than those indicated by Liu and Wong-Riley [29]. Nevertheless, as some degree of relationship existed between both variables, we classified them into light, moderate and dark neurons according to the level of enzyme activity; and into small, medium and large with regard to their size. At 3 months (young adult) enzyme activity levels were about the same as in a previous study [22]. On the whole, the percentage of light neurons is low in the dLGN, falling from 3 months (22.5%) to 28 months (10.2%). According to the criteria proposed by Kageyama and Wong-Riley [18] for cat dLGN, this decline could be related to a decrease in the population of interneurons in old age. The neurons predominating from adulthood (3 months) up to old age (24–28 months) are the moderate ones, whose numbers increase with age. Thus, at 28 months they represent 74.5% of neurons, 52.2% of which are medium-sized. On the other hand, dark neurons are significantly fewer (15.3%) in the 28 month old animals. The fact that there is a decrease in CO activity and an increase in moderate neurons in old age could be interpreted as reflecting a decrease in the
bioenergetic competence of the neurons of this nucleus. Further studies are required for a better understanding of age related functional changes in nerve cells.

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References


